Defence from the wild

Specialised metabolism in tomato glandular trichomes

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Summary

Glandular trichomes are small, hair-like structures found on the above-ground surfaces of about 30% of higher plants. Consisting of one or more glandular cells, oftentimes on top of a basal-and a stalk cell, glandular trichomes function as micro-organs designated to produce, store and/or secrete so-called specialised metabolites. These specialised metabolites generally play a role in the interaction of a plant with its biotic environment. There are several examples where they act in plant defence against small herbivores as they can have deterrent, antifeedant or toxic effects or attract the herbivore's natural enemies.

Members of the tomato genus (*Solanum* sp.) are known to produce a wide array of specialised metabolites in their glandular trichomes, consisting of blends that differ both qualitative and quantitative between species and accessions. Most of this diversity can be found amongst wild species, of which some accessions are resistant to (often invasive) herbivorous insects or harmful micro-organisms. In contrast, the diversity in specialised metabolites produced in cultivated tomato (*S. lycopersicum*) is relatively low and cultivars are often susceptible to various pests and pathogens. Since wild species can be crossed to cultivars, their germplasm offers a great source of natural resistance that is potentially metabolite-based. However, as glandular trichomes of resistant accessions often produce complex mixtures of specialised metabolites, our knowledge regarding the function of the individual components is only limited. Moreover, while much progress has been made on the identification of biosynthetic enzymes of specialised metabolites, regulatory mechanisms controlling their synthesis and the accumulation inside the trichome are still largely unknown.

In this PhD thesis I explore the natural variation in specialised metabolism in trichomes of wild tomatoes to (1) identify metabolites conferring insect resistance from complex blends they produce, (2) unravel their biosynthetic pathway and (3), gain insight into additional factors that are necessary for their accumulation inside glandular trichomes.

Many plant-produced metabolites have been reported to exert insecticidal effects, but these are often leaf or root extracts that are tested at (unnaturally) high concentrations. Hence there are only a few examples of individual specialised metabolites having a validated anti-insect effect *in planta*. **Chapter 2** reviews these examples including their site of synthesis and what is known about their underlying regulatory mechanisms. Furthermore, the possibilities and pitfalls of integrating such resistance traits in crops for successful application in the field are being assessed. **Chapter 3** describes the composition of volatiles and acylsugars produced by glandular trichomes of 19 different tomato accessions, belonging to 10 different species, using GC-MS and LC-MS methodologies. We subsequently developed a statistical pipeline employing a random forest (RF) machine learning algorithm to link the abundance of these specialised metabolites to plant resistance against whitefly (*Bemisia tabaci*) and thrips (*Frankliniella occidentalis*). This analysis showed that resistance is indeed insect specific. The RF algorithm predicted two acylsugars, i.e. S3:15 and S3:20, to be causal to the toxicity phenotype for whiteflies while no metabolite could be connected to resistance against thrips.

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In **Chapter 4,** the RF approach was applied to link volatiles produced by trichomes of an F2 population, made from a *S. lycopersicum* elite cultivar and the whitefly resistant *S. habrochaites* accession PI127826. This resulted in the prediction of two germacrenes (i.e. germacrene-B and germacrene-D) and a then unknown oxygenated sesquiterpene, all primarily produced by the wild parent, associated to whitefly resistance. In **Chapter 5,** the structure of the oxygenated sesquiterpene was clarified to be 9-hydroxy-zingiberene (9HZ) which is synthesised in trichomes of *S. habrochaites* accessions PI127826 and LA2167 together with another oxygenated sesquiterpene 9-hydroxy-10,11-epoxyzingiberene (9H10epoZ). Using a combination of genetic, biochemical and computational approaches, we found that both metabolites are products of a successive oxidation of the sesquiterpene 7-epizingiberene (7epiZ) by a single cytochrome P450 from *S. habrochaites*, ShCYP71D184 (ShZO). Bioassays with purified metabolites demonstrated that particularly 9H10epoZ is toxic to whiteflies while unexpectedly 7epiZ, known to be a whitefly repellent, does not affect survival of the insect.

In **Chapter 6** we found that marker-based introgression of the full 7epiZ pathway including *ShZO* for the conversion into 9HZ and 9H10epoZ, into a cultivar genetic background was successful. Unexpectedly however, the *S. lycopersicum* x *S. habrochaites* (PI127826) F1 hybrid produced only minor concentrations of terpenes compared to the wild parent. A segregation analysis of terpenoid levels produced in the F2 generation showed that, in addition to the biosynthetic genes, at least two recessive, yet unknown, loci from PI127826 need to be introgressed in order to obtain plants producing relevant high-levels of terpenes. Additional experiments showed that high-level terpene biosynthesis by type-VI trichomes largely relies on the metabolic flux through its precursor pathway in the plastid (MEP pathway) which seems to be repressed in trichomes of the cultivated tomato. The activity of this pathway is altering the accumulation of terpenes in type-VI trichomes and consequentially for the volume of the storage cavity in which they are stored.

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In **Chapter 7**, we present a methodology to modulate metabolic fluxes through isoprenoid precursor pathways in trichomes by genetic engineering. We targeted a prenyltransferase from chicken (*GgFPS*), involved the synthesis of sesquiterpene precursors, to the plastid and expressed it in cultivated tomato under a trichome-specific promoter. Trichomes of these transgenic plants contained elevated levels of the sesquiterpene precursor FPP, a strongly reduced total terpene profile and were less attractive for whiteflies. These results appear to confirm the dominant role of MEP-pathway in terpene biosynthesis in tomato. Finally, the major findings from this research are discussed in **Chapter 8**. In this chapter I will go into detail about structural features of specialised metabolites that may render them toxic properties and discusses the regulation of terpenoid synthesis and the organisation of (intercellular) storage; processes that seem particularly important during early stages of trichomes development to accumulate high levels of terpenes.

Nederlandse Samenvatting

Glandulaire trichomen zijn minuscule klierhaartjes die te vinden zijn op de bovengrondse delen van planten. Deze klierhaartjes, bestaande uit een "steel" met daarbovenop één of meerdere kliercellen, welke fungeren als "micro-organen" die gespecialiseerde moleculen, oftewel metabolieten, in hoge concentraties kunnen produceren en uitscheiden. Veel van dit soort moleculen spelen een rol in de afweer tegen vraatinsecten door hun toxische of afstotende werking op de plaag, of doordat de natuurlijke vijand van het insect door de productie ervan tot de plant wordt aangetrokken.

Tomatenplaten (*Solanum* sp.) staan erom bekend een brede variatie aan bovenbeschreven afweerstoffen in hun glandulaire trichomen te produceren. Het gaat hier dan echter voornamelijk over de wilde (niet eetbare) tomatensoorten, die daardoor vaak resistent tegen vraatinsecten en andere schadelijke organismen blijken te zijn. De cultuurtomaat (*S. lycopersicum*) wordt voornamelijk gecultiveerd om zijn eetbare vrucht en is op die eigenschap veredeld. De cultuurtomaat produceert geen, tot relatief lage concentraties gespecialiseerde metabolieten in zijn glandulaire trichomen met verhoogde gevoeligheid voor vraatinsecten en andere ziekteverwekkers als gevolg; een probleem dat telers over het algemeen proberen op te lossen middels het gebruik van synthetische pesticiden. Doordat wilde tomatensoorten te kruisen zijn met de cultuurtomaat zouden resistentie-eigenschappen uit wilde soorten in principe ingekruist kunnen worden. Echter, doordat wilde soorten een complexe mix van gespecialiseerde metabolieten in hun glandulaire trichomen produceren, is het veelal nog onduidelijke welke hiervan daadwerkelijk voor afweer zorgen. Daarbij is het ook vaak nog onbekend hoe de afweerstoffen in de trichoom worden aangemaakt, op welke manier de kliercellen dit proces reguleren en hoe de stoffen vervolgens in de trichoom worden opgeslagen.

In dit proefschrift onderzoek ik de natuurlijke variatie in de aanmaak van afweerstoffen door glandulaire trichomen van wilde tomatensoorten om (1) stoffen te identificeren die dienen ter afweer tegen vraatinsecten (2) de biosynthese van deze stoffen te ontrafelen en (3) inzicht te krijgen in de processen die zorgen voor een efficiënte aanmaak en opslag in de trichoom.

In **Hoofdstuk 2** wordt er op basis van de beschikbare literatuur, bekeken welke endogene afweerstoffen van planten er tot nu bekend zijn. Hierbij worden hun werking op insecten, de biosynthese en regulatie daarvan besproken. Ook wordt er in dit hoofdstuk aandacht besteed aan de wijze waarop we deze "natuurlijke vormen van afweer" zouden kunnen toepassen ter bescherming van onze voedselgewassen. **Hoofstuk 3** beschrijft de samenstelling van afweerstoffen in de glandulaire trichomen van 19 verschillende wilde- en cultuurtomaten. Vervolgens is er een geavanceerd statistisch model toegepast om de aanwezigheid van stoffen te koppelen aan resistentie tegen twee verschillende, economisch relevante plaaginsecten; de witte vlieg (*Bemisia tabaci*) en trips (*Frankliniella occidentalis*). Uit deze analyse blijkt dat de resistentie van (wilde) tomaten verschilt per insect en worden twee stoffen geïdentificeerd die te linken zijn aan resistentie tegen de witte vlieg; acylsuikers S3:15 en S3:20.

In **Hoofdstuk 4** wordt het statistische model opnieuw toegepast, ditmaal specifiek om de vluchtige stoffen die door trichomen van één van de wilde tomaat accessies, *S. habrochaites* (PI127826), worden gemaakt in detail te onderzoeken. Het model voorspelt drie zogenaamde terpenen als betrokken bij de resistentie tegen witte vlieg in PI127826. De stoffen worden vervolgens geïdentificeerd als germacreen-B, germacreen-D plus een dan nog onbekend terpeen-derivaat. In **Hoofdstuk 5** wordt het onbekende terpeen-derivaat nader onderzocht en geïdentificeerd als 9-hydroxy-zingiberene (9HZ) welke samen met een tweede terpeenderivaat, 9-hydroxy-10,11-epoxyzingiberene (9H10epoZ), in type-VI glandulaire trichomen van de wilde accessie wordt aangemaakt en opgeslagen. Met een combinatie van genetische en biochemische analyses en computermodellen wordt er aangetoond dat beide derivaten omzettingsproducten zijn van hetzelfde molecuul, namelijk het sesquiterpeen 7-epizingiberene (7epiZ). Hierbij wordt ook het eiwit geidentificeerd dat verantwoordelijk is voor deze omzetting: ShCYP71D184 (ShZO), een enzym dat alleen in de wilde tomatensoort gemaakt wordt. Verdere analyses wijzen uit dat 7epiZ, eerder in ons laboratorium geïdentificeerd als een afstotende geurstof, zelf niet dodelijk is voor de witte vlieg en dat met name derivaat 9H10epoZ toxisch is voor het insect.

In **Hoofstuk 6** blijkt dat hoewel de biosynthese genen om 7epiZ en de terpeen-derivaten 9HZ en 9H10epoZ te produceren ingekruist kunnen worden vanuit de wilde tomaat, de hybride F1-generatie (PI127826 x cultuurtomaat) slechts minieme hoeveelheden afweerstoffen in de trichomen produceert. Omdat de concentraties van belang zijn voor de mate van resistentie, wordt er in de volgende (F2-) generatie van nakomelingen uitgezocht welke factoren onderliggend kunnen zijn aan het dempende effect. Uit analyse van de F2-populatie, welke segregeert voor 7epiZ en 9H10epoZ concentraties, blijkt dat er, naast de genen voor de productie van de terpenen, nog ten minsten twee *recessieve* eigenschappen nodig zijn om de productie van terpenen in type VI trichomen hoog te houden. Er wordt geconcludeerd dat de cultuurtomaat de productie en opslag van grote hoeveelheden terpenen in de trichoom lijkt te onderdrukken. Deze onderdrukking heeft waarschijnlijk effect op de biosyntheseroute van terpenen in de plastide: één van de intracellulaire organellen binnen de kliercellen van een trichoom. De mate van activiteit van deze route blijkt ook gekoppeld te zijn aan het volume van het compartiment waarin de terpenen worden opgeslagen.

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Hoofstuk 7 beschrijft een methode om de biosyntheseroute van terpenen in de trichoom aan te passen door middel van genetische modificatie. Als voorbeeld brengen we *GgFPS*, een kippenvariant van het *Farnesyl diPhosphate Synthase* gen betrokken bij terpeenproductie in zowel kippen als tomaat, in de trichoom tot expressie. Daarnaast dirigeren het eiwit naar de plastide. Tomatenplanten met deze genetische aanpassing blijken een sterk gereduceerd bouquet aan aantrekkelijke terpenen te produceren en de planten zijn daardoor minder goed te vinden voor de witte vlieg. De resultaten uit dit onderzoek bevestigen ook de hypothese dat de plastide een cruciale rol speelt in de biosynthese van terpenen. In **Hoofdstuk 8** wordt de kennis verworven uit bovenstaande studies bediscussieerd en wordt er beredeneerd welke eigenschappen een trichoom zou moeten bezitten om een plant bescherming te kunnen bieden tegen vraatinsecten. Aan bod komen de effectiviteit van verschillende molecuul-structuren, de regulatie van terpeen biosynthese en de opslagcapaciteit hiervan binnen de trichoom.

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