

# Recent progress in secondary metabolism of plant glandular trichomes

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**Abstract** Trichomes can be found on the surfaces of the leaves, stems, and other organs of many angiosperm plants. Plant trichomes are commonly divided into two classes: glandular trichomes and non-glandular trichomes. Glandular trichomes produce large quantities of specialized natural compounds of diverse classes and are regarded as ‘chemical factories’ due to their impressively efficient biosynthetic capacities. This efficiency makes glandular trichomes an excellent experimental system for the elucidation of both the biosynthesis and the mechanisms of regulation of natural product pathways. The development of various -omics techniques has greatly accelerated experimental procedures that are typically used in combination with trichome studies. The purpose of this review is to provide an introduction to the methods and technologies used for the investigation of glandular trichomes, to summarize current progress in the field, and to highlight the potential applications of trichome studies in metabolic engineering using the strategies of synthetic biology.

**Key words:** Glandular trichomes, specialized natural products, -omics techniques, synthetic biology.

## Introduction

Glandular trichomes (GT) populate the aerial surface of approximately 30% of vascular plant species. Sometimes, one can find several types of glandular trichomes on a single leaf; for example, there are at least 4 different glandular trichomes on tomato leaves (type I, IV, VI, and VII, the glandular type I and type VI trichomes are usually dominant) (Glas et al. 2012). These various types of glands have been proposed to play key roles in plant interactions with growth environments. Structurally, plant glandular trichomes can be dissected into stalk cells, secretory cells, and the storage cavities where the final chemical products are stored (Figure 1A). One of the most remarkable features of glandular trichomes is their capacity to synthesize and store large amounts of secondary metabolites. Owing to this unique chemical property, glandular trichomes protect plants against a number of abiotic/biotic stresses including UV exposure, herbivore attack, and pathogen infection. As they are not essential for plant development, GT provide a unique opportunity to study complex and specialized metabolic pathways. It is noteworthy that many GT-produced chemicals have significant commercial value as pharmaceuticals or natural pesticides. For this reason, the prospect of exploring

GT as “chemical factories” to produce high-value plant natural products has recently captured the attention of plant biochemists and biotechnologists alike. Realization of this goal will be facilitated by genome-scale research focused on the identification of genes that control the biochemical functions of GT. The breadth of GT studies at the molecular level have extended rapidly from the Lamiaceae (e.g. *Mentha piperita* and *Ocimum basilicum*) to Asteraceae (e.g. *Artemisia annua*, *Helianthus annuus* and *Chrysanthemum cinerariaefolium*), Solanaceae (e.g. *Nicotiana tabacum* and *Solanum lycopersicum*), Leguminosae (e.g. *Medicago truncatula* and *Medicago sativa*) and Cannabaceae (e.g. *Cannabis sativa* and *Humulus lupulus*) (Aziz et al. 2005; Fridman et al. 2005; Marks et al. 2009; Nagel et al. 2008; Ramirez et al. 2013; Schillmiller et al. 2012; Schillmiller et al. 2009; Wang and Dixon 2009; Wang et al. 2008; Wang et al. 2009).

## Gene discovery from plant glandular trichomes using system biology approaches

It is well-known that the secondary metabolites produced by plants are derived from primary metabolites such as amino acids, fatty acids, and sugars. The type and quantity of the particular primary metabolites imported

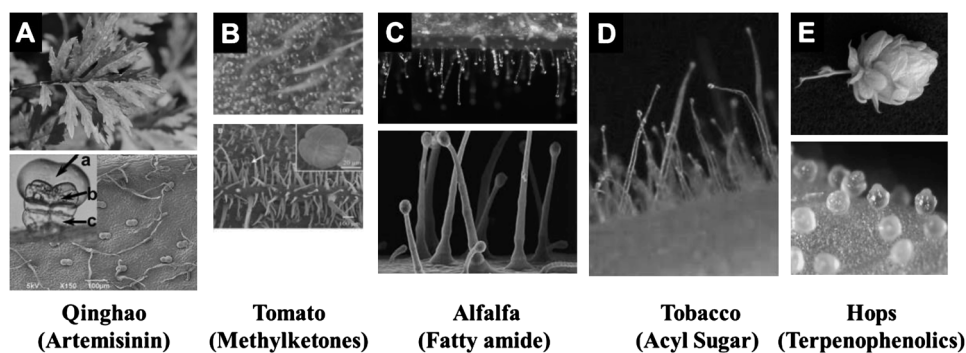


Figure 1. Examples of plant glandular trichomes and the major chemicals biosynthesized in these organs. A. *Artemisia* glandular trichome: a, storage cavity; b, secretory cells; c, stalk cells; B. Type VI trichomes of tomato; C. Alfalfa glandular trichomes; D. Tobacco glandular trichomes; E. Hops female flower and its glandular trichomes.

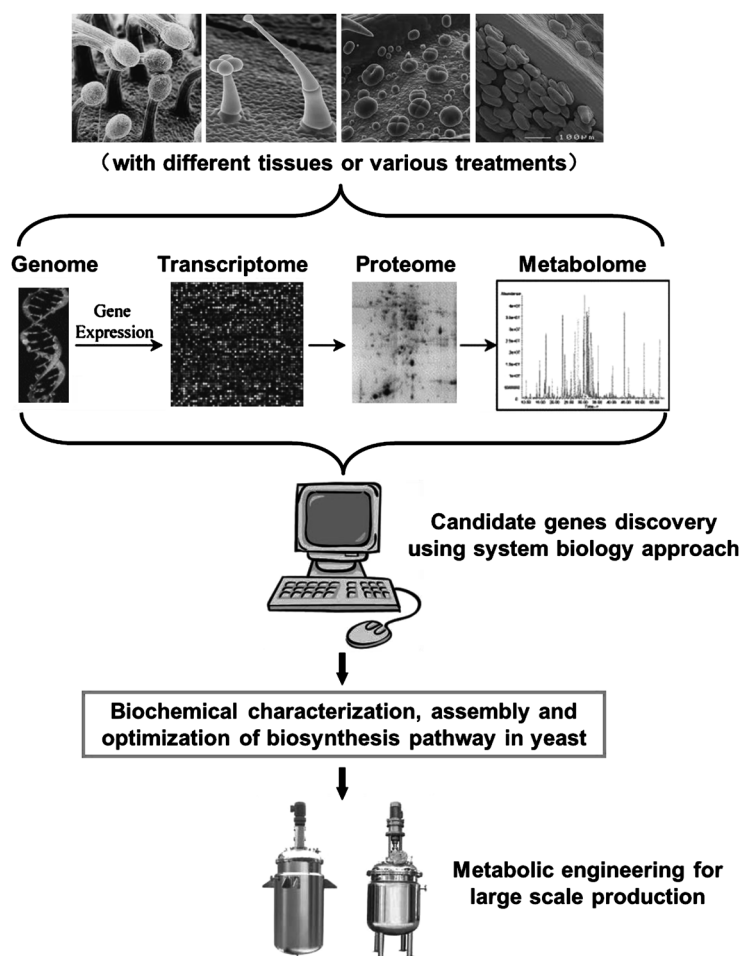


Figure 2. Pipeline for the discovery of novel biosynthetic genes from plant glandular trichomes and their potential applications for metabolic engineering. Biochemical characterization of candidate genes plays a key role in this procedure.

into glandular trichomes is a determining factor for the secondary metabolites that may be biosynthesized, due to the inability of glandular trichomes to carry out *de novo* biosynthesis of such precursors from photosynthesis. With the rapid development of “-omics” techniques, especially the lower cost of sequencing, more and more genome and transcriptome data have become available (The plant trichome databases: [www.planttrichome.org](http://www.planttrichome.org)

and [http://bioinfo.bch.msu.edu/trichome\\_est](http://bioinfo.bch.msu.edu/trichome_est)). Mega-sequencing data mining has thus become a critical component for the discovery of novel genes involved into the biosynthesis pathways of interest. Although the amount of such data has developed to a vast scale, two classical criteria are still used for novel gene elucidation using the “reverse genetics” strategy: (1) positive correlation between candidate gene expression

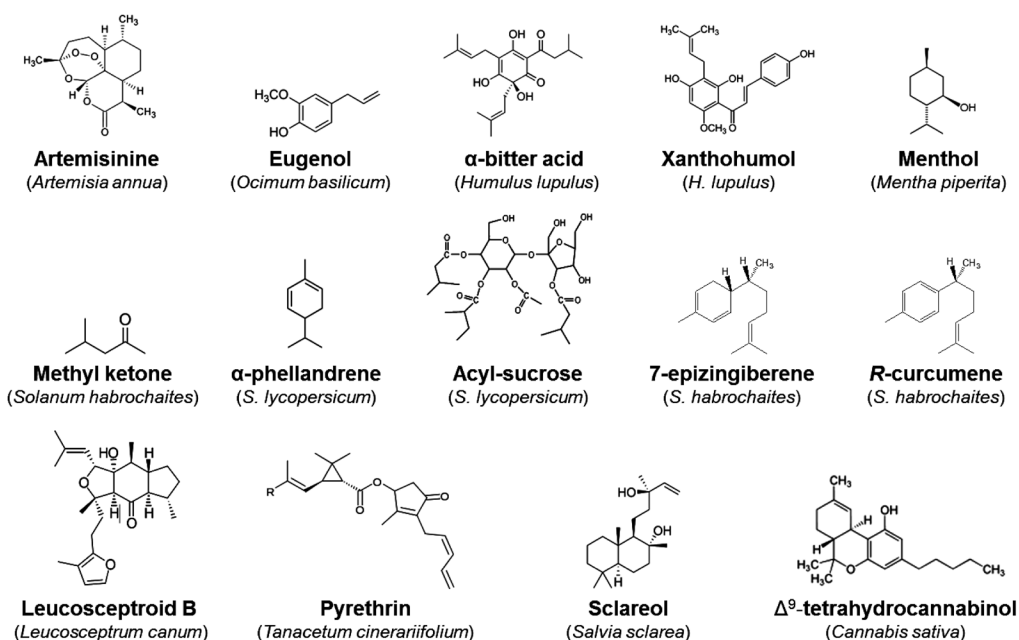


Figure 3. Representative chemicals (with their origin) mentioned in this review.

levels and the accumulation levels of metabolites of interest; (2) the accumulated biochemical knowledge about the enzymes which may catalyze the enzymatic step of interest. For example, the cytochrome P450 monooxygenase enzymes are always considered as candidate enzymes for mono-oxidation of aromatic rings (Figure 2). By using a genome-wide association analysis, one can expect to identify all gene candidates for each enzymatic step in the biosynthesis pathway of interest. This strategy has been successfully applied to many cases of novel gene functional elucidation when using plant glandular trichomes as an experimental subject (Dai et al. 2010; McDowell et al. 2011; Schillmiller et al. 2010b; van Bakel et al. 2011). It is noteworthy that there are only a few studies that have focused on the genes related to glandular trichome development and the transcription factors that regulate trichome-specific biosynthesis pathways (Ma et al. 2009; Spyropoulou et al. 2014; Yu et al. 2012). It is the same case in studying metabolite transportation of glandular trichomes (Choi et al. 2012). This dearth most likely results from the lack of successful transformation systems for the plant species that are typically used to investigate trichomes.

### Trichome-targeted metabolic pathway elucidation

Based on the chemical structures, plant secondary metabolites are typically classified into several groups: terpenoids, phenylpropanoids/polyketides, acyl sugars, and fatty acid derivatives (Figure 3), among others. Recent progress in the understanding about these groups of plant secondary metabolites will be summarized here;

particular emphasis will be given to compounds now known to be biosynthesized in glandular trichomes.

### Terpenoids

Terpenoids are the largest class of plant natural products, with more than 50,000 compounds identified to date. Terpenoids (isoprenoids) are any compounds that are derived from the isomeric 5-carbon building blocks isopentenylidiphosphate (IPP) and dimethylallyldiphosphate (DMAPP). IPP and DMAPP are generated by the plastidic MEP pathway and the cytosolic mevalonic acid pathway (Chen et al. 2011). The short-chain prenyltransferases catalyze the subsequent condensation of DMAPP and IPP to form longer prenyldiphosphates, which are the direct precursors in terpenoid biosynthesis. Based on the number of C5 units, terpenoids are classified into several classes: hemiterpene (generated from DMAPP), monoterpene (generated from NPP (nerylidiphosphate) or GPP (geranylidiphosphate)), sesquiterpene/triterpene (generated from *cis*- or *trans*-FPP (farnesylidiphosphate)), diterpene (generated from GGPP (geranylgeranylidiphosphate)), and sesterterpene (generated from GFPP (geranylarnesylidiphosphate)). Following catalysis by terpenoid synthase (TPS) enzymes, plant terpenoids are often further decorated with additional functional units by various enzymes such as P450s and methyltransferases. Recent studies suggest that the MEP pathway is likely the main contributor to IPP/DMAPP production in plant glandular trichomes (Besser et al. 2009; Wang et al. 2008; Wang et al. 2009).

The first systematic and successful trichome study was performed in the laboratory of Dr. R. Croteau. Using a

small EST (expressed sequence tag) database as a starting point, they elucidated almost all of the enzymes involved in the menthol (produced in the glandular trichomes of *Mentha piperita*) biosynthesis pathway at the molecular level, including heterodimeric geranyl diphosphate synthase (using traditional enzyme purification, followed by peptide sequence-based gene cloning) (Burke et al. 1999), the monoterpene synthase limonene cyclase, and P450 hydroxylases and dehydrogenases (Croteau et al. 2005). Benefitting from these increases in understanding, they were able to increase the essential oil yield of transgenic peppermint by up to 78% over wild-type controls in field conditions by overexpressing 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR gene) and down-regulating expression of the (+)-menthofuran synthase gene (Lange et al. 2011). Compared to the intensive study of plant TPS genes, short-chain prenyltransferases have been overlooked for a long time. Two unusual short-chain prenyltransferases responsible for neryl diphosphate (NPP, in cultivated tomato M82) and *Z,Z*-FPP (in *cis*-configuration, in *Solanum habrochaites* LA1777) were recently identified from tomato glandular trichomes (Sallaud et al. 2009; Schillmiller et al. 2009). The corresponding TPS genes (phellandrene synthase 1, PHS1 and Santalene and Bergamotene Synthase, SBS) linked to *cis*-prenyltransferase were also functionally identified in these studies. The cloned *cis*-prenyltransferase gene and terpene synthases gene formed a functional gene cluster, which probably evolved by duplication and divergence of TPS genes, together with alterations in substrate specificity to enable utilization of *cis*-prenyl diphosphates in *Solanum* (Matsuba et al. 2013). The glandular trichomes of different wild tomatoes also showed a huge chemical-diversity. Bleeker et al. found that the 7-epizingiberene and *R*-curcumene produced in the glandular trichomes of a wild tomato (*Solanum habrochaites* PI127826) showed toxicity and were repellent to herbivores. The herbivore-susceptibility of cultivated tomato partially results from the lost capacity of 7-epizingiberene production. Bleeker et al. introduced the biosynthesis pathway of 7-epizingiberene in the glandular trichomes of tomato cultivar 'Moneymaker' by coexpressing *ShZIS* (7-epizingiberene synthase) and *zFPP* synthase driven by trichome-specific promoters. The transgenic cultivated tomato re-gained resistance to several tomato pests without observed penalties in growth or development (Bleeker et al. 2011; Bleeker et al. 2012).

Sesquiterpene lactones are characteristic constituents of the Asteraceae plants. These bitter chemicals often contain an  $\alpha,\beta$ -unsaturated- $\gamma$ -lactone structural feature that is associated with their bio-activity. Artemisinin (Qing-hao-su in Chinese), which contains an unusual peroxide bridge, is the most well-known sesquiterpene

lactone and is used as an anti-malarial treatment. Detailed information about artemisinin biosynthesis has been covered comprehensively in a previous review (Covello et al. 2007). The biosynthetic pathway of pyrethrin (a sesquiterpene lactone commonly used as a botanical insecticide that is produced in the glandular trichomes of *Tanacetum cinerariifolium* fruits) was recently elucidated from a pyrethrum trichome EST library and includes germacrene A synthase, germacrene A oxidase, and costunolide synthase (Ramirez et al. 2013).

Although the sesterterpenoids (C<sub>25</sub> terpenes) were initially discovered from marine sponges, there are a few reports of their occurrence in the glandular trichomes of Lamiaceae plants. The main sesterterpenoids, Leucosceptroid A and Leucosceptroid B, clearly show insecticidal bioactivity to beet armyworm (*Spodoptera exigua*) and cotton bollworm (*Helicoverpa armigera*) (Luo et al. 2010). To date, we know little about sesterterpenoid biosynthesis in plants, although it has been proposed that GFPP (geranyl farnesyl diphosphate) is a direct precursor for sesterterpenoids (Luo et al. 2010). Because of their biological activity and structural diversity, elucidation of the sesterterpenoid biosynthesis pathway in plants is worthy of further study (Wang et al. 2013).

#### **Phenylpropanoids and polyketides**

The phenylpropanoids are a diverse family of plant secondary metabolites that are synthesized from the amino acid phenylalanine and are often detected in the glandular trichomes of many plant species. The peltate glands of basil (*Ocimum basilicum*, a member of the Lamiaceae) contain high concentrations of phenylpropenes (e.g. eugenol, chavicol, and their methylated derivatives); this plant has served as a model system to study phenylpropene biosynthesis. Pichersky and his colleagues have extensively studied the phenylpropene biosynthetic pathway, starting from an EST database containing 1,215 ESTs (Gang et al. 2001). Several structural genes, which were enriched in the gland-targeted EST database, were functionally identified, including methyltransferase (Gang et al. 2002) and eugenol synthase (a member of NADPH-dependent reductases, which converted coniferyl acetate ester to form eugenol) (Koeduka et al. 2006; Vassao et al. 2006). However, the biosynthesis of the direct substrate of eugenol synthase, coniferyl acetate ester, remains to be elucidated in basil glandular trichomes.

Flavonoids, one type of polyketides, represent one of the largest and most well-studied classes of plant specialized metabolites; it has been estimated that there are several thousand such compounds. The most well-known flavonoids are pigments, due to their visibility in most flowers, fruits, and seeds. The knowledge of the

core flavonoid pathway was generated from studies of the Arabidopsis *tt* mutants (*transparent testa*). The first enzyme of the flavonoid pathway, chalcone synthase, produces naringenin chalcone, from which all flavonoids derive (Winkel-Shirley 2001). Although the central pathway for flavonoid biosynthesis is conserved in plants, various enzymes such as isomerases, reductases, hydroxylases, methyltransferases, glycosyltransferases, and acyltransferases are known to modify the basic flavonoid skeleton, leading to the species-specific flavonoids. Flavonoid glycosides were found exclusively in the secretory products of glandular trichomes of *Phillyrea latifolia* leaves when exposed to high levels of sunlight, which suggested the flavonoids in glandular trichomes probably provide UV-protection for plants (Agati and Tattini 2010; Tattini et al. 2000).

There are also many other types of polyketide in addition to flavonoids in plant glandular trichomes. For example, hemp (*Cannabis sativa*) trichomes produce and store large amounts of cannabinoids which exhibit psychoactive and medicinal activities (ElSohly and Slade 2005). The biosynthesis of cannabinoids starts with the condensation of hexanoyl-CoA and three malonyl-CoA molecules to form olivetolic acid, catalyzed by a tetraketide synthase (TKS). The TKS was previously designated as olivetol synthase because no olivetolic acid could be detected in the *in vitro* biochemical reaction, and olivetol and other pyrones were always produced (ElSohly and Slade 2005). Recently, Page's research group discovered an enzyme (olivetolic acid cyclase, OAC), which is inactive itself, but works with TKS to produce olivetolic acid. Interestingly, these two proteins did not physically interact each other to form a protein complex, as was initially hypothesized (Gagne et al. 2012). Hop (*Humulus lupulus* L.), which also belongs to the Cannabaceae family, is an essential component, along with barley and yeast, for the beer brewing industry. Many studies have demonstrated that hop terpenophenolics (a term for both bitter acids and prenylated flavonoids), which are specifically produced in hops glandular trichomes, exert diverse biological activities and have a high potential for drug development. In hop, the initial step of the terpenophenolics biosynthetic pathway is catalyzed by a type III polyketide synthase such as HIVPS (valerophenone synthase) or chalcone synthase (CHS), which utilize different CoA-activated acids as starter units and malonyl-CoA as an extension unit (Okada et al. 2004). However, the routes to the CoA-activated substrates in hops trichomes have been overlooked. Recently we extensively studied the upstream branches of the bitter acid biosynthetic pathway, and found that the mitochondrial thioesterase genes (*HITE4*) and cytosolic branched short chain fatty acid CoA ligase genes (*HICCL2* and *HICCL4*) play critical roles in linking the BCAA (branched chain amino acid)

degradation pathway and the bitter acid pathway (Xu et al. 2013).

### **Fatty acid derivatives**

Methylketones naturally function as defense compounds against various insects (Williams et al. 1980). Methylketones (with carbon chains ranging from 7 to 15 carbons) are formed by the hydrolysis of acyl-ACPs, intermediates of the fatty acid biosynthesis pathway, and then subsequently decarboxylation of the resulting 3-ketoacids in plant cells. Comprehensive chemical analysis showed that the type VI trichomes of *Solanum habrochaites* (subspecies *glabratum*) specifically biosynthesize high levels of methylketones, predominantly the C11, C13 and C15 compounds, while LA1777 (a cultivated tomato species) trichomes contain no detectable methylketones. Pichersky's lab screened out the candidate genes (*MKS1* and *MKS2*) involved in the methylketone pathway by comparing the trichome-target EST database of both of these tomato species. Consistent with the location of fatty acid biosynthesis in plant cells, *MKS1* is a plastidic protein (Yu et al. 2010). Due to the potential of methylketone as a biofuel, the *MKS2* gene was integrated into *E. coli*, and the best production was around 380 mg/l in a fatty acid-overproducing strain (Goh et al. 2012). However, a similar strategy in plant systems did not generate satisfactory results, probably due to the limited availability of substrates (Yu and Pichersky 2014). This result also pointed out the much higher complexity of plant systems as compared to microbial systems in metabolic engineering.

### **Acyl-sugars**

Acyl sugars typically consist of aliphatic acyl groups of varying chain length esterified to hydroxyl groups of glucose or sucrose; these are always detected in the glandular trichomes of Solanaceae plants such as tomato and tobacco. The resulting polyester acyl sugars secreted from glandular trichomes provide a strong protection from insect attacks, either directly or indirectly (Schillmiller et al. 2012; Weinhold and Baldwin 2011). The investigation of acyl sugar biosynthesis has pended for some time on the identification of the gene encoding UDP-glucose fatty acid : glycosyltransferase, which catalyzed the first acylation reaction of glucose; this was functionally identified from tomato in 2000 (Li and Steffens 2000). Recently, *SIAT2* (a BAHD acetyltransferase), which is responsible for tetra-acyl sugar acetylation, was cloned using a map-based cloning strategy coupled with a high-throughput acyl sugar analytical platform (Schillmiller et al. 2010a; Schillmiller et al. 2012). *SIAT2* is expressed specifically in the head of type I/IV trichomes, where the acyl sugars produced (Schillmiller et al. 2012).

## Translation of the knowledge from plant glandular trichomes into microbes

As mentioned above, many chemicals biosynthesized and stored in GT have high economic value; they are frequently used as medicines, fragrances, or flavors. However, the tiny biomass of plant GTs prevents the practical application of GT themselves for bio-production of these compounds. Further, chemical synthesis of these compounds is often not economical and is not environmentally-friendly. Synthetic biology (here defined as the design and construction of biological systems for producing natural products) can overcome these limitations. Biosynthetic modules need to be defined in plants, which serve as building blocks for reconstruction of biosynthetic pathway of target chemical. These modules of glandular-trichome-origin can then be transferred into micro-organisms so that value-added plant natural products can be synthesized in microbes at a large scale. It will save time to search the biological modules because the metabolic pathway in glandular trichomes have been selected (and thus improved) for a long time to reach their current high efficiency. Among the microbes used for biotechnological applications, baker's yeast (*Saccharomyces cerevisiae*) has several advantages for producing plant natural products: it is easy to manipulate; there are many publically-available “-omics” resources that can be used to increase researchers' capacity to predict the behavior of engineered yeast cells; and many plant genes, especially those encoding membrane-bound proteins, require a eukaryotic cell environment for expression.

Yeast cell naturally produce DMAPP/IPP via the MVA pathway, and high endogenous amounts of FPP make yeast cells an excellent system for producing high-value sesquiterpenoids. For example, Ro et al. reconstructed the upstream pathway of the antimalarial artemisinin in yeast by co-expressing amorphadiene synthase (ADS) and a cytochrome P450 (CYP71AV1, which catalyzes three oxidation steps from amorphadiene to artemisinic acid). The resulting strain produced up to 100 mg/l artemisinic acid after several rounds of optimization to increase the availability of FPP (Ro et al. 2006). The latest version of artemisinic acid-producing yeast strains (around 25 g/l production under optimized fermentation conditions). Together with an efficient chemical conversion to artemisinin, these strains have been applied to industrial production (Paddon et al. 2013; Westfall et al. 2012). It is noteworthy that all of the plant genes (ADS, CYP71AV1, CYB5 (cytochrome *b5*), ADH1 (alcohol dehydrogenase), and ALDH1 (artemisinic aldehyde dehydrogenase)) used for the final engineered yeast were elucidated from a trichome-targeted *A. annua* cDNA library. The increased production of artemisinic acid by addition of ADH1 and ALDH1 in

engineered yeast also suggested that the reactions from amorphadiene to artemisinic acid were catalyzed by CYP71AV1, ADH1, and ALDH1 in order, rather than by CYP71AV1 alone.

Ambergris is an expensive fragrance ingredient; the diterpenoid ambroxide is responsible for the odor of ambergris. In the fragrance industry, ambroxide was semi-synthesized from sclareol, a diterpene-diol. Most of the commercially-produced sclareol is derived from cultivated clary sage (*Salvia sclarea*), and subsequent extraction from the plant material. In clary sage, sclareol mainly accumulates in essential oil-producing trichomes that densely cover flower calices (Caissard et al. 2012). Caniard et al. recently cloned and functionally characterized two new diterpene synthase (diTPS) enzymes for the complete biosynthesis of sclareol using a transcriptome database obtained by deep 454-sequencing of cDNA from clary sage calices. These two diTPS (*SsLPPS* and *SsSS*) converted geranylgeranyl diphosphate (GGPP) to sclareol. The biosynthesis pathway of sclareol was reconstructed by co-expressing these two diTPS in yeast, although no yield information was reported (Caniard et al. 2012). Another research group further reconstructed the sclareol biosynthetic pathway in engineered *Escherichia coli*; the yield of sclareol reached around 1.5 g/l under optimal fermentation conditions (Schalk et al. 2012). Both studies provide a basis for the development of a sustainable and cost-efficient route for the production of sclareol.

In addition to its use in terpenoid production, yeast has for a long time been engineered for polyketide biosynthesis. However, yeast cell lack much of the CoA-ester starter substrates needed for plant polyketide synthesis. We recently re-constructed the initial steps of the bitter acids pathway from hop trichomes by co-introducing the aforementioned CoA ligase gene (*HICCL2/HICCL4*) and *HIVPS* in a yeast system; the phloroglucinol production was around 2 mg/l, and more than 95% of final product was secreted into the culture medium. This engineered yeast also offers a bio-platform to further characterize the remaining enzymatic steps in the bitter acid biosynthesis pathway, e.g. the prenyltransferase and monooxygenase catalyzed reactions (Xu et al. 2013). Following the outline in Figure 4, we will generate a yeast strain producing bitter acid/xanthohumol in the near future. Likewise, Gagne et al. have co-expressed TKS and OAC from hemp in yeast. When these cells were fed exogenous hexanoic acid, olivetolic acid (0.48 mg/l) was produced (Gagne et al. 2012).

Despite these advances, only partial biosynthetic pathways from plant glandular trichomes have been reconstructed in yeast hosts to date, mostly due to the complex pathways with many unelucidated steps. On the other hand, these engineered yeast systems could be

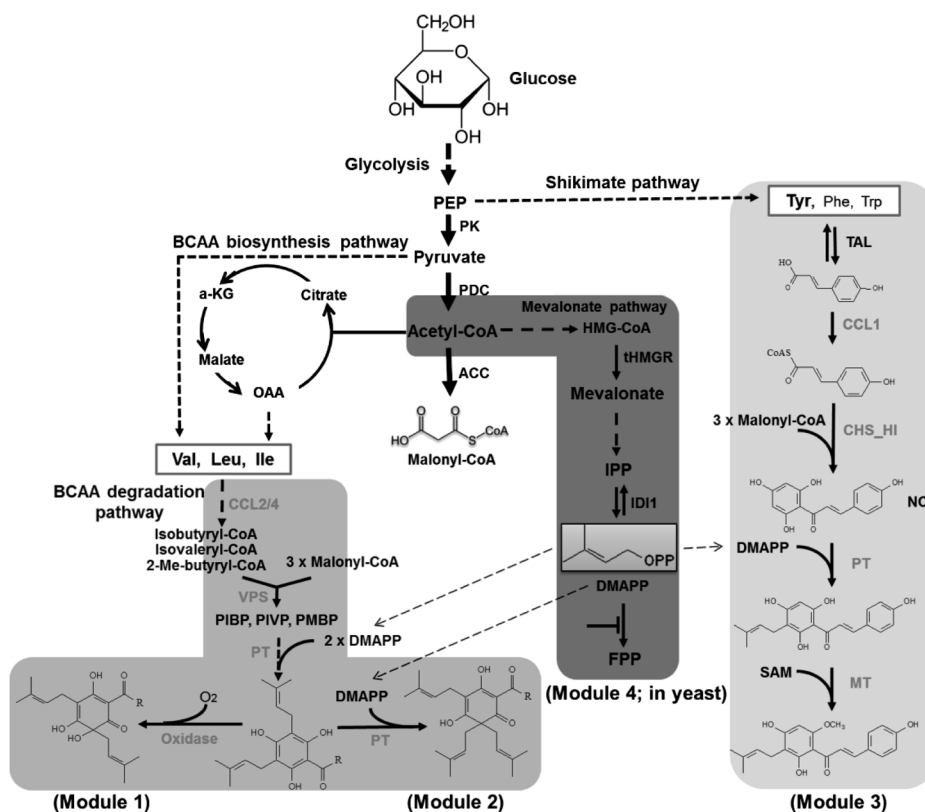


Figure 4. The blueprint of reconstructing the bitter acids and xanthohumol pathways in engineered yeast. The genes highlighted in gray will be derived from a hops trichome cDNA library, and the pathway of module 4 will be optimized for the production of DMAPP, which is the direct substrate for the prenyltransferase enzymes.

the starting point for functional identification of direct down-stream enzymes. To optimize the final production in yeast systems, future endeavors will also focus on the adaptation of the cellular context of the host organism beyond simply the functional identification of genes from glandular trichomes (interested readers are referred to Keasling 2010; Paddon and Keasling 2014).

## Conclusion and future prospective

Thus far, many kinds of glandular trichomes have been studied to elucidate the biochemical pathways of the major trichome-accumulated compounds. These studies have made significant contributions to our understanding of specialized plant metabolism, particularly in the areas of terpenoid and phenylpropanoid metabolism. Naturally, many chemicals biosynthesized and stored in glandular trichomes are toxic to various kinds of herbivores, viruses and fungi. This suggests a high potential for the use of trichome metabolites for drug development for humans. Glandular trichomes are thus attractive targets for metabolic engineering and breeding. Moreover, the successful semi-synthetic production of artemisinin in engineered yeast makes it feasible to transfer any kinds of trichome-targeted biosynthetic pathway into

appropriate yeast strain to produce chemicals of interest at industrial scales. To achieve this long-term goal, it is crucial to understand how whole biosynthetic pathways work in plant glandular trichomes. On the other hand, any intermediate engineered yeast system could be the starting point for functional identification of direct down-stream enzymes. This strategy for the functional characterization of novel plant enzymes circumvents many of common problems with in vitro enzyme assays, such as the purification of recombinant proteins and difficulties with substrate availability. Lower cost DNA sequencing and continual improvements in the resolution and detection limits of mass spectrometry and other technologies from analytical chemistry are expediting biochemical pathway discovery in glandular trichomes. Thus more industrial yeast strains producing value-added chemicals of glandular-trichome-origin can surely be expected in near future.

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