Running title: Glandular trichomes development and bioengineering

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Update review

Plant glandular trichomes: natural cell factories of high biotechnological interest

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Glandular trichomes: from developmental aspects to metabolic engineering approaches.

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Abstract:

Multicellular glandular trichomes are epidermal outgrowth characterized by the presence of a head made of cells that have the ability to secrete or store large quantities of specialized metabolites. Our understanding of the transcriptional control of glandular trichome initiation and development is still in its infancy. This review points to some central questions that need to be addressed to better understand how such specialized cell structures arise from the plant protodermis. A key and unique feature of glandular trichomes is their ability to synthesize and secrete large amounts, relative to their size, of a limited number of metabolites. As such, they qualify as true cell factories, making them interesting targets for metabolic engineering. In this review, recent advances regarding terpene metabolic engineering are highlighted with a special focus on *Nicotiana tabacum*. In particular, the choice of transcriptional promoters to drive transgene expression and the best ways to sink existing pools of terpene precursors are discussed. Bioavailability of existing pools of natural precursor molecules is a key parameter and is controlled by so-called “crosstalks” between different biosynthetic pathways. As highlighted in this review, the exact nature and extent of such crosstalks are only partially understood nowadays. In the future, awareness of, and detailed knowledge on, the biology of plant glandular trichome development and metabolism will generate new leads to tap the largely unexploited potential of glandular trichomes in plant resistance to pests, and lead to the improved production of specialized metabolites with high industrial or pharmacological value.
**Introduction**

Trichomes, the epidermal outgrowths covering most of aerial plant tissues, are found in a very large number of plant species, and are composed of single-cell or multicellular structures. These structures are divided into two general categories: they can be glandular or non-glandular depending on their morphology and secretion ability. Glandular trichomes can be found on approximately 30% of all vascular plant species (Fahn 2000) and in a single plant species several types of trichomes (both glandular and non-glandular) can be observed. Glandular trichomes are characterized by the presence of cells that have the ability to secrete or store large quantities of secondary (also called “specialized”) metabolites, which contribute to increasing the plant fitness to the environment (see Box 1 for details).

Two main types of glandular trichomes stand out: peltate or capitate, which differ according to their head size and stalk length. Capitate trichomes typically possess a stalk whose length is more than half the head height, whereas peltate trichomes are defined as short stalked (uni- or bicellular stalk) trichomes with a large secretory head made of four to eighteen cells arranged in one or two concentric circles. Capitate trichomes are very variable in their stalk cell number and length, glandular head morphology as well as secretion pattern, and can be classified into various types (Glas et al. 2012).

A key and unique feature of glandular trichomes is their ability to synthesize and secrete large amounts, relative to their size, of a limited number of specialized metabolites; mainly terpenoids (Gershenzon et al. 1992; Gershenzon and Dudareva 2007), but also phenylpropanoids (Gang et al. 2001; Deschamps et al. 2006; Xie et al. 2008), flavonoids (Voirin et al. 1993; Tattini et al. 2000), methylketones (Fridman et al. 2005), and acyl sugars (Kroumova and Wagner 2003; Schilmiller et al. 2010; Weinhold and Baldwin 2011).

Over the long term, the ability to modulate the density and productivity of such secreting structures in plants would be of great biotechnological interest. This requires the identification and characterization of the genes initiating, regulating and driving the development of such glandular structures. Awareness of, and detailed knowledge on, the biology of plant glandular trichome development and metabolism will generate new leads to turn trichomes into biochemical factories using metabolic engineering approaches (Tissier 2012a), tap their largely unexploited potential in plant resistance to pests, and lead to the improved production of important specialized metabolites (Lange and Turner 2013; Lange et al. 2011).
*Plant glandular trichomes: an interesting paradigm to study plant cell differentiation*

To modulate the density of glandular trichomes in the epidermis or their productivity for biotechnological purposes, a detailed understanding of the molecular genetic framework governing their development and patterning in the plant epidermis would be beneficial. Glandular trichomes have mostly been studied to decipher the biochemical pathways of the compounds they produce and secrete (Champagne and Boutry 2013; Lange and Turner 2013) and have thereby contributed to advancing our understanding of the secondary metabolism in plants.

*A detailed understanding of glandular trichome initiation and development is currently missing*

All multicellular organisms face the challenge of coordinating cell proliferation with cell differentiation and patterning. Defects in this coordination can lead to incorrect tissue formation, malformed organs, and cancerous growth. Glandular trichomes exemplify this coordination challenge: they are elaborate, highly organized, and polarized cell structures whose morphogenesis is modulated by an intricate array of molecular processes controlling the different steps of their patterning on the leaf epidermis and subsequent differentiation. Therefore, glandular trichomes can be used as a paradigm to tackle basic questions about the development and differentiation of specialized multicellular secretory structures in plants.

A complete circuit for glandular trichome formation and patterning in the leaf epidermis will require a sound understanding of their initiation process in the plant protodermis and of the subsequent developmental steps leading to the formation of a polarized and specialized multicellular structure, of the genes that regulate cell division, participate in cell signaling, and promote specialized cell fate (Figure 1). It also requires an understanding of how this developmental regulatory network affects cellular biological targets such as the core cell cycle machinery. A particularly large gap in our current knowledge is the identification of regulators of entry into the glandular trichome cell fate and of progression through the pathway.

Research on non-glandular trichomes has been very fruitful in *Arabidopsis thaliana*. It has generated a developmental framework for unicellular trichome formation and identified over 30 genes involved in the initiation and development of non-glandular trichomes. We chose not to review the development of trichomes in *A. thaliana* as this has been thoroughly reviewed (Pattanaik et al. 2014; An et al. 2011; Tominaga-Wada et al. 2011; Balkunde et al.
2010; Matías-Hernández et al. 2016). Unlike the situation of A. thaliana, which contains a single type of unicellular, non-glandular trichome, our understanding of the molecular genetic aspects of glandular trichome development is still in its infancy but currently improving due to recent progresses in -Oomics and genome editing technologies as well as to a growing focus from several research teams to characterize this process in different plant species (Liu et al. 2016; Bergau et al. 2015; Bosch et al. 2014; Li et al. 2004; Dai et al. 2010). The (draft) genome sequences of a number of plant species with glandular trichomes are now available. A non-exhaustive list includes different tomato species (Solanum lycopersicum and Solanum pimpinellifolium (the tomato genome consortium, 2012), Solanum pennellii (Bolger et al. 2014)), potato (Solanum tuberosum (Xu et al. 2011)), cucumber (Cucumis sativus (Huang et al. 2009)), tobacco (Nicotiana tabacum, (Sierro et al. 2014; Edwards et al., 2017)) and its related species (Nicotiana sylvestris and Nicotiana tomentosiformis (Sierro et al., 2013)), hot pepper (Capsicum annuum, (Kim et al., 2014)), mint (Mentha longifolia, (Vining et al., 2017)), Cannabis (Cannabis sativa, (van Bakel et al. 2011)) and hop (Humulus lupulus, (Natsume et al. 2015)).

Since most of them can be genetically transformed, studying the molecular genetics of trichome development in these species has become much easier (among others by using genome editing or RNAi technologies). Particularly for species where genetics is poorly developed (e.g. mint), the combination of transcriptomics at different stages of trichome development (see below) and of genome editing or RNA interference technology should be a powerful approach to address the function of candidate genes.

**Trichome-specific data exist but cell stage-specific data are crucially needed to advance our understanding of glandular trichome development**

Extensive glandular trichome-specific EST resources were generated for a variety of (non-model) plant species (Tissier 2012b; Dai et al. 2010; Akhtar et al. 2017; Chen et al. 2014; Jin et al. 2014; Soetaert et al. 2013; Trikka et al. 2015). Dedicated trichome-related open access resources exist that help researchers mine this vast and increasing amount of trichome-related data. One such resource is the TrichOME database (Dai et al. 2010) which hosts functional -omics data, including transcriptomics (ESTs/unigene sequences) metabolomics (mass spectrometry-based trichome metabolite profiles) as well as trichome-related genes curated from published literature from various species. These include members of Lamiaceae (Mentha piperita, Salvia fruticosa, Cistus creticus, Ocimum basilicum), Solanaceae (Nicotiana benthamiana, Nicotiana tabacum, Solanum habrochaites, Solanum...
lycopersicum, and Solanum pennellii), Asteraceae (Artemisia annua), Fabaceae (Medicago sativa, Medicago truncatula) and Cannabaceae (Cannabis sativa, Humulus lupulus).

These trichome-specific expression data are particularly useful to identify trichome-specific genes involved in particular biosynthetic pathways as well as in other trichome-related processes. A key question is how to extract the most significant data out of this massive and growing amount of bulk information to advance our understanding of specific aspects of glandular trichome biology (Tissier 2012a)? From a developmental perspective, one of the main limitations of such resources is that most of these trichome-specific expression data were derived from mature glandular trichomes (which are usually easier to isolate than developing structures) or from a population of trichomes at mixed developmental stages. Indeed, glandular trichome initiation and differentiation occur at different times in different locations within a single leaf. Therefore, even very young leaves contain a trichome population of mixed developmental stages (Bergau et al. 2015). Given the way most EST resources were generated up to now, expression of genes playing a role in early developmental steps may be, in the best case, underestimated or, in the worst case, even not detected. This is particularly the case of genes active in the initiation phase (selection of trichome initials) or early on in the development.

In addition to trichome-specific EST resources, numerous glandular trichome-specific gene promoters have been reported in the literature for a variety of plants including (but not restricted to): Antirrhinum majus, A. annua, Cucumis sativus, H. lupulus, Mentha sp., N. tabacum, S. lycopersicum, S. habrochaites (Ennajdaoui et al. 2010; Wang et al. 2002; Sallaud et al. 2012; Choi et al. 2012; Gutiérrez-Alcalá et al. 2005; Spyropoulou et al. 2014; Okada and Ito 2001; Shangguan et al. 2008; Vining et al. 2017; Liu et al. 2006; Kim et al. 2008; Wang et al. 2011; Wang et al. 2013; Laterre et al. 2017; Jaffe et al. 2007; Kortbeek et al. 2016); thoroughly reviewed in (Tissier 2012b). It is worth noting that most of these trichome-specific promoters are active in mature glandular trichomes (mostly in glandular cells at the tip of the trichome). Their activity during early trichome development has been barely analyzed.

Molecular data pointing to genes playing a specific role in glandular trichome development already exist, especially concerning some transcription factors, cell cycle regulators as well as receptors involved in phytohormones-induced signalling cascades. Several transcription factors belonging to different protein families and playing a role in glandular trichome development have indeed been identified: AmMIXTA, a MYB transcription factor from Antirrhinum majus whose ectopic expression in tobacco induces the development of additional long glandular trichomes (Glover et al. 1998); GoPGF, a basic
helix-loop-helix (bHLH) transcription factor from *Gossypium sp.*, acting as a positive regulator of glandular trichome formation, its silencing leading to a completely glandless phenotype (Ma et al. 2016); AaHD1, a homeodomain-leucine zipper transcription factor required for jasmonate-mediated glandular trichome initiation in *Artemisia annua* (Yan et al. 2017); AtGIS, a C2H2 zinc-finger transcription factor from *A. thaliana* whose ectopic expression in tobacco regulates glandular trichome development through gibberellic acid signaling (Liu et al. 2017); AaMYB1, a MYB transcription factor from *Artemisia annua* whose overexpression induces the formation of a greater number of trichomes (Matías-Hernández et al. 2017), CsGL3, a HD-Zip transcription factor whose mutation leads to a glabrous phenotype in cucumber (Cui et al. 2016).

In tomato, several genes required for proper development and function of different types of glandular trichomes have been reported. The *woolly* (*Wo*) gene, encoding a class IV homeodomain-leucine zipper protein homolog to the *A. thaliana* GL2, and a B-type cyclin gene, *SlCycB2* (possibly regulated by *Wo*), control the initiation and development of type I trichomes. Mutant alleles of *Wo* triggered a hairy phenotype due to the overproduction of type I trichomes, while suppression of *Wo* or *SlCycB2* expression by RNAi decreased their density in tomato (Yang et al. 2011a; Yang et al. 2011b). Another mutation (hairless) affects the SRA1 (Specifically Rac1-associated protein) subunit of the WAVE regulatory complex. SRA1 controls the branching of actin filaments and is required for the normal development of all trichome types of tomato, which suggests that proper actin-cytoskeleton dynamics is a basal requirement for normal trichome morphogenesis (Kang et al. 2016). Mutations of some enzymes involved in secondary metabolism also impact trichome density and/or metabolic activity of glandular trichomes. For example, in the anthocyanin-free (af) mutant, loss of function of the chalcone isomerase (*SlCHI1*) triggers a reduction of type VI trichome density and metabolic output (Kang et al. 2014) while downregulation of DXS2, a MEP enzyme, increases their density (Paetzold et al. 2010). The molecular mechanisms through which these genes affect trichome density are currently unknown.

The tomato *Wo*’ allele was shown to promote abnormal multicellular trichome differentiation when ectopically overexpressed in *N. tabacum* (Yang et al., 2015). This hints at conserved transcriptional networks among Solanaceae species. However, the trichomes in this *Wo*’ overexpressor line failed to develop glandular heads and appeared as rather aggregated and undifferentiated structures. Whether a WD40-bHLH-MYB regulatory mechanism similar to the one in *A. thaliana* also controls glandular trichome development in Solanaceae is still unclear (Serna and Martin 2006; Yang et al. 2015). A recent RNA-seq
analysis of *N. tabacum* trichomes showed that orthologs of *A. thaliana* genes involved in
trichome formation via the WD40-bHLH-MYB regulatory mechanism (such as TTG1, GL2,
GL3, and several MYB transcription factors) are expressed in these structures (Yang et al.
2015). However, their transcriptional levels were not significantly altered in response to the
overexpression of a *Wo* transgene, which induced a clear trichome proliferation phenotype.
On the contrary, homologs of genes (*Wo* and *SlCycB2*) involved in trichome formation in
Asterids (Yang et al. 2011a; Yang et al. 2011b) were significantly upregulated in *Wo* transgenic *N. tabacum* plants (Yang et al. 2015).

Differentiation of long-stalked glandular trichomes may be initiated and controlled in
*N. tabacum* by the activity of another MYB transcription factor. Indeed, ectopic expression of
*MIXTA* from *Antirrhinum majus* (Glover et al. 1998) or of its *Gossypium hirsutum* ortholog
*CotMYBA* (Payne et al. 1999) resulted in the development of excess long-stalked trichomes. It
is therefore likely that another unidentified *N. tabacum* MYB gene, an ortholog to *AmMIXTA*
and *CotMYBA*, plays a role in the development of long-stalked glandular trichomes in this
species. Whether this MYB gene needs to be part of a regulatory complex to initiate and
promote glandular trichome development is not yet known.

Five trichome-related mutants of the genes *CsGL3*, *TRIL*, *MICT*, *TBH*, and *CsGL1*, all
of them encoding homeodomain-leucine zipper transcription factors from different
subfamilies, have been reported in *Cucumis sativus* (Chen et al. 2014; Zhao et al. 2015; Cui et
al. 2016; Li et al. 2015; Pan et al. 2015; Wang et al. 2016). Based on the observed
phenotypes, a molecular mechanism underlying the development of multicellular trichomes in
this species has been proposed in a recent review (Liu et al. 2016) and seems to confirm that
the transcriptional control of multicellular trichome in *C. sativus* differs from the one
observed in *A. thaliana*.

The development of glandular trichome is tightly regulated by the integration of
diverse environmental and endogenous signals. In this respect, some phytohormones,
especially jasmonate (JA) and possibly gibberellins (GA) elicit glandular trichome
development via signaling cascades and the activation of trichome-specific transcriptional
regulators (Tian et al. 2014; Bosch et al. 2014; Koo and Howe 2009; Li et al. 2004; Liu et al.
2017; Bose et al. 2013; Yan et al. 2017). In *Mentha arvensis*, exogenous application of GA
resulted in a moderate increase in trichome density and diameter of the gland, suggesting a
positive, although moderate, effect of GA on trichome initiation and development in mint
(Bose et al. 2013). For example, reduced JA levels (through silencing of OPR3, a key enzyme
in the biosynthesis of the precursor of JA) led to impaired glandular trichome development in
tomato: the density of type VI trichomes was drastically reduced and their metabolite content was different from that of the wild-type (Bosch et al. 2014). The JA receptor JASMONIC ACID INSENSITIVE 1 (JAI1), the tomato ortholog of the ubiquitin ligase CORONATINE INSENSITIVE1 in A. thaliana is involved in this JA-mediated signaling cascade (Li et al. 2004; Bosch et al. 2014; Katsir et al. 2008; Kang et al. 2010). It remains to be investigated whether a synergistic effect between GA and JA signalling, similar to that observed in A. thaliana (Qi et al. 2014) also promotes glandular trichome development.

**Time course analysis of glandular trichome development coupled to cell type- and stage-specific expression data as a way to advance our understanding of glandular trichome development**

The development of multicellular glandular trichomes proceeds through the enlargement of single epidermal cells, followed by several cell divisions to generate a structure perpendicular to the epidermal surface (see Figure 2) and specific types of glandular trichomes seem to have a well-defined developmental plan (Tissier 2012a; Bergau et al. 2015). This highly regulated differentiation program also includes a polarized and localized cell wall lysis and remodelling (Bergau et al. 2015).

Within a given plant species, it would be interesting to dissect the developmental sequence of glandular trichome formation (including a time course analysis) and to sort the cells at specific stages using marker assisted cell sorting. The difficulty resides in the specific isolation of cells at early developmental stages. Recently, flow cytometry was used to specifically separate young and mature type VI trichomes from the wild tomato species S. habrochaites based on their distinct autofluorescence signals. This allowed the analysis of their transcriptomic and metabolomic profile in a cell stage specific way (Bergau et al. 2016).

Such systematic dissection of the development of glandular trichomes seems very promising but could be refined. Ideally, instead of autofluorescence (which may span various developmental stages), a series of trichome-specific transcriptional promoters driving the expression of a fluorescent marker (used as cell-stage marker) and closely associated to well defined developmental stages should be used. Such genetic resources are not yet available in the field of glandular trichome development. Focus should be set on identifying markers of entry into the glandular trichome pathway, as well as those labelling subsequent early differentiation steps. Some published gene promoters, like the one of AmMYBML3 (Jaffe et al., 2007), are already known to specifically label developing trichomes and could be used to drive the expression of a fluorescent reporter protein. Flow cytometry-assisted cell (or
nucleus) sorting would then permit to characterize the transcriptomic changes in a stage-specific way, in a similar manner as what was done to characterize stomatal development (Adrian et al. 2015). In an iterative fashion, the data could be mined to identify additional marker genes, so that only a few markers are necessary to initiate such transcriptomic studies.

As an alternative approach, ectopic overexpression of some transcription factors is known to induce glandular trichome development (Yang et al., 2015, Payne et al., 1999). Inducible overexpression of such transcription factors could be used in RNA-seq assays as a molecular switch to identify genes acting during early developmental stages (Yang et al., 2015) that could in turn be used as cell-stage markers. Further research is definitely needed to identify key genes driving the post-embryonic development of glandular trichomes, and to generate a molecular toolbox facilitating more applied genetic engineering approaches.

**Glandular trichome development: a diversity of model species!**

The field of glandular trichome development lacks a unique and robust model system. This is partly due to the difficulty of finding an appropriate model system. Glandular trichomes are extremely diverse in terms of shape, cell number, and type of secreted compounds, and may not be the result of a single evolutionary event (Serna and Martin 2006). This implies that their development may not be under similar transcriptional control in different plant families, or even within a single plant species between different trichome types (Serna and Martin 2006). The current view is that no single species can serve as a unique model to study the biology of glandular trichomes, but certain species or families of species progressively emerge as references for certain types of trichomes, such as Lamiaceae for peltate trichomes or Solanaceae for capitate trichomes as suggested in Tissier (2012a). So far, published data suggest that multicellular trichome formation probably occurs through different transcriptional regulatory networks than those regulating trichome formation in *A. thaliana*, so that mere orthologous relationships may not be inferred (Payne et al. 1999; Serna and Martin 2006; Yang et al. 2015; Liu et al. 2016).

**Turning glandular trichomes into chemical factories**

Plant specialized metabolites have been used for centuries as a source for fragrances and medicine. Since the discovery of their molecular structures and the elucidation of their biosynthesis pathways, breeders and chemists have been trying to select the best compounds by crossing species and varieties. For small molecules, chemical synthesis is another option...
once the structure of the molecule has been determined. However, the size and complexity of
the stereochemistry of some plant metabolites make their chemical synthesis extremely
complicated, and expensive.

The rise of molecular genetics and a better understanding of the genomes has changed
the way breeders work: they now use molecular genetic screening approaches to help them
select the best breeding candidates and descendants. This speeds up the selection process and
optimizes the breeding program.

Among plant specialized metabolites, terpenoids are the most abundant in term of
quantity and diversity (reviewed in (Croteau et al. 2000; Bouvier et al. 2005; Gershenzon and
Dudareva 2007)). Some of them are renowned not only for their economic value but also for
their molecular complexity.

Metabolic engineering of terpenoids emerged as a new method to produce naturally
occurring products. Microorganisms have been heavily used for the heterologous expression
of plant metabolites (reviewed by (Kirby and Keasling 2009; Keasling 2010; Marienhagen
and Bott 2013)), and nowadays plants have also become a host of interest for the heterologous
or homologous production of some plant specialized metabolites (reviewed by (Aharoni et al.
2005; Dixon 2005; Wu and Chappell 2008)).

The biosynthesis of isoprenoids in plants is quite unique and many reviews have
already covered the different aspects of their production and regulation (Bouvier et al. 2005;
Hemmerlin et al. 2012; Lipko and Swiezewska 2016). Plants synthesize the common
precursor for isoprenoids, isopentenyl diphosphate (IPP), and its allylic isoform dimethylallyl
diphosphate (DMAPP), by two distinct and compartmentalized pathways, the cytosolic
mevalonate (MVA) pathway and the plastidial methylerithritol 4-phosphate (MEP) pathway
(Figure 3). Specialized terpenoids are generally synthesized by either the MEP or the MVA
pathway depending on their length: sesquiterpenes (C15) and triterpenes (C30) mainly derive
from the MVA pathway, while monoterpenes (C10) and diterpenes (C20) derive from the
MEP pathway.

**Metabolic engineering in Nicotiana tabacum**

* N. tabacum is an interesting model system for metabolic engineering of terpenoid
compounds because it synthesizes an important pool of natural precursors (IPP/DMAPP) and,
besides the essential metabolites derived from the isoprenoid biosynthesis pathways, it
produces a very high amount of a limited range of specialized metabolites. These consist of a
few types of terpenoids, namely sesquiterpenes (Back and Chappell 1996; Starks et al. 1997, Ralston et al. 2001), as phytoalexins specifically produced in response to a pathogen attack (Stoessl et al. 1976), and diterpenes, constitutively synthesized and secreted by the head of glandular trichomes (Keene and Wagner 1985; Kandra and Wagner 1988; Guo and Wagner 1995; Wang and Wagner 2003) (Figure 3). Capsidiol, one of the main phytoalexin sesquiterpenes produced in response to a fungal attack, and cembratriendiol, one of the main diterpenes found in the cuticle, derive from the MVA and the MEP pathway, respectively (Huchelmann et al. 2014). Both types of metabolites are not required for the growth and development of the plants, and do not involve a complex metabolic pathway. Changing the fate of the metabolic fluxes normally used for sesquiterpenes and diterpenes production looks, in theory, quite simple. As the phytoalexin sesquiterpenes are only synthesized in response to a pathogen attack, the main pool of terpenoid precursors available under normal conditions is that involved in diterpene synthesis.

**Terpenoids engineering with constitutive and ubiquist promoters**

Metabolic engineering of tobacco plants to produce various terpenoids is widely described, and reviews already covered the methods and the final metabolic profiles (reviewed in (Verpoorte and Memelink 2002; Lange and Ahkami 2013; Moses et al. 2013)). Most of the engineered metabolites were synthesized using the original precursor pools, derived from the MEP pathway for mono- and diterpenes and from the MVA pathway for sesqui- and triterpenes. Promoters used to drive expression of the transgenes were ubiquist and constitutive. Although successful, the amount of metabolites produced in transgenic tobacco lines is usually relatively low (in the range of a few ng g\(^{-1}\) FW (Lücker et al. 2004; Wei et al. 2004; Farhi et al. 2011)), compared to the endogenous production of capsidiol (up to 100 µg g\(^{-1}\) FW (Dokládal et al. 2012)), or of diterpenes (up to 75 µg cm\(^{-2}\) depending on the variety (Severson et al. 1984)). To be economically viable, engineering of terpenoids in plant should reach yields comparable to those occurring naturally.

As mentioned previously, sesquiterpenes (C\(\text{15}\)) and triterpenes (C\(\text{30}\)) are considered to derive from the (cytosolic) MVA pathway while monoterpenes (C\(\text{10}\)) and diterpenes (C\(\text{20}\)) are derived from the (plastidial) MEP pathway. However, such a distinction is actually not so strict given the existence of a crosstalk between the MVA and MEP pathways, which consists of an exchange of prenyl diphosphates between the cytosol and the plastids (reviewed in (Hemmerlin et al. 2012)). More and more metabolites have been shown not to derive from a
strictly cytosolic or plastidial pool of precursors but the exact way the crosstalk works is still unclear (Box 2). As an example, sesquiterpenes can be synthesized using a plastidial pool of IPP (Dudareva et al. 2005; Bartram et al. 2006). Monoterpenes and diterpenes can also have mixed origins (Itoh et al. 2003; Wungsintaweekul and De-Eknamkul 2005; Hampel et al. 2007).

New strategies emerged for the engineering of terpenoids in plants, which consisted of targeting the overexpressed enzymes to different subcellular localizations to take advantage either of the MVA- or of the MEP-derived pool of precursors (Wu et al. 2006). Using such a strategy, engineering of sesquiterpenes and monoterpenes was monitored in tobacco (Wu et al. 2006). To produce sesquiterpenes (patchoulol or amorpha-4,11-diene), these authors expressed in tobacco the corresponding terpene synthase and a farnesyl diphosphate synthase (FDS) fused (or not) to a chloroplast targeting sequence to exploit either the MEP- or the MVA-derived pool of precursors, respectively. In this pioneering study, expression of the transgenes was driven by ubiquist promoters (a different one for each transgene). Addressing the enzymes to the cytosol only led to a low yield of sesquiterpenes (a few ng.g$^{-1}$ FW as in previous studies), while addressing the enzyme to the plastids (to take advantage of the MEP-derived pool of IPP/DMAPP) led to a much higher yield (up to 25 µg.g$^{-1}$ FW). Those impressive results are also quite surprising as sesquiterpenes are naturally synthesized in the cytosol while the engineered production is higher when the enzymes are localized in plastids.

Monoterpenes production in the cytosol was achieved using the same strategy to express the monoterpene synthase and the geranyl diphosphate synthase. However, in this case, the localisation of the enzymes seemed to be less important, as both engineering strategies (using the MVA- or the MEP-derived precursors) led to roughly the same amount of R-(+)-limonene (400-500 ng.g$^{-1}$ FW) (Wu et al. 2006).

Modifying the fate of isoprenoid precursors can lead to severe phenotypes, which might be expected given the importance of isoprenoids in plant growth and development, and the fact that expression was ubiquist. Tobacco lines producing the highest quantity of sesquiterpenes were indeed severely affected: they exhibited chlorosis and dwarfism. This phenotype was also observed in metabolic engineering of the ginsenoside saponin in tobacco (Gwak et al. 2017). In this case, production of the saponin led to a severe phenotype, including dwarfism, change in flower and pollen morphology and impaired seed production (Gwak et al. 2017). Similarly, dwarfism was observed upon metabolic engineering of tobacco chloroplast to produce artemisinic acid (Saxena et al. 2014). All these approaches used ubiquist promoters to drive expression of the transgenes. Because of the similarity of the
phenotypes observed (dwarfism, chlorosis, decreased seed production) between the different reports, cytotoxicity of the new metabolites is probably not the only cause. The problem could lay in the constitutive expression of transgenes, which might result in plant depletion of its essential terpenoid precursors (IPP/DMAPP). For plant metabolic engineering to be efficient, there is a necessity to better control the spatio-temporal expression of the transgenes. One strategy to limit the effect of sinking essential IPP pools consists of specifically targeting the expression of the genes in a cell type-specific way. In this respect, glandular trichomes are an ideal expression system.

**Terpenoids engineering specifically in trichomes**

From a metabolic point of view, glandular trichomes are of particular interest, as they are involved in the synthesis, storage, and/or excretion of specialized metabolites, making these compounds easily available. The carbon metabolism of glandular trichome in Solanaceae has evolved to support high metabolite production (Balcke et al. 2017). Some genera, such as Nicotiana, produce up to 15% of their leaf biomass in the trichomes (Wagner et al. 2004). The C_{20} terpenoids are exported to diffuse within the cuticle and mediate resistance to insects and fungi (Chang and Grunwald 1976; Severson et al. 1984; Wang and Wagner 2003; Sallaud et al. 2012). Rerouting the diterpenes production from cembrane and labdane types (naturally produced in tobacco) to other diterpenes has been performed by addressing the terpene synthase directly to the plastids with a trichome specific promoter.

**Diterpenes engineering**

One of the best examples is the heterologous production of taxadiene in trichomes of wild tobacco, *Nicotiana sylvestris* (Rontein et al. 2008; Tissier et al. 2012). Taxadiene, particularly taxa-4(5),11(12)-diene, is the precursor of Paclitaxel, a potent anti-cancer diterpene usually extracted from the bark of *Taxus* species (Hezari et al. 1995; Koepp et al. 1995). Several groups have already tried to synthesize this precursor ubiquitously in plants (reviewed in (Soliman and Tang 2015)), but the production of the diterpene triggered growth defects in tomato (Kovacs et al. 2007), and a lethal phenotype in *A. thaliana*, most probably because of an over-used GGPP pool for diterpene synthesis (Besumbes et al. 2004). As a result, the MEP-derived primary metabolites were not synthesized in sufficient amounts to sustain normal growth. Only few attempts led to the production of taxadiene in plants without
any defect like in ginseng (Cha et al. 2012) and *Nicotiana benthamiana* (Hasan et al. 2014) by
the ubiquitous overexpression of the taxadiene synthase, but the amount produced was quite
low.

Expression of taxadiene synthase specifically in *N. tabacum* trichomes, using the
transcriptional promoter of cembratrien-ol synthase (CBTS) led to the production in the
exudate of 5-10 µg/g fresh weight of taxadiene, representing only 10% of the total taxadiene
production, suggesting a problem of excretion (Tissier et al. 2012; further discussed in Box 3).
However, the amount produced is impressive compared to the 27 µg/g dry weight obtained in
the best stable transgenic *N. benthamiana* line (Hasan et al. 2014). Hasan et al. (2014) could
increase the production in *N. benthamiana* up to 48 µg/g dry weight by, in addition to
constitutively overexpressing the taxadiene synthase, silencing the expression of the gene
coding for the phytoene synthase, and thereby increasing the GGPP availability. In *N.
tabacum*, silencing the genes coding for CBTS did not increase the production of taxadiene
(Tissier et al. 2012). Compared to the cultivated tobacco (*N. tabacum*), the wild tobacco (*N.
sylvestris*) naturally synthesizes, as diterpenes, cambranoids but no labdanoids. Using the
CBTS promoter to drive the expression of the 8-hydroxy-copalyl diphosphate synthase and the
Z-abienol synthase specifically in *N. sylvestris* trichomes, synthesis of the labdanoid, Z-
abienol, reached a yield of 30 µg·g⁻¹ FW, without any major impact on the production of
cembranoids, nor on the plant development (Sallaud et al. 2012). Similarly, casbene, another
diterpene from *Ricinus communis*, was also produced in *N. tabacum* trichomes (Tissier et al.
2012), demonstrating that these structures can really be used as a biofactory for the production
of a diverse set of diterpenes. While most of the cambrane and labdane diterpenes are
normally found in the cuticle or in the exudate, only a small fraction (10%) of casbene or
taxadiene was found in the exudate, suggesting the necessity of associating a transporter when
engineering a metabolic pathway (Box 3).

**Triterpenes engineering**

Could *N. tabacum* trichomes be used as a production platform for terpenoids other
than diterpenes? In *N. tabacum* trichomes, the main isoprenoid-derived metabolites are
diterpenes from the MEP-pathway. Metabolic engineering in trichomes might take advantage
of this crosstalk to produce, for example, triterpenes instead of the naturally occurring
diterpenes by diverting the plastidic pool of IPP towards the cytosol to produce the
compounds of interest (Figure 4). Some plants, such as *Solanum habrochaites*, produce some
sesquiterpenes directly in the plastids. The corresponding enzymes, involved in the biosynthesis of those C_{15}, a sesquiterpene synthase and a farnesyl diphosphate synthase, evolved to be localized to the plastids (Sallaud et al. 2009). Engineering the production of MVA-derived metabolites such as sesquiterpenes (C_{15}) or triterpenes (C_{30}) in the plastids using specifically MEP-derived IPP and DMAPP precursors may require the corresponding enzymes (normally localized to the cytosol or to the ER) to be engineered to get targeted to the plastids (Figure 5).

Triterpenes, the C_{30} family, are of particular interest for their substantial carbon content, making them good targets for biofuel production (Gübitz et al. 1999; Khan et al. 2014) as well as for pharmacological purposes. The biosynthesis of triterpenes requires several enzymatic steps (reviewed in (Phillips et al. 2006; Thimmappa et al. 2014)). The production of squalene is the limiting regulatory step to produce triterpenes. Modifying the production and fate of squalene, a key precursor of phytosterols, is risky if this modification affects the whole plant as it can lead to dwarfism and loss of fertility (reviewed by (Clouse 2002; Schaller 2003, 2004)). Using the CBTS promoter, the production of squalene was achieved in *N. tabacum* trichomes (Wu et al. 2012). The production was greater when the two enzymes for squalene production, squalene synthase and farnesyl diphosphate synthase, were targeted to the chloroplast. Even though the expression of the transgenes was expected to be restricted to trichomes, those transgenic plants that expressed squalene at the highest level displayed strong phenotypes such as dwarfism and chlorosis, which is similar to the phenotypes observed upon ubiquist expression of transgenes impacting the pool of IPP precursors (Wu et al. 2006; Saxena et al. 2014; Gwak et al. 2017).

Using the same approach, the production of linear triterpenes typical of the green algae *Botryococcus braunii* was achieved in *N. tabacum* trichomes (Jiang et al. 2016). Once again, the best production rate was when the enzymes were targeted to the plastid, which suggests that the best strategy for triterpene production is to exploit the plastid pool of IPP/DMAPP. However, as for squalene production (Wu et al. 2012), the synthesis of triterpenes led to chlorosis and dwarfism. Plastidial membranes are quite fragile and the balance of sterols is important for the stability of the plastids (Babiychuk et al. 2008). Thus, the production of squalene or other triterpenes in the plastids might disrupt the chloroplast integrity and lead to chlorosis. Since trichomes are thought to be dispensable, the breakdown of chloroplasts is not expected to have major consequences on the whole plant. A possible explanation for this phenotype is that the promoter was not completely trichome specific, probably due to the presence of 35S enhancers upstream of the promoter (Wu et al. 2012; Jiang et al. 2016) or to
some positional effects of the transgene affecting the specificity of the promoter. Cell type
specificity of the transgene(s) expression should therefore be strictly ascertained in transgenic
lines, and not solely based on the assessment of cell-type specificity of the promoter via the
analysis of transcriptional reporter lines. Another plausible explanation to such phenotype
could be that, although trichomes are not essential, they may transmit a stress signal to other
leaf tissues leading to the observed phenotypes in the transgenic lines (Wu et al. 2012).

Metabolic engineering in tobacco has great potential. The amount of terpenoids that
the plant can naturally produce is impressive. Using the plastidial pool for production of
terpenoids seems much more efficient than using the cytosolic one (Wu et al. 2006). However, ubiquist expression alters plant development and directly impairs the yield. To be
economically viable, the yield of terpenoids should be increased with no or only a slight
impact on the plant phenotype. Lack of excretion of the potentially cytotoxic metabolites is
another issue (discussed in Box 3). In addition, comprehension of the exchange of prenyl
diphosphates between the plastids and the cytosol (Box 2) should also be investigated so as to
reroute the plastidic flux towards the cytosol, and avoid disrupting the stability of the
chloroplast with a change in sterol profile (Babiychuk et al. 2008).

Conclusion

Nowadays, plant biologists are trying to go beyond the A. thaliana model and to move
to non-model plant species. In this respect, the study of glandular trichome biology is greatly
benefitting from such change of model system. Recent advances in DNA sequencing, –omics
technology, and reverse genetics, including plant genome editing now offer new technical
resources to investigate such biological aspects in a wide variety of species.

Our current understanding of both the development of glandular trichomes and of the
biosynthetic pathways going on in these structures is improving but at this point is still quite
fragmentary. However, an increasing number of research groups are now focusing on various
aspects of glandular trichome biology including developmental aspects and bioengineering.

Understanding the way glandular trichomes develop to finally turn into highly
efficient biochemical factories in the epidermis of non-model plant species is of key
importance and could lead to more applied outcomes. This calls for basic research to address
those fascinating aspects. It is now time to consider the real biotechnological potential of
glandular trichomes as biochemical factories, and use up-to-date technology to fully exploit
the cellular machinery. Increased knowledge on these fundamental aspects will in the mid-
term allow researchers to tap the up-to-now largely unexploited biotechnological potential of
glandular trichomes to engineer plants that would exhibit increased resistance to pests or that would produce compounds of immense industrial/pharmaceutical interest (molecular pharming).

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**Figure legends:**

Figure 1: Glandular trichome initiation and development, a process with many unknowns.

A differentiating protodermal cell integrates both environmental and endogenous signals. Such signal integration results in the selection of a pool of trichome cell precursors which will initiate a specific developmental program. In these trichome initials, cell-specific transcriptional control of gene expression and cell cycle regulation results in the onset of a controlled cell division and trichome morphogenesis program, most of which is still not so well understood in the case of glandular trichomes. It probably also involves some cell-cell signaling promoting the “one cell-spacing rule” which allows a specific patterning of trichomes in the epidermis. Morphogenesis of the trichome glandular head also necessitates extensive remodeling of the cell wall. The extent of endoreduplication in glandular trichomes is still mostly uncharacterized. The illustration shows a modified confocal picture of a long glandular trichome initial from *Nicotiana tabacum*. Chloroplasts are shown in green, propidium iodide-stained cell walls in magenta, and nuclei in cyan.

Figure 2: Glandular trichome initiation and development in *Nicotiana tabacum*.

A-F/ Confocal microscopy pictures showing the early steps of glandular trichome development. The number of cells forming the developing glandular trichome is shown at the bottom of each frame. A differentiating protodermal cell enlarges and forms a protuberance (A), the cell nucleus migrates to the tip of the protuberance (B), cell division takes place (C) forming a structure made of two cells (D). The upper cell protruding from the epidermis then undergoes an asymmetric division forming one large cell (which will form the multicellular stalk after several rounds of controlled cell division) and one small cell (which will give rise to the multicellular glandular head) (E). A developing trichome made of five cells is shown in
(F). Magenta: cell wall (propodium iodide staining), Cyan: nuclei (DAPI staining), Green: chloroplasts (Chlorophyll a autofluorescence).

G/ Scanning electron micrograph showing the typical cell architecture of a mature long glandular trichome.

**Figure 3: Isoprenoid metabolism in *Nicotiana tabacum* cells.**
The red square represents the major MEP isoprenoid metabolism in plastids of developed trichomes. Phytohormones are indicated in blue and specialized metabolites in violet. MEP pathway, methylerthritol phosphate pathway; MVA pathway, mevalonate pathway; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid.

**Figure 4: Terpenoid metabolism in engineered tobacco trichomes for triterpene production in the cytosol.**
The gray pathway represents the normal biosynthetic route for triterpenes and sterols. The enzymes are targeted to the cytosol to enhance the crosstalk and sink the plastids from its precursors. Overexpressed enzymes are denoted in dark blue. New products deriving from the engineering metabolism are denoted in light blue. MEP pathway, methylerthritol phosphate pathway; MVA pathway, mevalonate pathway; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FDS, FPP synthase; SQS, squalene synthase; SQE, squalene epoxidase; OSC, 2,3-oxidosqualene cyclase; CYP450, cytochrome P450

**Figure 5: Terpenoid metabolism in engineered tobacco trichomes for triterpene production in plastids.**
The gray pathway represents the normal biosynthetic route for triterpenes and sterols. The enzymes are targeted to directly produce the triterpenes in the plastids. Overexpressed enzymes are denoted in dark blue. New products deriving from the engineering metabolism are denoted in light blue. Sinking plastidial isoprenoid pool might provoke undesirable consequences. Potentially affected metabolites are denoted in orange. MEP pathway, methylerthritol phosphate pathway; MVA pathway, mevalonate pathway; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FDS, FPP synthase; SQS,
squalene synthase; SQE, squalene epoxidase; OSC, 2,3-oxidosqualene cyclase; CYP450, cytochrome P450; ABA, abscisic acid.

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Figure 1: Glandular trichome initiation and development, a process with many unknowns.

A differentiating protodermal cell integrates both environmental and endogenous signals. Such signal integration results in the selection of a pool of trichome cell precursors which will initiate a specific developmental program. In these trichome initials, cell-specific transcriptional control of gene expression and cell cycle regulation results in the onset of a controlled cell division and trichome morphogenesis program, most of which is still not so well understood in the case of glandular trichomes. It probably also involves some cell-cell signaling promoting the “one cell-spacing rule” which allows a specific patterning of trichomes in the epidermis. Morphogenesis of the trichome glandular head also necessitates extensive remodeling of the cell wall. The extent of endoreduplication in glandular trichomes is still mostly uncharacterized. The illustration shows a modified confocal picture of a long glandular trichome initial from *Nicotiana tabacum*. Chloroplasts are shown in green, propidium iodide-stained cell walls in magenta, and nuclei in cyan.
Figure 2: Glandular trichome initiation and development in *Nicotiana tabacum*.

A-F/ Confocal microscopy pictures showing the early steps of glandular trichome development. The number of cells forming the developing glandular trichome is shown at the bottom of each frame. A differentiating protodermal cell enlarges and forms a protuberance (A), the cell nucleus migrates to the tip of the protuberance (B), cell division takes place (C) forming a structure made of two cells (D). The upper cell protruding from the epidermis then undergoes an asymmetric division forming one large cell (which will form the multicellular stalk after several rounds of controlled cell division) and one small cell (which will give rise to the multicellular glandular head) (E). A developing trichome made of five cells is shown in (F). Magenta: cell wall (propodium iodide staining), Cyan: nuclei (DAPI staining), Green: chloroplasts (Chlorophyll a autofluorescence).

G/ Scanning electron micrograph showing the typical cell architecture of a mature long glandular trichome.
Figure 3: Isoprenoid metabolism in *Nicotiana tabacum* cells. The red square represents the major MEP isoprenoid metabolism in plastids of developed trichomes. Phytohormones are indicated in blue and specialized metabolites in violet. MEP pathway, methylerythritol phosphate pathway; MVA pathway, mevalonate pathway; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid.
Figure 4: Terpenoid metabolism in engineered tobacco trichomes for triterpene production in the cytosol.

The gray pathway represents the normal biosynthetic route for triterpenes and sterols. The enzymes are targeted to the cytosol to enhance the crosstalk and sink the plastids from its precursors. Overexpressed enzymes are denoted in dark blue. New products deriving from the engineering metabolism are denoted in light blue. MEP pathway, methylerythritol phosphate pathway; MVA pathway, mevalonate pathway; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FDS, FPP synthase; SQS, squalene synthase; SQE, squalene epoxidase; OSC, 2,3-oxidosqualene cyclase; CYP450, cytochrome P450.
Figure 5: Terpenoid metabolism in engineered tobacco trichomes for triterpene production in plastids.
The gray pathway represents the normal biosynthetic route for triterpenes and sterols. The enzymes are targeted to directly produce the triterpenes in the plastids. Overexpressed enzymes are denoted in dark blue. New products deriving from the engineering metabolism are denoted in light blue. Sinking plastidial isoprenoid pool might provoke undesirable consequences. Potentially affected metabolites are denoted in orange.

MEP pathway, methylerythritol phosphate pathway; MVA pathway, mevalonate pathway; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; FDS, FPP synthase; SQS, squalene synthase; SQE, squalene epoxidase; OSC, 2,3-oxidosqualene cyclase; CYP450, cytochrome P450; ABA, abscisic acid.
OUTSTANDING QUESTIONS

- How diverse is the transcriptional control of glandular trichome development in plants? Is there a single transcriptional network at least partially shared between different types of glandular trichomes, or did these structures evolve independently in different (groups of) species (via some evolutionary convergence)?

- Can we engineer trichomes to produce potentially toxic compounds without consequences for the rest of the plant? This also raises the question of finding appropriate gene promoters to drive the different transgenes in plants. Special emphasis should be set on identifying strictly cell-type specific promoters to avoid undesirable effects on plant growth and development.

- To which extent can we exploit the existing crosstalk between the plastidial and cytosolic isoprenoid biosynthesis pathways? Are the cytosolic and plastidial pools of precursors somehow connected via these crosstalks so that depletion of one pool could be compensated by the other one, or are they rather independent?

- How important is it to include transporters in metabolic engineering approaches to help secrete out the compounds of interest (thereby avoiding cytotoxicity) or channel the precursors to the appropriate cell compartment?

- What about the primary metabolism occurring in glandular trichome cells that supports such an intense secondary metabolism? What are the carbon, nitrogen, and energy sources? How do they reach the trichome head cells?

ADVANCES

- Given the recent progress in –omics technology and genome editing (CRISPR/Cas9), the time has come to investigate up-to-now overlooked aspects of glandular trichome biology in non-model species down to a molecular level. For example, given these technical advances, the study of the molecular genetic aspects governing glandular trichome development has now been greatly facilitated in numerous species.

- Advances in mass spectrometry now allow the simultaneous determination of hundreds of metabolites in a small number or even in single cells, with a sensitivity in the attomole ($10^{-18}$ mole) range. Such advances in metabolic profiling are highly interesting for glandular trichomes that, depending on the plant species and trichome type, may not always be easy to isolate for chemical analysis.

- Metabolic engineering of specialized metabolites in glandular trichomes still faces many experimental challenges, including ways of efficiently dealing with the potential cytotoxicity of the synthesized molecules (for example via the expression of some specific ABC transporters), the necessity to limit the negative impact of such engineering at the whole plant level (via the use of cell type-specific gene promoters).

- Importantly, the bioavailability of the pool of precursors to be used needs to be addressed, as those precursors are sometimes stored in a different subcellular compartment (e.g., the cytosolic vs plastidial pools of isoprenoid precursors). This could necessitate the identification/cloning of appropriate transporters between the plastidial envelope and the cytosol to facilitate the exchange of precursor molecules between these compartments.
Box 1: Trichome metabolites are involved in plant defense

Our understanding of the metabolomic profiles of glandular trichomes is greatly increasing (Aschenbrenner et al. 2015; Bombo et al. 2016; Spring et al. 2015; Wang et al. 2015; Muravnik et al. 2016; Venditti et al. 2016). Recently, technological advances have even made it possible to obtain the metabolic profile of a single trichome cell (Nakashima et al. 2016). However, the exact role of glandular trichomes in mediating plant interactions with their environment has only started to be investigated at a molecular level.

Terpenoids are the most abundant class of metabolites found in trichomes and have important properties in terms of plant defense and interaction with herbivores (Glas et al. 2012). In petunia, the production of insecticidal steroids is important for plant defense, as silencing of a trichome-specific steroid transporter increased the sensitivity to pests (Sasse et al. 2016). Metabolic engineering of terpenoids in tomato trichomes can also increase the resistance of plants to phytopathogens (Bleeker et al. 2012).

Methylketones are a class of medium length carbon chain molecules that are involved in the defense against pests (Williams et al. 1980; Maluf et al. 1997). While Solanum habrochaites, a wild tomato, produces a large amount of those metabolites in its trichomes, those molecules are almost absent in their cultivated counterpart, Solanum lycopersicum. Engineering the cultivated tomato trichomes to efficiently produce methylketones could lead to an increased resistance to pests. The trichome-specific expression of genes coding for two methylketone synthases from S. habrochaites, the enzymes involved in methylketone biosynthesis (Yu et al. 2010), only led to a small increase in the methylketone content in S. lycopersicum (Yu and Pichersky 2014).

Acyl sugars are produced in the glandular trichomes of most species of the Solanaceae family (Kroumova and Wagner 2003; Luu et al. 2017). In tomato and wild tomato, these molecules render the leaf sticky and, by physically trapping pests, play a role in defense against herbivorous attacks (Puterka et al. 2003; Simmons et al. 2004). In Nicotiana attenuata, the role of acyl sugars is totally different as these trichome-derived compounds attract the moth Manduca sexta, the larvae of which feed on the wild tobacco. Frass from the larvae contain volatile acyl chains hydrolyzed from the acylsugars that in turn attract Pogonomyrmex rugosus, one of their natural predators (Weinhold and Baldwin 2011). These compounds also protect the plant from pathogenic fungi (Luu et al. 2017).

Flavonoids are metabolites synthesized in the glandular trichomes of various species (Tattini et al. 2000; Valkama et al. 2003; Liakopoulos et al. 2006; Spring et al. 2015), and have been shown to also play a role in plant defense. Rather than a protection against pathogens, these flavonoids are used as a protection against UV irradiation in plants exposed to high solar radiation, such as olive (Olea europaea), sunflower (Helianthus annuus) and the Mediterranean plant Phillyrea latifolia (Tattini et al. 2000; Liakopoulos et al. 2006).

The metabolites produced in plant trichomes thus fulfill a variety of roles in plants that all contribute to increasing the fitness of the plant to the environment, thereby increasing plant survival.
Box 2: The crosstalk between isoprenoid biosynthesis pathways

Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the common precursors of the two isoprenoid biosynthetic pathways (the mevalonate, MVA, and the methyerythritol phosphate, MEP, pathways) in plants. Several chemical and biochemical analyses already demonstrated that the crosstalk involves the exchange of IPP/DMAPP (Hemmerlin et al. 2003; Bick and Lange 2003; Flügge and Gao 2005; Jozwiak et al. 2017). Also geranylgeranyl diphosphate synthesized in the plastids is used for protein geranylgeranylation in the cytosol by the protein geranylgeranyltransferase type-I (Gerber et al. 2009). Geranyl diphosphate synthesized in the plastids can exit in the cytosol where it is converted into farnesyl diphosphate (FPP) by FPP synthase (Adam et al. 1999) or is directly used by terpene synthases to produce monoterpenes (Gutensohn et al. 2014). Farnesol or one of its derivatives has been shown to inhibit the MEP pathway, and is so expected to cross the plastid envelope (Huchelmann et al. 2016). Even though the MEP pathway was discovered in plants in the early 1990’s (Arigoni et al. 1997; Schwarz 1994), reviewed by (Rohmer 2003) and the crosstalk, roughly ten years later (Hemmerlin et al. 2012; Hemmerlin et al. 2003), the precursor exchange mechanism has not been discovered yet. To fully understand the importance of the crosstalk and use its features for metabolic engineering purposes, the identification and characterization of proteins involved in transport is needed.

Box 2 Figure: Transport of terpenoid precursors through the plastid envelope.

Exchange of isopentenyl diphosphate (IPP) and/or dimethylallyl diphosphate (DMAPP) between the cytosolic and plastid pathways is possible via an unknown mechanism. Other exchanges have been shown to take place, although the transporters have not been identified. The C10 geranyl diphosphate (GPP) and the C20 geranylgeranyl diphosphate (GGPP) are known to exit the plastids. Farnesol (Fol), a C15 molecule, or one of its metabolites (symbolized by a question mark) is known to enter the plastids. Other abbreviations are FPP, farnesyl diphosphate; FDS, FPP synthase; MEP, methyerythritol phosphate; PGGT-I, protein geranylgeranyltransferase type I; Protein-GG, geranylgeranylated protein.
Box 3: The input of metabolite transporters

Terpenoids can be cytotoxic, and because of this most are usually secreted out of the cells, but only a few studies have reported on how these metabolites get transported. Although they are expected to easily diffuse across membranes, active transport might still be required to keep the internal concentration as low as possible to avoid toxicity (Adebesin et al., 2017; Widhalm et al. 2015). *Nicotiana plumbaginifolia* trichomes secrete large amounts of diterpenes involved in the biotic stress response. Pleiotropic Drug Resistance (PDR) 1, an ATP-binding cassette (ABC) transporter localized to the plasma membrane was shown to be involved in diterpene extrusion (Jasinski et al., 2001; Crouzet et al., 2013). The secretion of diterpenes by tobacco trichomes seems not only to be dependent on PDR1 but also on another class of proteins, the lipid transfer proteins (LTPs). *N. tabacum* plants over-expressing *NilTP1* have a greater content of cembratriene-diol in the leaf exudate, while the *NilTP1*-silenced line has a slightly lower content of this diterpene in the leaf exudate (Choi et al. 2012). As another example, Wang et al. (2016a) transiently expressed the metabolic pathway of artemisinin, a sesquiterpene, in *Nicotiana benthamiana*. The expression of both a PDR transporter and an LTP resulted in the increased secretion of artemisinin precursors. Recent studies in yeast and mammalian cells showed that cholesterol is transported through the cytosol, from the endoplasmic reticulum to the plasma membrane, by LTPs (reviewed by Mesmin and Antonny 2016).

*Petunia hybrida* produces insecticidal molecules such as petuniasterone and petuniolide (Elliger et al. 1988; Elliger et al. 1990). A screening for *P. hybrida* PDR genes expressed in trichomes showed that *PhPDR2* is mainly expressed in the trichomes and leaf margin. A metabolite screening on plants silenced for *PhPDR2* showed a link between the transporter and the accumulation of the steroidal compound as well as herbivore sensitivity (Sasse et al. 2016). Although direct transport of the steroids by PDR2 has not been shown, the transporter has a role during the response to herbivores and the mobilization of the metabolites in the trichomes and leaf margins. Another ABC transporter, PhABCG1, was recently found to actively mediate the passage of volatile compounds across the plasma membrane of petunia petals (Adebesin et al., 2017).

The role of PDR in import or export of metabolites was also investigated in *Artemisia annua*. T-shaped glandular trichomes from the sweet wormwood are involved in the synthesis and accumulation of β-caryophyllene, a sesquiterpene. In this species, AaPDR3, an ABC transporter, was shown to actively transport sesquiterpene (Fu et al. 2017).

The identification of secondary metabolite transporters is only at its beginning, and many transport mechanisms still need to be investigated. But based on the recent studies, it can be hypothesized that combining the appropriate LTPs and plasma membrane transporters to trichomes producing squalene and/or triterpenes in the plastids, or overproducing metabolites in the cells, would help prevent cytotoxicity. As a prerequisite, appropriate transporters and LTPs will have to be identified.


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