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Defence from the wild

Specialised metabolism in tomato glandular trichomes

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Chapter 1

General Introduction

Plant defence and the repercussion of domestication

In nature, plants are the most abundant source of food and are continuously under attack by other organisms including fungi, bacteria, insects and herbivorous animals. Besides causing physical damage, insects can act as vectors for plant viruses that may lead to severe disease or death. From an evolutionary point of view, this hostile environment has pushed plants to evolve effective defence strategies, fending off these aggressors while surviving to produce the next generation. The defence strategies evolved against insects include physical adaptations like spikes, spines, wax layers or thickened cell-walls that serve as an obstruction impeding close contact, penetration or ingestion of plant tissues. In addition, plants have evolved strategies to protect themselves with chemical agents that are often toxic, deterrent or repellent to the attacking entity. Nevertheless, our domesticated crop plants, habitually grown in monocultures, are often highly susceptible to pest infestations. In 2020, the Food and Agriculture Organisation of the United Nations estimated that annually 20-40% of the global crop yield is lost due to pest infestations with an economical cost of 270 billion US dollars. So, while self-defence is naturally present in plants, present-day food and ornamental crops have not retained (much) of these particular traits during domestication.

Around 13,000 years ago, humans started domesticating wild plants which ultimately gave rise to the crop plants we know today (Diamond, 2002). During the domestication process, people selected plants mainly for traits related to ease of harvest, nutritional value and yield. Describing his view on the variation under domestication Charles Darwin already noted: *“one of the most remarkable features in our domesticated races is that we see in them adaptation, not indeed to the animal's or plant's own good, but to man's use or fancy”* (Darwin, 1859). For many crops, selection has been on the tissue to be consumed e.g. the fruit, seeds or leafy parts. A classical and well-studied example is the domestication of maize from the wild grass teosinte, where the small inedible ears of teosinte considerably increased in size and holding many large and edible kernels (Wang *et al.*, 2005). The selection of traits for *man's fancy*, has (unconsciously) led to the crop's dependency on mankind for its existence; a phenomenon we know as the domestication syndrome. In case of plant defence, this often means the innate plant defence systems are reduced in cultivated species compared to their wild ancestors (Meyer, DuVal, Jensen, 2012). In some cases, traits were actively selected against, e.g. spikes and thorns on, or toxic chemicals in edible leaves and fruits. Furthermore, defence mechanisms might also have been lost “as a side-effect” in the absence of selection pressure. In a general sense, this resulted in crops being efficient and nutritious food-producers but left them rather vulnerable to herbivory and pathogen infection.

By the application of synthetic pesticides for crop protection, which started to develop in the 16th century with the use of highly toxic mercury and arsenic, and much later in the 1870's through the first modern pesticide DDT, mankind was able to efficiently protect their crops (Zacharia, 2011; Abubakar *et al.*, 2020). Moreover, during the 'green revolution' that started in 1944, the use of synthetic pesticides accelerated in order to resolve the global demand for food (Zacharia, 2011). The adverse effect of these synthetic and often persistent pesticides on non-target organisms, including beneficial insects, fish, birds and even humans, became visible over following decades, receiving large-scale public attention first after the alarming publication of Rachel Carson's *Silent Spring* in 1962 (Carson, 1962). To date, the negative impact of several

widely used insecticides on the environmental ecology and human health are recognised by policy makers and global health organisations, leading to restrictions or prohibition of their usage. For example, the European Union banned the use of anti-insect neonicotinoids (i.e. clothianidin, thiamethoxam and imidacloprid; EU regulation No. 485/2013) and has strong aspirations towards a 'toxic-free environment' regarding the use of pesticides in agriculture (EU COM(2020) 667 document No. 52020DC0667). However, with an estimated global population of 9.7 billion in 2050, and thereby an increasing demand for food, the agricultural sector has to rise to this challenge.

An additional factor complicating the vulnerable state of food crops is the limited genetic diversity, as early plant breeders likely domesticated crops from only a small number of individual plants. To date, the resulting genetic bottleneck makes it difficult, if not impossible, to breed for novel (defence) traits using solely cultivated germplasm. Wild relatives, on the other hand, still hold a lot of genetic variation in different ecotypes as, opposite of cultivars, they have been intercrossing for millennia and have adapted to local environments. At the start of the 20th century, scientist put forward to use of wild species to introduce new traits in cultivated material as a way to crop improvement and diversification (Bessey, 1906). Successful examples of employing wild traits for crop improvement include the introgression of a quantitatively trait locus (QTL) from a wild species of wheat to a cultivar elevating the protein levels in the grains (Kovacs *et al.*, 1998), improving fruit quality in tomato (Rick, 1974) and drought tolerance in chickpea (Hajjar and Hodgkin, 2007).

Besides plant-and fruit characteristics, the diversity among wild species could be used to identify traits that improve resistance against pathogens or herbivores (Lenne and Wood, 1991). Resistance against pathogens and viruses introgressed from wild species regularly involve resistance (R) genes triggering a strong immune response after pathogen perception, in many cases providing partial or full resistance. While > 300 R-genes have been identified against pathogens, the contribution of R-genes to insect resistance seems very limited (Broekgaarden *et al.*, 2011; Kourelis and Van Der Hoorn, Renier AL, 2018). One of the known R-gene associated with insect resistance is the nematode resistance-gene *Mi* from tomato (*Solanum lycopersicum*) encoding an NBS-LRR protein which was introgressed from wild relative *S. peruvianum* (Kaloshian *et al.*, 1998). *Mi*, probably interacting with other proteins, is thought to impede phloem-feeding insects like whiteflies and aphids from finding a suitable feeding site after probing for sieve elements, thereby limiting their feeding (Kaloshian *et al.*, 1998; Gouveia *et al.*, 2018). The temperature sensitivity of *Mi*-mediated resistance makes plants lose resistance at higher temperatures making its application in the field instable (El-Sappah *et al.*, 2019). In general, plant defence against herbivorous insects seems to rely largely on structural and biochemical defences. The latter is often complex, polygenic and moreover intertwined with the plant's physiology, complicating the discovery of the underlying mechanisms and their genetics. Introgression of such resistance traits into cultivated species is therefore regarded to be a challenging process (de Vos and van Doorn, 2013). However, the bans on synthetic pesticides currently urge breeders to look for alternative ways to protect crops and utilise the existing genetic variation in wild germplasm in order to use the wide variety of anti-insect metabolites present in wild germplasm.

In this thesis, I aim to explore metabolite-based insect-resistance present in the wild tomato germplasm and gain insight in their genetic basis. Tomato is an important vegetable crop, with an estimated world-wide production of 180 million tonnes in 2018, having a market value of 93 billion US dollars (United Nations Food and Agriculture Organisation; www.fao.org) and serves as a research model for many other fruit crops. Tomato cultivars are, however, susceptible to a plethora of pests including insect-transmitted bergomoviruses, tospoviruses and toradoviruses including the recently emerged and devastating tomato brown rugose fruit virus (ToBRFV; Luria *et al.*, 2017). The tomato genus is rich in species and accessions, as intensively studied in the last century by Charles Rick, and its resistance traits could serve as a source of breeding material (Rick, 1973; Rick and Chetelat, 1995). However, as mentioned above, resistance traits can be complex and therefore a full understanding of the nature of these mechanisms are necessary to effectively implement them.

Tomato and trichome-based defence

Originating from the Andes in South America, tomato was domesticated in South or central America before being imported to Europe in the 16th century (Peralta, Spooner, Knapp, 2008). Our tomato likely originated from the red berry-like fruit bearing *Solanum pimpinellifolium* evolving to *S. lycopersicum* L. var. *Cerasiforme* around 7000 years ago, which was used to domesticate to the current tomato cultivated species *S. lycopersicum* L. var. *lycopersicum* (Razifard *et al.*, 2020). The original population that was brought to Europe consisted of only a limited number of individuals which may have caused a massive genetic bottleneck. It is estimated that the genomes of tomato cultivars account only for <5 % of the genetic variation present in the germplasm the tomato genus (Miller and Tanksley, 1990). For the identification of novel traits such as insect resistance, tapping into the wild tomato germplasm can therefore be a viable, though challenging approach. There are 12 wild tomato species within the *Solanum* section *Lycopersicon*, relatives of *S. lycopersicum*, including *S. pennelli*, *S. habrochaites*, *S. cheesmaniae*, *S. galapagense*, *S. pimpinellifolium*, *S. neorickii*, *S. chmielewskii*, *S. arcanum*, *S. huaylense*, *S. corneliomulleri*, *S. peruvianum*, and *S. chilense* (Peralta, Spooner, Knapp, 2008). All tomato species appear to have glandular trichomes; glandular hairs producing a wide array of structurally diverse protective metabolites including terpenoids, acylsugars, methyl ketones and defence proteins like protease inhibitors and polyphenol oxidases (Glas *et al.*, 2012). Many such chemicals, commonly referred to as specialised metabolites (Pichersky and Lewinsohn, 2011), have been documented to play a role in direct and indirect defence against herbivorous insects. Still, only a few examples introducing trichome-based defence traits from wild to cultivated tomato have been documented (Bleeker *et al.*, 2012; Leckie, De Jong, Mutschler, 2012; Therezan *et al.*, 2021). For each of these examples, however, the transfer of the trait appears to be incomplete, e.g. by not producing the appropriate metabolite or by not producing sufficiently high levels to gain resistance levels as in the wild parent. As our full understanding about these traits is still lacking, up to date none of them have been commercialised yet.

Engineering for improved crop protection using wild resources

Metabolite-based insect resistance is complex, with the involvement of multiple biosynthetic and regulatory genes, each with their own contribution. When a trait involves multiple genes, the number of possible genotypes quickly increases, increasing the chance of losing (part of) the trait

in the breeding process. In addition, the resistance phenotype can vary depending on (a)biotic environmental factors and developmental stage of the plant and metabolites may have different (dose-dependent) effects depending on the insect species, developmental stage or behaviour. Therefore, in order to effectively introduce a metabolite-based resistance trait in tomato, or any plant, one should understand the biological mechanism and be able to connect it to the underlying genetics.

When using wild resources to improve defence, the first step is to find out which metabolite(s) is most effective against the insect to be targeted. Secondly, the biosynthetic pathway should be characterized biochemically, and their underlying genes should be identified. Specialised metabolites are often phytotoxic, especially when produced in high amounts and should therefore be transported, secreted and/or stored properly to avoid autotoxicity. Therefore, the third step is to define the necessary (cellular) physiological requirements for their biosynthesis. Finally, the new cultivar should be able to synthesise the specialised metabolites. Hence, epistatic effects, artefacts, unexpected feedback inhibition or unintended biochemical interactions could occur with the introduction of metabolic traits in domesticated crops.

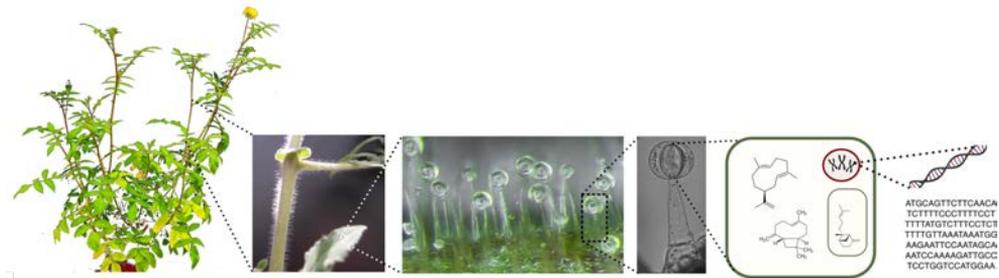


Figure 1. An illustrative overview of glandular trichomes on (wild) tomato plants. The stem-and leaf-surface of tomato plants are covered by trichomes and insect resistance found in wild tomato species can often be traced back to the production of specialised metabolites in glandular trichomes. As an example, type-VI glandular trichomes of a whitefly-resistant *Solanum habrochaites* predominantly producing terpenoids, are highlighted. These compounds are synthesised in the glandular cells on top of the trichome's stalk, as can be seen in the magnified photos, and secreted in the central storage-cavity to accumulate. Type-VI trichomes are therefore often referred to as 'oil bombs' as they hold large quantities of, often toxic, metabolites in an oily matrix. The genetic variation that exists amongst wild tomatoes results in divergent metabolic profiles in their trichomes which is used in this thesis to study the biosynthesis of anti-insect metabolites.

Defence from the wild: Thesis outline

In this thesis I will explore the fundamental characteristics of glandular-trichome based defences of wild tomato species and investigate the requirements for their transfer to cultivars. To this end the landscape of metabolite-based defences of plants will first be discussed in **Chapter 2**. Here, we will review the current knowledge on metabolite-based insect defences in plants. We will see that, although hundreds of metabolites have been identified that negatively impact pest insects (Nawrot and Harmatha, 2012), there are only a few examples known that show a well-founded metabolite-insect relationship in the field. We furthermore will go into detail about the synthesis of specialised metabolites, the known regulatory aspects, and the possibilities to utilise

these traits for crop protection. In **Chapter 3** we identified accessions with insect resistance and apply a machine learning approach to associate specialised metabolites with insecticidal properties using the natural variation in metabolite blends produced by glandular trichomes of wild tomatoes. After screening 19 different (wild) tomato accessions for their resistance to whiteflies and thrips, trichome densities and specialised metabolite profiles, we were able to pinpoint specific compounds likely to be involved in insect resistance. Equipped with this knowledge and tools, we applied the same approach to find toxic components in trichomes of the high-level sesquiterpene producing, and highly whitefly resistant, *S. habrochaites* accession PI127826 in **Chapter 4**. The outcomes of the study showed that from the metabolite blend of PI127826, an oxygenised sesquiterpene derivative is the most strongly connected volatile to the whitefly-resistance phenotype. In **Chapter 5** we characterise this molecule as 9-hydroxy-zingiberene (9HZ), an oxygenised form of the 7-epizingiberene, that is produced in PI127826 trichomes along with a second derivate 9-hydroxy-10,11-epoxyzingiberene (9H10epoZ). We show that the oxygenation of 7-epizingiberene (7epiZ) into these two molecules is performed by a single cytochrome P450 (*ShZO*) and that especially 9H10epoZ has a toxic effect on whiteflies. With the biosynthetic pathway for 9HZ and 9H10epoZ synthesis elucidated, and the aim to introduce this pathway into cultivated tomato, we investigated whether additional factors were required for high-level production of these compounds in glandular trichomes. In **Chapter 6**, we present the results from screening an interspecific F2 (sub)population, resulting from a cross between PI127826 and a *S. lycopersicum* elite breeding line, for high 7epiZ production. Testing different genetic models showed that the model involving 2 recessive and 2 dominant loci from PI127826 best fitted the observed segregation pattern of the “high terpene level” phenotype. The work furthermore uncouples glandular trichome density from productivity; two traits that seem to segregate independently and together determine the total quantity of metabolites produced. While studying the productivity of glandular trichomes, pathway inhibition assays indicate that the plastidial precursor pathway is essential to accumulate high levels of terpenes. In **Chapter 7** we describe a method to investigate glandular-trichome metabolism by way of genetic engineering. As a proof of concept, we modulated the metabolism in the terpenoid precursor pathway in the plastids of *S. lycopersicum* trichomes and obtained plants with an altered volatile bouquet which are less attractive for whiteflies. Finally, **Chapter 8** discusses the main results of this thesis. Here I will go into details about the metabolic and physiological characteristics that must be met for glandular trichomes to function as an effective defensive micro-organ.

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