Defence from the wild

Specialised metabolism in tomato glandular trichomes

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Chapter 8

General Discussion

Foreword

The historically termed "secondary metabolites" were brought to attention by Fraenkel in 1959, stating their function in plant-insect interactions. When it became clear that these metabolites are not merely waste products of "primary metabolism", but are synthesised for a specific purpose, such as defence, the term "specialised metabolites" was introduced (Pichersky and Lewinsohn, 2011). Metabolite-based defence strategies evolved in many higher plants. Various species developed dedicated tissues containing glandular cells primarily producing specialised metabolites that are secreted and/or stored in specialised organelles or structures (**Chapter 2**). The estimated number of different specialised metabolites produced by plants is overwhelming (>200,000 different structures; Yonekura-Sakakibara and Saito, 2009; Pichersky and Lewinsohn, 2011) and our knowledge regarding their biological function or biosynthesis spans only a fraction of that. In this thesis I have zoomed in on glandular trichomes of wild tomato, of which some appear to be involved in insect resistance. I tried to shed light on the biological role and biosynthesis of specialised metabolites, with a focus on terpene synthesis by type-VI trichomes. The accumulation of terpenes seems restricted in trichomes of *Solanum lycopersicum* compared to trichomes of some *S. habrochaites* accessions. On leaves of these wild tomatoes, a single trichome can accumulate 35 times more mono-and sesquiterpenes than a cultivar trichome (**Chapter 6**) and can add up to 12% of the leaf's dry weight (Frelichowski Jr, J. E. and Juvik, 2005). Although glandular trichomes are "dedicated tissues", the synthesis and storage of specialised metabolites seems highly controlled. In the following paragraphs I will discuss how our findings contribute to our understanding of specialised metabolism in glandular trichomes and assess the bottlenecks to overcome the implementation in breeding for insect resistance.

Natural variation as a tool for metabilte selection

The use of comparative metabolomics can be a first step to disentangle chemotype-phenotype relationships, and this has resulted in the discovery of multiple plant-signalling compounds in the past (Kuhlisch and Pohnert, 2015). One of the ideas that came up during the work on this thesis was to build a toolbox with a potentially more generic applicability. We chose a broad panel of wild tomato accessions with the idea that datasets could be interchanged and re-used to identify other metabolite-pest relationships. In collaboration with industrial partners, we screened the 19 accessions used in **Chapter 3** for *Phytophthora infestans* and *Botrytis cinerea* resistance which resulted in completely different resistance scores than found for thrips or whitefly (unpublished data). Applying the random forest (RF) pipeline using the existing acylsugar and volatile terpene data may identify defence metabolites against these micro-organisms. Additionally, the insect-phenotype data of the accessions in **Chapter 3** could also be implemented in the search for non-trichome defence metabolites. Leaf-based metabolites, for example, have been suggested to play a role in resistance against thrips (Bac-Molenaar *et al.*, 2019) and specialised metabolites secreted in the phloem could potentially affect phloem-feeding whitefly (Will, Furch, Zimmermann, 2013). After measuring such additional metabolites the RF-pipeline could be applied on the existing phenotypic datasets. It should be noted that this approach only addresses the constitutive defences of the tomatoes as herbivory-induced changes are not considered. Also, plants grown during different experiments may produce altered specialised metabolite profiles, due to variation in environmental conditions (Escobar-Bravo *et al.*, 2018). However, as trichomes are the plant's first line of defence they should be armed before

insect arrival and one would therefore expect defence metabolites to be present under changing circumstances.

Natural variation is commonly used in association studies linking metabolites to other plant characteristics like (resistance) phenotypes or genotypes (Meihls *et al.*, 2013; Chen *et al.*, 2014). In our studies, the broad metabolic diversity was intended to increase the chance of identifying a toxic metabolite and to reduce false-positive metabolite-phenotype correlations through undetected or co-occurring metabolites. However, high diversity in metabolite profiles with only a limited number of samples can lead to reduced statistical power also known as "the curse of dimensionality" (Bellman, 1957; Smilde *et al.*, 2013). As extensively discussed in **Chapter 3**; the choice of germplasm is crucial. In hindsight, the biochemical diversity in trichomes of the accessions used in that chapter was probably too high in relation to the number of accessions, especially regarding their volatile profiles of resistant accessions. Illustrative are the rather unique volatile profiles of the three most resistant accessions LA0716, PI127826 and LA1777 (**Chapter 3**, Fig. 3B). Reducing the number of wild species e.g., by selecting only *S. habrochaites* and cultivated accessions, may create more overlap in profiles of the resistant class, providing the power to calculate sound relationships. By using a segregating population from parents with contrasting phenotypes, as done for whitefly in **Chapter 4**, we indeed reduced the qualitative variation in volatile profiles while still having a genetically diverse set of plants. Indeed, this resulted in a RF-model of higher accuracy and volatiles associated with whitefly resistant class **(Chapter 3**, Fig. 5; **Chapter 4**, Fig 3). After predicting an association, the role of metabolites should always be validated. In our case, we developed a controlled bioassay using purified compounds to validate metabolite toxicity (**Chapter 5,** Fig. 6) and additional evidence was collected by having plants lacking the functional allele of the biosynthetic enzyme (**Chapter 5**, Fig. S17).

Specific functional groups determine the action of a metabolite

As discussed in **Chapter 2**, while there are many reports on the insecticidal properties of essential oils and plant extracts, there is only limited information about the individual biologically active compounds. With a handful of effective molecules now identified, the question is raised whether there are particular features of the (specialised) metabolites that exert a toxic effect. As noticed in in **Chapter 3**, the two acylsugars predicted to have a negative effect on whitefly survival are mainly composed of 5-carbon side branches; S3:15 (5,5,5) and S3:21 (5,5,11). Our findings were in line with the results of Leckie *et al.* (2016) who found that acylsugar extracts of tomato trichomes containing mainly of 5-carbon fatty-acid branches (i.e. 3-methylbutanoic acids) particularly reduced whitefly fecundity (Leckie *et al.*, 2016) . While the exact mode of action of acylsugars is still unknown, early observations on the physiology of insects sprayed with acylsugar exudates, resulted in the hypothesis that acylsugars cause a disruption of the insect cuticle or membranes (Neal Jr *et al.*, 1994; Puterka and Severson, 1995). The combination of sugar moiety, number of fatty-acid chains and their length determines the final hydrophilic-lipophilic balance (HLB) of the molecule and together these determine the efficiency of the acylsugar to integrate and disrupt cellular membranes or cuticle structures (Egan, 1976; Zheng *et al.*, 2015). As the optimal HLB also depends on the composition of the encountered matrix, i.e. the insect surface, the efficacy of acylsugars is expected to vary for the

insect species and/or life stage. Having the ability to counteract multiple different herbivores and micro-organisms may explain why tomato accessions invest in the production of such a wide array of different acylsugars (**Chapter 3** and others e.g. Lybrand *et al.*, 2020), and might be the driving force behind the divergent evolution of acyltransferases that has occurred in Solanaceae species (Fan, Leong, Last, 2019). Testing fractionated acylsugar extracts followed by insect bioassays would be a great first step to shed light on the bioactivity of individual acylsugars. In addition, the mode of action regarding membrane integrations could be tested, for example on insect cell cultures followed by membrane integrity assays. Separating acylsugars from the complex mixture as found in trichome extracts is not trivial due to their similar structures, and our first attempts using silica columns were unsuccessful. Separation using a preparative HPLC system could constitute a follow-up approach and provide both the information and material to perform biological experiments with individual acylsugar moieties. Additionally, using introgression lines predominantly producing a subset of acylsugar types (Leckie, De Jong, Mutschler, 2012; Smeda *et al.*, 2016; Ben-Mahmoud *et al.*, 2018) or acyltransferases mutants could help establish a relationship between specific acylsugars and toxicity. These genetic approaches are nevertheless challenging as the enzymes in acylsugar biosynthesis show a high degree of enzymatic promiscuity (Moghe *et al.*, 2017; Fan, Leong, Last, 2019).

Interestingly, it appears that volatiles with toxic properties are often modified (sesqui)terpenes or other oxygenated hydrophobic hydrocarbons. In this thesis, we described the toxic effects of 9H10epoZ while this was not found for 7-epizingiberene (**Chapter 5**). Similarly, the carboxylic acid derivatives of santalene and bergamotenes (SCAs) were shown to display toxic properties to lepidopteran larvae (Frelichowski Jr, James E. and Juvik, 2001). Preliminary experiments show that SCAs are also toxic to whiteflies (Figure 1A) while their survival seems not to be affected by their unmodified santalenes/bergamotene precursors (Therezan *et al.*, 2021). Other oxygenated terpenoids that exert a validated toxic effect on insects include the diterpene 17-hydroxygeranyllinalool and triterpene cucurbitacin-C as discussed in **Chapter 2** and the sesquiterpene aldehyde polygodial (Hardie et al., 1992). In addition, the fatty acid derived methyl ketone 2-tridecanone, first reported to have toxic effects on spider mites (Chatzivasileiadis and Sabelis, 1997), is probably toxic to thrips larvae as well. Although this compound was detected in several *S. habrochaites* accessions in **Chapter 3**, the RF model in did not report it as significant, possibly due to experimental limitations (i.e. too little overlapping accessions) as described above. Bioassays with pure compounds however showed dose-dependent effect of 2-tridecanone on the mortality of thrips larvae (Figure 1B).

The structure of a (small) hydrophobic molecule with a functional group able to form hydrogen bonds (e.g. alcohols, aldehydes, ketones) seems to cause membrane disorganisation or impaired cellular functions. Small hydrophobic terpenes can integrate into phospholipid bilayers, spacing the phospholipids, thereby increasing membrane fluidity and weakening the hydrogen-bonded network of the headgroups (Camargos *et al.*, 2010). Moreover, the polarity of terpene derivatives may affect membrane charges (Castelli *et al.*, 2005), or interfere with the second messenger function of signalling phospholipids of at the surface of the membrane (Park *et al.*, 2011). In addition, oxygenated terpenes may, while their apolar part is anchored in the membrane, disrupt hydrogen bonds within membrane-bound proteins, essential for their folding and function. The

latter is exemplified by the ketone groups of several mono-terpenoids that render them toxic to insects by inhibiting acetylcholine-esterase activity (López and Pascual-Villalobos, 2010; Herrera *et al.*, 2015). The insecticidal and antimicrobial activity of 9H10epoZ, also in relation to 9HZ, may be explained by the presence of the epoxide group. The three-atom ring structure of an epoxide is known to be highly reactive to the nucleophilic amino (-NH2) and thiol (-SH) groups and can cause damage to DNA, RNA and protein structures. The hydrophobicity of the molecule in combination with the electrophilic epoxide group are suggested representative features of toxic epoxides (Manson, 1980; González-Pérez *et al.*, 2012) and could explain the potency of 9H10epoZ to serve as a cytotoxin.

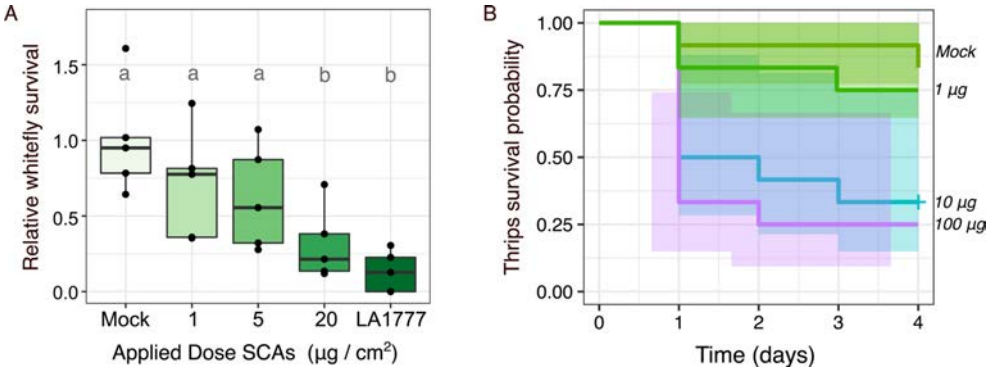

Figure 1. Survival of whitefly and thrips exposed to purified specialised metabolites in controlled bioassays. (**A**) Survival of whitefly (*Bemisia tabaci*) after 3 days on *S. lycopersicum* cv. Moneymaker leaf discs treated with different concentrations of sesquiterpene carboxylic acids (SCA's) purified from *S. habrochaites* LA1777 extracts, solvent (Mock) and a leaf disc taken from LA1777. The survival is expressed relative to the average survival in mock treatment. Letters indicate significant groups according to. For further details on the experimental design see Methods section of Chapter 5. (**B**) Survival assays of first-instar thrips larvae (*Frankliniella occidentalis*) on *S. lycopersicum* cv. Moneymaker leaf discs treated with different concentrations of 2-tridecanone. The experimental setup is adopted from the setup described in the Methods section of Chapter 3. The survival of the larvae was observed each day for four days in total. The survival probability is expressed as the proportion of larvae alive compared to the start of the experiment (day 0).

Although the exact mechanisms are not fully elucidated yet, the modification of terpenes that cause polarity, or charge, seems to play an important role in their biological activity. Additionally, the concentration of metabolites encountered by the insect will imminently have an effect on its cytotoxicity. Hence, as all other living organism, insect cell-membranes are party composed of sterols, hydroxylated triterpenes, that are required for an appropriate cell physiology (Jing and Behmer, 2020). The emergence of dedicated structures, like trichome cavities or glandular ducts, enables the plant to store bioactive compounds extra-cellularly in high concentrations and prevent auto-toxicity. Otherwise, modifications like glycosylation are crucial to prevent auto-toxic effects (Heiling *et al.*, 2021). We have estimated the concentration of 9H10epoZ in the glandular head of a type-VI trichome on *S. habrochaites* to be 1M (**Chapter 5**). Physical contact will make the trichome's head cell rupture, resulting in high concentrations dispersed over the insect's body and cytotoxic effects.

A combined system of repellence and toxicity for durable resistance

Insects have evolved several ways to overcome toxic effects of xenobiotic compounds. These adaptations include detoxification by modification, solubilisation and/or transportation followed by excretion or sequestration in specific tissues (Heidel-Fischer and Vogel, 2015) or by creating tolerance by evolving insensitive (protein) targets of the toxin (Dobler *et al.*, 2012). When it comes to breeding for resistance, evolution of such adaptations in the pest should ideally be slowed down. One of the conclusions made in **Chapter 2** is that stacking multiple metabolite-based defence traits would be a prerequisite for a durable approach in breeding and application in agriculture. I suggest combining a repellent volatile compound with a feeding deterrent, a toxin, or a disruptor of insect-development as an effective strategy for durable resistance. **Chapter 5** shows that PI127826 indeed seems to follow this approach by producing the repellent volatile 7-epizingiberene, which oxidises to *R*-curcumene in contact with air, together with a (semi-volatile) toxic derivative, i.e. 9H10epoZ. As whiteflies are repelled by *R*-curcumene (Bleeker, Diergaarde *et al.*, 2011), plant-insect contact is initially prevented lowering the selection pressure on whitefly to adapt to the toxin, 9H10epoZ, they encounter on the leaf surface. Vice versa, adaptation of whitefly through becoming insensitive to the repellent volatile is put off by the presence of a toxin.

The interesting question remains whether whitefly has evolved the olfactory response to 7epiZ to avoid this specific hostile environment. From our early experiments it appeared that the ability to detect and respond to 7epiZ is innate, as whiteflies reared on cucumber exhibited the repellence response when encountering wild tomato (Bleeker *et al.*, 2011). Also, the recent finding that the odorant binding protein 1 (OBP1) in the antenna of *B. tabaci* specifically binds *R*-curcumene from a *S. habrochaites* volatile blend (Zhan *et al.*, 2021), suggests the mechanism to detect this "warning for danger" cue is specific

The native whitefly biotypes in the Americas ("New World" biotypes, previously biotype A) split off from their African ancestors ~29 million years ago (Boykin *et al.*, 2007; Mugerwa *et al.*, 2018). The invasive biotypes MEAM1 and MED, previously biotype B and Q respectively, used in many studies including this thesis, originate from the Middle East/Asia and Mediterranean respectively and invaded the Americas from the 1980s (De Barro *et al.*, 2011). Because tomato originates from the Andes, it is unlikely that direct co-evolution between the invasive biotypes and this (wild) tomato occurred. Also, we are not aware of any reports describing 7epiZ/*R*-curcumene being produced by other plant species. Generally, OBPs can bind to multiple ligands, albeit with different affinities (Venthur and Zhou, 2018) e.g., OBP1was shown to bind β-ionone (Li *et al.*, 2019). It is therefore more likely that olfactory response of whitefly has adapted to volatiles structurally related to 7epiZ/*R*-curcumene possibly warning for an analogous toxic environment.

Is terpene precursor biosynthesis suppressed in *S. lycopersicum* trichomes?

Our work and that of many others highlights the importance of sufficient quantities of specialised metabolites that tomato should produce to confer resistance. In **Chapter 6** we established that there should be 2 recessive and 2 dominant alleles from PI127826 required to accumulate high levels of terpenes. In turn this indicates that the *S. lycopersicum* germplasm holds 2 dominant alleles repressing terpene accumulation. We furthermore concluded that the plastidial MEP

pathway especially prevails in type-VI trichomes of PI127826. **Chapter 7** also highlights the importance of this pathway to produce volatile terpenes in cultivated tomato. Redirecting the metabolic flux by introducing a targeted FPS-gene to the plastid, resulted in elevated FPP, apparently at the cost of monoterpene precursors as these plants no longer produced monoterpenes. This also resulted in a depletion of precursor in the cytosol as cytosolic-sesquiterpene levels dropped as well (**Chapter 7**). It furthermore showed that FPP cannot be transported from the plastid into the cytosol, supporting the hypothesis that it is the IPP/DMAPP precursor able to cross the plastidial membrane, feeding volatile terpene biosynthesis in both compartments. Finally, we observed that, while driven by the same promoter, introducing a foreign *FPS* gene (*GgFPS*) results in higher transcript levels compared to the introduction of an extra copy of the native version (*SlFPS*) implying that (post)transcriptional regulation of the pathway occurs in trichomes. The repression of terpene accumulation in *S. lycopersicum* trichomes may thus be acting on the MEP pathway e.g. by transcriptional inhibition. Several MEP-pathway genes indeed show a lower (i.e. 1.5 to 3-fold) expression and protein levels in cultivated tomato compared to *S. habrochaites* LA1777 (Balcke *et al.*, 2017) and we obtained comparable results comparing gene expression of the MEP-pathway in trichomes of PI127826 and the elite line of **Chapter 6** (unpublished data). Terpene levels in trichomes of both LA1777 and PI127826 are >35 higher than in those of cultivars, raising the question if higher gene expression suffices for a high accumulation of terpenes. A recent study overexpressing the transcription factor *SlSCL3* in *S. lycopersicum* upregulated the expression of precursor genes that are generally regarded as rate-limiting (*DXS*, *ACAT*, *HMGR*, *HMGS*) by 3-to 10-fold. However, terpene content in the trichomes was "only" ~2 fold higher (Yang, C., Marillonnet, Tissier, 2021). Elevated expression of MEP-pathway genes indeed leads to higher amounts of terpenes, but this does not seem to be the only bottleneck for "high terpene accumulation" in cultivated tomato.

Interestingly, the explorative metabolomics, transcriptomics, and proteomics studies on type-VI trichomes done by Balcke *et al*. (2017) showed that 1-deoxylulose 5-phosphate (DXP), the first dedicated product of the MEP pathway, is 14 times higher in *S. lycopersicum* LA4024 trichomes in comparison to *S. habrochaites* LA1777. In addition, the level of IPP in LA4024 trichomes is a stunning 75 times higher compared to LA1777. The relatively low levels of these important intermediates in LA1777, and at the same time the high level of end products, supports the hypothesis of a high metabolic flux through the MEP pathway in *S. habrochaites* trichomes with a rapid turn-over of IPP/DMAPP to sesquiterpenes. On the contrary, while precursor molecules seem abundantly available in *S. lycopersicum* they do not appear to be further metabolised into terpenes by cytosolically or plastidial localised terpene synthases. I therefore suggest that one of the bottlenecks in the metabolic flux towards terpene accumulation lies downstream of the MEP/MVA precursor pathways. The accumulation of MEP-intermediates can have inhibiting effects on gene expression in the pathway (Xiao *et al.*, 2012; D'alessandro, Ksas, Havaux, 2018) which could explain the lower expression levels observed in *S. lycopersicum* trichomes. To understand this, it is important to know what keeps trichomes of cultivars from utilising the reservoir of IPP/DMAPP.

Previous studies showed that products of the MVA/MEP pathway can start to accumulate when downstream metabolism is blocked or impaired. For example, silencing of the plastidial isoprene

synthase in poplar resulted in accumulation of IPP the leaves (Ghirardo *et al.*, 2014) and silencing of the amorpha-4,11-diene synthase in *Artemisia annua* resulted in an accumulation of FPP in trichomes (Catania *et al.*, 2018). In trichomes of cultivated tomato, however, the activity of prenyl-transferases or terpene synthases do not seem to be the bottleneck. First because the F1 (Elite x PI127826) we created (**Chapter 6)** produced higher quantities of plastidial terpenes through allelic variants from the cultivar encoding NDPS and PHS1 compared to those from the wild parent encoding zFPS and ZIS. Furthermore, we found a majority of F2 plants (**Chapter 6)** to have low 7epiZ levels in their trichomes while they were selected for the presence of *zFPS*/*ZIS*. Moreover, transgenic *S. lycopersicum* lines over-expressing *zFPS* and *ZIS* under trichome type-VI specific promoters only produced minute amounts of 7epiZ compared to monoterpenes (Bleeker *et al.*, 2012). It therefore seems unlikely that the bottleneck for utilising IPP/DMAPP is primarily due to the *S. lycopersicum* alleles of biosynthetic enzymes downstream of the MEP pathway. It should be noted that the origin of excess IPP in *S. lycopersicum* as described by Balcke *et al*., (2017) is cytosolic and/or plastidial. By feeding those plants with labelled carbon, e.g. 1-^{13}C glucose, one could distinguish the MVA from the MEP-generated IPP/DMAPP (Lichtenthaler, Rohmer, Schwender, 1997) which could give more clarity if there is a specific cellular compartment where terpene synthesis is held up. Nevertheless, together with the data on gene transcription of the precursor pathways, it seems that a different bottleneck, possibly imposing a stronger effect, is withholding *S. lycopersicum* type-VI trichomes from accumulating large amounts of terpenes.

Transport and storage capacity to facilitate terpene accumulation

As described above, terpenoids and other specialised metabolites may exert several cytotoxic effects and therefore should not accumulate inside living cells, hence the development of specialised structures for extracellular storage. Feedback mechanisms will prevent overaccumulation in the metabolite-producing cell by rebalancing carbon distribution to pinch off the biosynthetic pathway, reducing terpene synthesis and thereby protecting the cellular integrity. An example of such feedback was recently shown in *Arabidopsis* where the diterpene β-cyclocitral binds and inhibits DXS activity thereby downregulating its own synthesis (Mitra *et al.*, 2021). Besides direct feedback, local accumulation of the product may lead to reduced enzyme activity through steric hinderance. By the transport of (end) products away from the site of synthesis, these inhibiting mechanisms will not be triggered, keeping optimal conditions for ongoing production.

In type-VI trichomes, MEP-pathway derived terpenes must cross both the plastidial and the plasma membrane before being secreted into the storage cavity. The way terpenes are transported remains elusive, but various mechanisms have been suggested to include lipid vesicles, lipid transfers proteins (LTPs) and/or ABC-transporters (Figure 2; Crouzet *et al.*, 2013; Wang *et al.*, 2016; Tissier, Morgan, Dudareva, 2017). That the increased transport of terpenoids to extracellular spaces can dramatically increase their accumulation was shown by the ectopic expression of *Aa*LTP3 and ABC-transporter *Aa*PDR2 in artemisinin-producing *Nicotiana benthamiana* plants (Wang *et al.*, 2016). The seemingly optimal transport of terpene cargo into the storage cavity in *S. habrochaites* trichomes may be distorted in cultivated tomato due to less functional alleles for components such as LTPs and/or ABC transporters. However, that case we

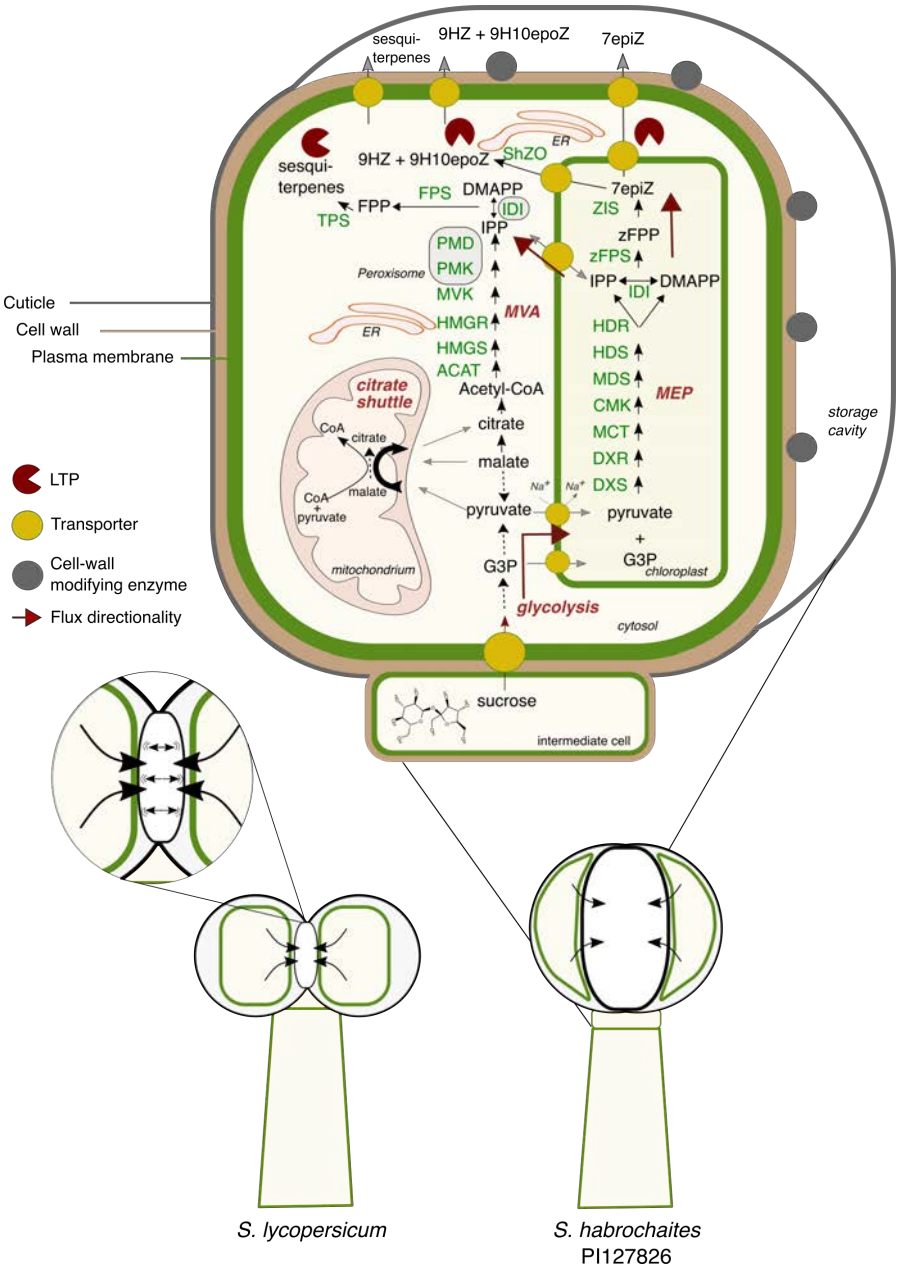

Figure 2. Overview of sesquiterpene synthesis in type-VI glandular trichomes of PI127826. Glandular cells of PI127826 import sugars from the leaf that get metabolised to G3P and pyruvate as starting material for the MEP pathway, providing IPP/DMAPP precursors for sesquiterpene synthesis in both the cytosol and the plastid. A lower amount of pyruvate is used to produce Acetyl-CoA via the citrate shuttle, serving as input for the MVA pathway. Terpenes have to cross the cell-membrane and cell wall to get secreted into the storage cavity, which is likely aided by LTPs and ABC-transporters. Cell-wall modifying enzymes, localised at the side of the cell-wall facing the cavity, are loosening the cell-walls to facilitate cavity expansion. Metabolites are written in black letters, enzymes in green letters, pathways are indicated in red letters and cellular compartments in italics. Solid arrows indicate a single

enzymatic step; dashed arrows indicate multiple enzymatic steps. Enzyme abbreviations: 1-Deoxy-d-xylulose 5-phosphate synthase (DXS); 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR); 2-C-methyl-d-erythritol 4-phosphate cytidylyltransferase (MCT); 4-(cytidine 5′ -diphospho)-2-C-methyl-*D*-erythritol kinase (CMK); 2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MDS); (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS); (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; (HDR); isopentenyl diphosphate isomerase (IDI); *cis*-farnesyl diphosphate synthase (zFPS); ZIS; 7-epizingiberene synyhase (ZIS); 7-epizingiberene oxidase (ShZO); acetyl-CoA C-acetyltransferase (ACAT); 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS); 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR); mevalonate kinase (MVK); phospho-mevalonate kinase (PMK); diphospho-mevalonate decarboxylase (PMD); farnesyl diphosphate synthase (FPS); terpene synthase (TPS). Metabolite abbreviations: glyceraldehyde 3-phosphate (G3P); isoprene diphosphate (IPP); dimethylallyl diphosphate (DMAPP); farnesyl diphosphate (FPP); *cis*-farnesyl diphosphate (zFPP); 7-epizingiberene (7epiZ); 9-hydroxy-zingiberene (9HZ); 9-hydroxy-10,11-epoxyzingiberene (9H10epoZ).

could expect intermediate phenotypes in hybrid, rather than the extremely low levels we observed. Therefore, less functional alleles cannot be one of the predicted dominant factors restricting terpene biosynthesis in *S. lycopersicum*. Nevertheless, they might play an additional role in this complex trait. There are several genes encoding LTPs and ABC-transporters that are highly expressed in trichomes of cultivated and wild tomato (our lab, unpublished results). The role of these transporters in glandular trichomes is a field yet to be discovered, but the trichome-specific expression profiles of some of them strongly suggests a role in (specialised) metabolic processes.

Besides the transport systems facilitating terpene accumulation in the cavity, the trichome's structural components may influence their capacity to store their metabolites. As can be observed in **Chapter 6**, cultivated tomato has 'clover'-shaped trichomes with small storage cavities while trichomes of PI127826 appear as 'spherical' created by the "inflated-balloon effect" of their large storage cavities. Fosmidomycin treatment resulted in PI127826 trichome cavities of similar size as the cultivar with individual gland cells more visible. However, reduction of the cavity did not result in the typical clover shape seen in the cultivar. This indicates that the shape of the head cell is not solely dependent on cavity expansion through terpene deposition, but is also influenced by other, probably structural components. One of these could be the content of the type-VI trichome's gland cells, which are predominantly filled with small vacuoles of yet unknown function and content (Bergau *et al.*, 2015). Interestingly, gland cells of wild tomato LA1777 seem to have more spaced, and smaller sized vacuoles than gland cells of cultivated tomato. The small vacuoles may provide the the flexibility to change shape as the cavity is being filled and expanding. Alternatively, or additionally, they may be involved in transport or temporal storage of terpenes, or other classes of specialised metabolites, such as flavonoids (Sugimoto *et al.*, 2022). Other structural components may include the cell-walls of the gland cells. Cell walls seem to be modified during trichome development which results in expansion of the cavity by demethylation of pectin polysaccharides specifically at the sites facing the cavity (Bergau *et al.*, 2015; Figure 2). Our unpublished data suggest that, indeed, genes encoding putative cell-wall modifying enzymes e.g. expansins and pectinases are highly expressed in trichomes of tomato. Fine-tuned modification of structural components could facilitate optimal metabolite storage, and thereby continuation of the gland cell's metabolic activity. As modifications of cell-walls can

have a significant effect on the cellular integrity, they may be strictly regulated and hence repressed in glandular trichomes of cultivars.

How active are fully developed trichomes?

An outstanding question remains if the gland cells of 'mature' trichomes, as used in most studies including this thesis, are still actively producing metabolites. The outgrowth of (glandular) trichomes starts very early in tissue development; fully developed type-VI glandular trichomes can already be found on leaf primordia of tomato (Figure 3A). Young leaflets (~1-2 cm long) hold over 99% fully developed trichomes (Bergau *et al*., 2015). Type-VI trichomes do not seem to (actively) release specialised metabolites from their storage compartments which are surrounded by cell-wall material (Widhalm *et al.*, 2015; Tissier, Morgan, Dudareva, 2017) unless when broken by mechanical force. It is therefore likely they retain most compounds during their lifespan. The connection of the gland cells to the stalk appears to be the 'weakest link', occasionally creating leaky apertures (Bergau *et al*., 2015). Type-VI trichomes can however stay intact throughout plant development and we have not observed any significant differences in terpenoid content or cavity volume in trichomes on young or (very) old leaves (this lab, personal communication Therezan, 2022). It raises the question how metabolically active fully developed trichomes still are, and how suitable they are as a model for studying trichome metabolism. When studying already developed trichomes, one should consider the possibility that the gland cells already stopped or slowed down activity of the specialised metabolic pathways. In peppermint, for example, it is estimated that glandular-trichome cavities are filled up with monoterpenes within only 20 to 30 hours, indicating the narrow time window in which (high) metabolic activity is required (Turner, Gershenzon, Croteau, 2000). As the transition from P1 to P2 leaf primordium of tomato takes approximately 48 hours (Hussey, 1963), I estimate that within this time frame tomato type-VI trichomes can transform from developing to being mature with fully filled glands (Figure 3B). Nonetheless, many of the enzymes involved in biosynthesis of terpenes (e.g. prenyl transferases, terpene synthases or cytochrome P450s) are still highly expressed in already developed trichomes (Schilmiller *et al.*, 2009; Bleeker, Spyropoulou *et al.*, 2011) (**Chapter 5)**. Earlier work in our lab showed that volatiles do "escape" in a constant manner from the trichomes into the tomato headspace (Bleeker *et al.*, 2009) and, as trichomes on young and old leaves contain similar quantities of terpenes, trichomes probably replenish what is lost.

Combining this information I suggest a model for the course of gland-cell activity during type-VI trichome development (Figure 3C, dashed lines). The activity of both "active" trichomes of *S. habrochaites* accessions, and the "lazy" trichomes of cultivars, peaks during the stage of secretion. During this phase, the metabolic activity is much higher in "active" trichomes, resulting in more terpenes accumulated in the storage cavity compared to "lazy" trichomes (Fig 3C, solid lines). In the post-secretory phase, when trichomes are fully developed, gland-cell activity significantly drops in active trichomes to levels that compensate for terpenes that escape or leak out. Lazy trichomes show a relative minor drop in activity after the secretory phase, to a residual level. The residual activity of active trichomes is probably still higher than in lazy trichomes as fully filled cavities may leak more metabolites. I postulate however, that the major difference in activity must be during the secretory developmental stage (Fig. 3B).

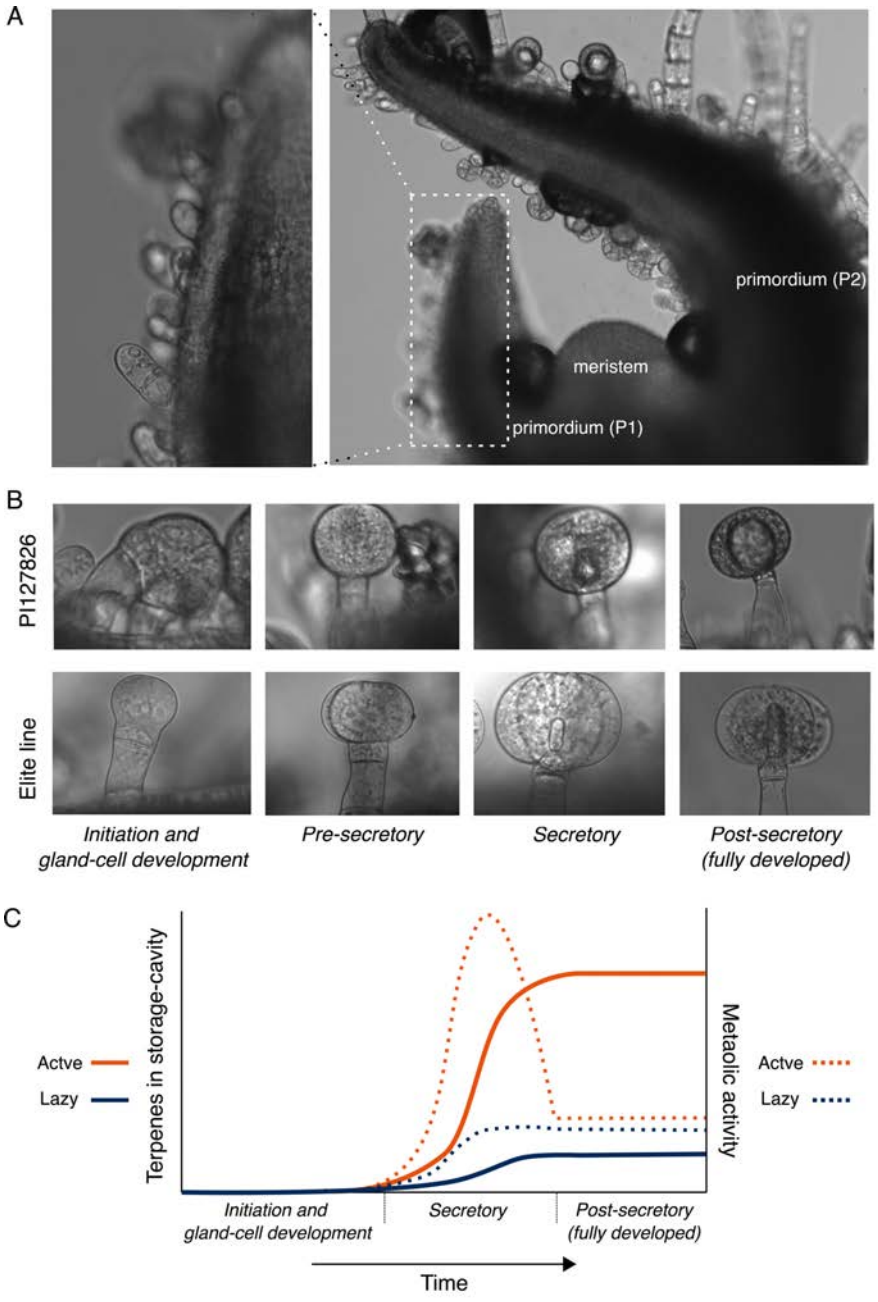

Figure 3. Trichome type-VI during development.

(**A**) Images of *S. habrochaites* PI127826 leaf primordia using bright field microscopy. The left photo shows a magnified image of the P1 primordium. (**B**) Type-VI trichomes in different stages of development that can be found on the P1 and P2 primordia of PI127826 (top) and the elite cultivar used in Chapter 6 (bottom). (**C**) Theoretical model of the quantity of terpenes in the storage cavity (solid lines) and metabolic activity of the gland cells (dashed lines) during different phases of development of active (orange) or lazy (blue) type-VI trichomes.

Interestingly, gene expression of several terpene synthases is upregulated in (cultivated) tomato trichomes after jasmonic-acid treatment and/or wounding, along with an increased detection of some terpenoids in the plant's headspace (Kant *et al.*, 2004; Ament *et al.*, 2004; van Schie, Haring, Schuurink, 2007; Falara *et al.*, 2011). This suggests that the ongoing (residual) metabolic activity and terpenoid production by the gland cells of fully developed trichomes can be boosted upon a stimulus. Such boosts of activity could be used to magnify differences in metabolic activity of maturated trichomes, as a tool to e.g. study MEP-pathway behaviour. Ideally, one would like to study trichomes in their most active phase, hence in their 'pre-secreting' or 'secreting' stage (Figure 3B, C). The factors that underlie high terpene accumulation in type-VI trichomes are probably predominantly exhibited during these stages and analysis of gene-expression or metabolite levels would give the most contrasting differences. While collection of sufficient such material will be one of the major bottlenecks, technical advances in e.g. in single-cell metabolomics (Nakashima *et al.*, 2016) and gene expression analysis (Schuierer *et al.*, 2017; Curion *et al.*, 2020) will help elucidating metabolic activity throughout development of the trichome.

Concluding remarks

Production of specialised metabolites requires several genes involved in their biosynthesis, transport and storage. It is therefore not surprising that also in trichomes, the species-to-species transfer of metabolite production is complex and subject to regulatory constraints. The wild tomato germplasm does, however, contain trichome-based defence traits, that can be identified as shown in this thesis. The next step, identification of the biosynthetic genes will become easier when more genomic sequences of wild tomatoes become available in the (near) future and gene annotations are improving. Still, big steps need to be made regarding our understanding of the regulatory mechanisms for synthesis and transport and we have seen that introducing new genes can lead to unexpected outcomes. In addition, our knowledge on the fundamental structures of (glandular) trichomes is still inconclusive, though they seem of major importance for its metabolic activity. Therefore, I believe more detailed research on the trichome's physiology and development can lead to improved understanding of their production capacity.

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