Host-plant resistance to western flower thrips in Arabidopsis

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Thesis

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Chapter one

General Introduction

Manus P.M. Thoen

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Rise and Fall of the Thunderflies

Thrips (a.k.a. thunderflies) are minute slender piercing-sucking insects represented by over 5000 species in the order Thysanoptera. The western flower thrips, *Frankliniella occidentalis,* is a devastating pest insect on numerous crop species worldwide. Besides the direct damage inflicted by feeding on cell contents, they transmit plant-pathogenic viruses leading to significant yield losses. Exploring and understanding the genetic basis of plant resistance mechanisms to thrips can greatly benefit the development of thrips-resistant crops. However, for the most part resistance mechanisms at the molecular level are still unclear. Screening plants for resistance to insects is generally costly in terms of space, time and labor. Also, the need for efficient large-scale phenotyping is increasing now that next-generation sequencing has rendered a wealth of genomic information. Combining quantitative genetics with high-throughput phenotyping of thrips behavior could reveal new genetic markers and genes that underlie thrips resistance in plants. Breeding for host-plant resistance with the help of these markers may then lead to a durable contribution to solving the *F. occidentalis* problem. This chapter will introduce the mechanisms that plants can use to chemically defend themselves against thrips, current methods of phenotyping for host-plant resistance to thrips and the role of quantitative genetics in elucidating novel resistance mechanisms in plants.

Rise of *Frankliniella occidentalis*

Thrips are insects that belong to the order Thysanoptera within the superorder Paraneoptera. The scientific name Thysanoptera derives from the Greek words *thysanos* (fringed) and *pteron* (wing), referring to their typical wing structure, although they are sometimes alternatively called 'thunderflies', due to the observed swarming behavior of these tiny insects, triggered by thunderstorms in hot summers. The Paraneoptera lineage (also including the orders Hemiptera, Psocoptera and Phthiraptera) is characterized by the progressive development of haustellate mouthparts, adapted in different orders within the Paraneoptera branch for different feeding strategies (Buckman, Mound et al. 2013). Thrips are unique in this branch (and among all insects species for this matter) in having an asymmetrical feeding apparatus, consisting of three haustellate stylets derived from the two maxillae and the left mandible (depending on the species, the right mandible is reduced or completely lost during embryogenesis). These stylets are housed in a conical mouth opening and can pierce epidermal cells and pollen of plants to suck up their contents with the help from the cibarial pump (Lewis 1984, Kindt, Joosten et al. 2003). The empty cells fill with air and become reflective, making them recognizable as

'silver scars'. The western flower thrips (WFT, *Frankliniella occidentalis* (Pergande) (Thysanoptera : Thripidae)) has established itself as a world-wide pest in the last decades. This highly polyphagous thrips species causes direct feeding damage on many different important crop species, and also indirect damage by transmitting tospoviruses that cause considerable damage to plants (Hunter and Ullman 1989). Starting from North America, *F. occidentalis* spread to all other continents, except Antarctica, from the 1970s onwards (Kirk and Terry 2003). Western flower thrips are difficult to control with pesticides, partly due to their small size and thigmotactic behavior, which causes them to hide deep in the flowers out of reach of most sprayed chemicals. In addition, their rapid life cycle in combination with high pesticide pressures has resulted in the emergence of pesticide resistance in *F. occidentalis* in the last two decades. The haplo-diploid mode of reproduction of *F. occidentalis* may also enhance this process, as genes associated with pesticide resistance are fully expressed in the haploid males (Cloyd and Bethke 2011). Countering thrips solely on the basis of pesticide applications is, therefore, no longer an option. A more refined and holistic solution is needed, combining different strategies in an Integrated Pest Management (IPM) approach. Key in the successful application of IPM is the use of non-insecticidal tactics like biological control and host-plant resistance. Other additional tactics can for instance be found in insecticide resistance management (IRM) programs, like rotation in the use of different insecticides (with different modes of action) and the monitoring of thrips resistance and population sampling to determine proper implementation of insecticide applications (instead of spraying pesticides in a proactive manner) (Gao, Lei et al. 2012). Over the past few decades, much work has been done on the development of efficient biological control agents. Several effective natural enemies of *F. occidentalis*, like predatory mites, have proven their effectiveness in greenhouses, although the success rate of biological control agents is dependent on the plant species (Ramakers 1988, Messelink, Van Steenpaal et al. 2006). In addition to biological control, host-plant resistance is a cornerstone in IPM, yet molecular mechanisms underlying host-plant resistance to thrips are for the large part still unknown, hampering the development of host-plant resistant crop varieties. This general introduction will focus on ways in which plants can chemically defend themselves against *F. occidentalis*, the different methods currently applied to assess host-plant resistance towards thrips and the role of quantitative genetics in elucidating novel resistance mechanisms occurring in natural plant populations. Elucidating the molecular architecture behind host-plant resistance to thrips, should pave the way for breeding programs that can develop cultivars resistant to *F. occidentalis*.

Plant defensive chemistry against *F. occidentalis*

The fact that plants are sessile organisms does by no means imply that they are helpless victims. Although they are harassed in some shape or form by members of all kingdoms of life, it is exactly this harassment that has shaped and defined the unique defensive arsenal of plants. Plants have evolved morphological features that hinder herbivory, like trichomes and leaf waxes (Panda and Khush 1995, Schoonhoven, van Loon et al. 2005), but likely even more important are the diverse chemical defenses (Schoonhoven, van Loon et al. 2005(Mithöfer and Boland 2012) that have evolved under the selection pressure of herbivores. *Frankliniella occidentalis* is often studied in relation to economically relevant (ornamental) crops, and some of these studies have revealed chemical resistance in plants against thrips herbivory. In chrysanthemum (*Dendranthema grandiflora*) using TLC and HPLC, isobutylamide was proposed as a candidate compound involved in thrips resistance (Tsao, Marvin et al. 2005). A different study, using nuclear magnetic resonance (NMR)-based metabolomics showed that thrips-resistant chrysanthemum varieties contained higher amounts of chlorogenic acid and additional bioassays with artificial diets confirmed the negative effect of this phenylpropanoid on the survival rate of juveniles (Leiss, Maltese et al. 2009). Although chlorogenic acid occurs in high levels in carrot plants (*Daucus carota* L.), NMR did not show differences in cholorogenic levels between resistant and susceptible carrots. Instead, the flavonoid luteolin, the phenylpropanoid sinapic acid and the amino acid β-alanine were correlated with thrips resistance in this plant species (Leiss, Cristofori et al. 2013). Using several wild and cultivated tomato species, the presence of acylsugars was correlated with lowered thrips feeding damage (Mirnezhad, Romero-Gonzalez et al. 2010). Using LC-MS on nine different pepper (*Capsicum*) cultivars showed a correlation with several tocopherols, alkanes, a sterol and a terpene in relation to thrips resistance, although no bioassays were done to confirm the activity of these compounds (Awang 2013). Studies with the model plant *Arabidopsis thaliana* using transcriptomics and different knockout mutants, revealed a prominent role for the phytohormone jasmonic acid (JA) in thrips resistance. Mutant plants hampered in their JA signaling pathway, were not able to trigger induced defenses leading to higher susceptibility in two-choice assays (De Vos, Van Oosten et al. 2005). The function of JA in thrips resistance is not restricted to Arabidopsis but is likely a general mechanism underlying resistance of plants to thrips. In Chinese cabbage (*Brassica rapa*), JA also played a crucial role in the induced defenses triggered by *F. occidentalis* (Abe, Shimoda et al. 2009). Depending on the plant species and analytic tools used, different candidate compounds have been brought forward that play a potential role in thrips resistance. This is perhaps not surprising, given the polyphagous nature of *F. occidentalis.* Correlations between specific chemical

compounds and thrips resistance are frequently found, but even when these compounds are further tested in *in vitro* bioassays, a profound understanding of how these metabolites directly influence thrips behavior is still lacking for the most part. Testing different germplasm for chemical profiles will likely bring forward interesting compounds, but we should be careful in mixing up 'correlation' with 'causality', especially when the activity of compounds was not confirmed .

Current methods to phenotype thrips resistance

There are currently several methods to determine the resistance of host plant genotypes to *F. occidentalis,* based on assessing plant damage, population size, and mortality or by monitoring insect behavior in various choice assay formats or using Electro Penetration Graphs (EPG) (Kindt et al. 2003). When thrips feed on the epidermal cell layers of various plant tissues, they leave behind typical silvery feeding spots, often referred to as 'silver scars'. Plant damage in terms of the number or total area of 'silver scars' can be used as a proxy for host acceptance (Abe, Shimoda et al. 2009, Leiss, Cristofori et al. 2013). Population size and/or mortality rate of thrips can be used as an end-point measurement of resistance, using whole plants, or *in vitro* using detached plant parts (Outchkourov, de Kogel et al. 2004). Extracts from plants can be screened using micro-titer plates filled with plant extracts covered with parafilm, that thrips can easily pierce through to feed and oviposit (Leiss, Cristofori et al. 2013).

To dissect overall host-plant resistance into component traits, focusing on insect behavior instead of host-plant performance might be more effective (Kloth, Thoen et al. 2012). Complex traits are often multi-genic. Exposing these component traits could lead to stronger signals in genetic studies, because there could be less confounding of multiple genetic mechanisms within one phenotype. This highlights the potential of accurate high-throughput phenotyping of insect behavior on host plants. Both for volatile and non-volatile cues two-choice assays in a single open arena lasting no longer than 24 hours have been used regularly (Outchkourov 2004; Yang et al. 2013). Distributions of insects are scored by eye at specific time intervals. Volatile cues determine the initial choice, while non-volatiles influence insect distributions across both samples that depend quantitatively on the content of the non-volatile compounds. For constitutive deterrents it was shown that *F. occidenatlis* established a stable differential distribution within the first 4-6 hours of the experiment (Outchkourov 2004; Yang et al. 2013). To establish the exclusive role of volatiles, Y-tube olfactometer systems have been the preferred setup (de Kogel, Koschier et al. 1999, Koschier, de Kogel et al. 2000). Plant volatiles are passed through a

Y-shaped glass tube, and one thrips at a time is scored for its preference for either arm and odor source. As an alternative to a Y-tube olfactometer, flight behavior has been analysed using wind tunnels (Davidson, Butler et al. 2006). Both methods can accurately determine the initial choice of thrips individuals, but it is hard to determine what happens after thrips arrive on a host plant. Manual determination of thrips settlement over time can be used on whole plants or leaf discs to elucidate thrips behavior after initial host-plant selection. This could elucidate the role of additional aspects like plant structure, secondary metabolites and induced defences in relation to thrips behavior (Koschier, Hoffmann et al. 2007, Yang, Stoopen et al. 2013). If detailed information on probing behavior is required, Electro Penetration Graph (EPG) could be applied. This system uses an electrical circuit to 'visualize' the probing behavior of piercing/sucking insects. It was originally designed to monitor probing behavior of aphids. The studies with thrips suggest it is not a very reliable screening method of host-plant acceptance for this order of insects (Kindt, Joosten et al. 2003, Kindt, Joosten et al. 2006). In summary, existing methods to quantify thrips feeding damage, behavior or life cycle have not yet been automated in any way or are not suitable for automation. Yet there is a strong need for methods allowing parallel-unattended screening of natural variation in resistance to thrips.

Exploring natural variation in Arabidopsis for resistance to thrips

The metabolome of most plants is characterized by a huge variety of secondary chemical compounds that can have negative effects on insect herbivores. It has been estimated that the plant kingdom makes more than 200.000 specialized metabolites, which could have evolved in the response to particular ecological challenges (Mithöfer and Boland 2012). Morphological and chemical defenses are evolved traits with a selective advantage against local threats. It is, therefore, likely that specific populations of a species with a worldwide distribution have adapted their defensive traits to the local herbivores.

The model plant *Arabidopsis thaliana* is often studied in relation to insect herbivory, especially when research questions are centered on gaining insight in plant defense at the molecular level (De Vos, Van Oosten et al. 2005, Bidart-Bouzat and Kliebenstein 2011). The popularity of *A. thaliana* has several reasons, namely the vast amounts of molecular tools and mutant lines that are available and its practical use (small plants, small genomes, short generation time and selfing reproduction strategy). However, natural variation in *A. thaliana* is still an underexploited area in plant science (Alonso-Blanco and Koornneef 2000), despite the ample opportunity it offers to answer some of our most fundamental and exciting questions concerning how and why key traits in plant defense have evolved. More and more information

on the molecular biology of plants is becoming available in the current ~omics era. Entire populations of model plants like *A. thaliana* have been completely genotyped. Phenotyping such a population for the ability to cope with biotic stresses such as insect herbivory, allows genome-wide studies in relation to insect resistance. In this way, novel naturally occurring defense mechanisms and strategies may be discovered, that can be utilized for further deepening our understanding of plant defense in relation to insect herbivory. Studying the genetic architecture on a genome-wide scale can take place in pedigree based quantitative trait loci (QTL) mapping studies or population based association studies (Mitchell-Olds 2010).

Pedigree based linkage mapping (or QTL mapping) is the classical approach in finding QTLs that relate to the trait of interest. In general, it involves genotyping and phenotyping the F2 progeny of two well-characterized parents, in order to associate specific regions of the genome with the trait of interest. The recombination events of such a population are often limited, resulting in a low-resolution map wherein significant QTLs can still encompass a region of well over 1000 kb, covering hundreds of candidate genes. In addition, the genetic diversity captured by such a population is very narrow, when compared with the diversity on the species level. With the dropping costs of genotyping, Association Mapping with large natural plant population has been put forward as a promising tool, utilizing the recombination events of millions of years in high resolution mapping population to find QTLs underlying the trait of interest. Both approaches have their advantages and disadvantages (Mitchell-Olds 2010).

Throughout this thesis I have worked mainly on a population that consists of 349 wild *A. thaliana* accessions that have been collected worldwide (Baxter, Brazelton et al. 2010). This population was established to capture most of the genetic diversity in this species from a larger Arabidopsis panel from over 1000 accessions. Although many different Arabidopsis populations exist, I will refer to this specific population of 349 accessions as 'The Arabidopsis HapMap population' throughout this thesis. The Arabidopsis HapMap population has been genotyped, (initially for 250.000 single nucleotide polymorphisms (SNPs), but the majority of these accessions have by now been fully resequenced) bringing with it a promising tool for ~omics research at the population level. Since all these accessions and the associated genetic information are freely available, many different laboratories in plant science contribute and collaborate while working on this population. In the last few years, the first studies were published that used this population. For example, this population was used to identify ATPase3 as the primary determinant of natural variation in leaf cadmium levels (Chao, Silva et al. 2012). A more recent study was conducted on drought response in this population, revealing novel effector genes involved in proline accumulation (Verslues, Lasky et al. 2013). Using the

Arabidopsis HapMap population, the worldwide genetic diversity in cadmium levels and drought response was explored with GWA mapping on the publicly available SNP datasets. Both studies show the benefits of working with Arabidopsis in a GWA context, as reverse genetics approaches can be relatively easily applied to validate the functional genes underlying significant SNPs. However, complex biotic interactions are often multi-genic, and robust and multiple phenotyping tools should be used to find clear association signals and validate the function of the potentially many candidate genes that will be identified through GWA (Kloth, Thoen et al. 2012). It is becoming more and more clear that phenotyping large panels of plants is the bottleneck in understanding genotype-phenotype relationships in quantitative genetics studies (Cobb, Declerck et al. 2013). Robust phenotyping is a necessity for characterizing the genetic factors that contribute to quantitative phenotypic variation in host-plant resistance to insects. In this regard, phenotyping host-plant resistance to herbivorous insects will require collaborations across different disciplines (biology, engineering, statistics, software development etc.), in order to enable high-throughput phenotyping of large plant populations. The development of such high-throughput phenotyping platforms to screen for thrips resistance will stimulate progress in exploring the natural variation still undiscovered in the Arabidopsis HapMap population.

Fall of the thunderflies

Finding new natural ways in which plants defend themselves against thrips, and understanding these ways on the molecular level, could pave the way for breeding new resistant crop varieties. This will in its turn diminish the amount of pesticides that are currently used to protect crops, and make way for a more environmentally friendly and sustainable practice of agriculture (Broekgaarden, Snoeren et al. 2011). Perhaps one of the most important aspects of a successful management is recognizing that the complete control of *F. occidentalis* is impossible. However, breeding for host-plant resistance towards this pest insect can be an essential step to manage thrips within acceptable limits, where additional management can be achieved in combination with biological control. Here, I bring forward the idea of exploiting natural variation in thrips resistance in the Arabidopsis HapMap population, by ways of genome wide association mapping (Figure 1). Screening the Arabidopsis HapMap population for thrips resistance, could lead to the discovery of naturally occurring resistance mechanisms in an unbiased approach. Using *A. thaliana* as a model species to study thrips resistance means that we can use all the molecular tools, cutting edge technology and thousands of readily available mutants from the Arabidopsis community. It will give relevant insights in the genetic architecture behind insect

resistance. Homologs of promising candidate genes could be screened for in crop plants. Furthermore, the techniques developed by screening the Arabidopsis HapMap population, could be implemented in GWA studies on relevant crop species that have been genotyped. Techniques include the statistical and computational models used for GWA, and the techniques developed to accurately screen a population of this size in a quick and reliable manner. A challenge in unraveling the quantitative genetics of host-plant resistance for the coming years is not only to understand the genetic basis of this complex trait, but also translate this knowledge into breeding programs that can yield new crop varieties (Cobb, Declerck et al. 2013). A true 'Fall of the Thunderflies' is not attainable, but managing thrips in such a way that there are no significant economic losses in the near future, is possible when IPM is successfully applied. The effective use of biological control is important, but in the coming years there is still much potential in breeding for host-plant resistance as an important pillar in IPM. There is a diverse and vast arsenal of naturally occurring defense mechanisms in plants for scientists and breeders to explore. Utilizing these natural resources of host-plant resistance, by exploring natural variation in a GWA approach, has great potential for finding new effective plant defenses against *F. occidentalis*.

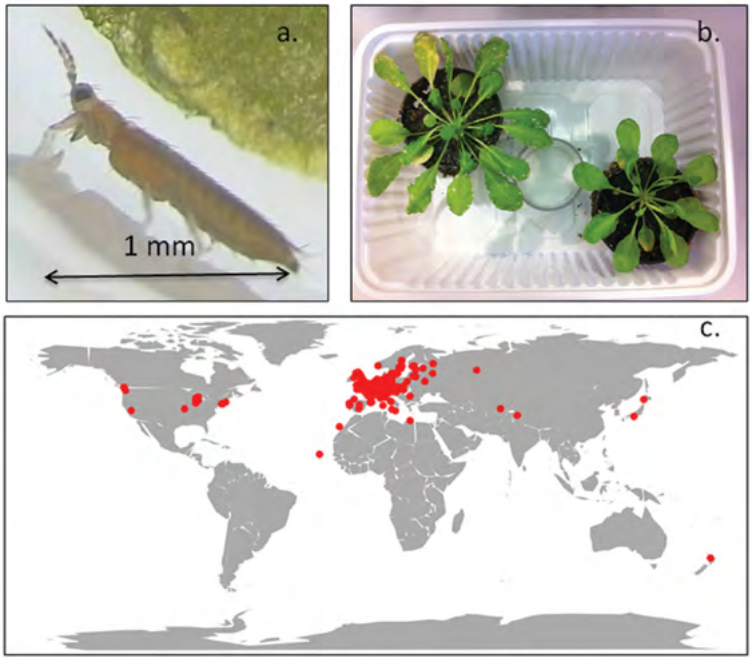


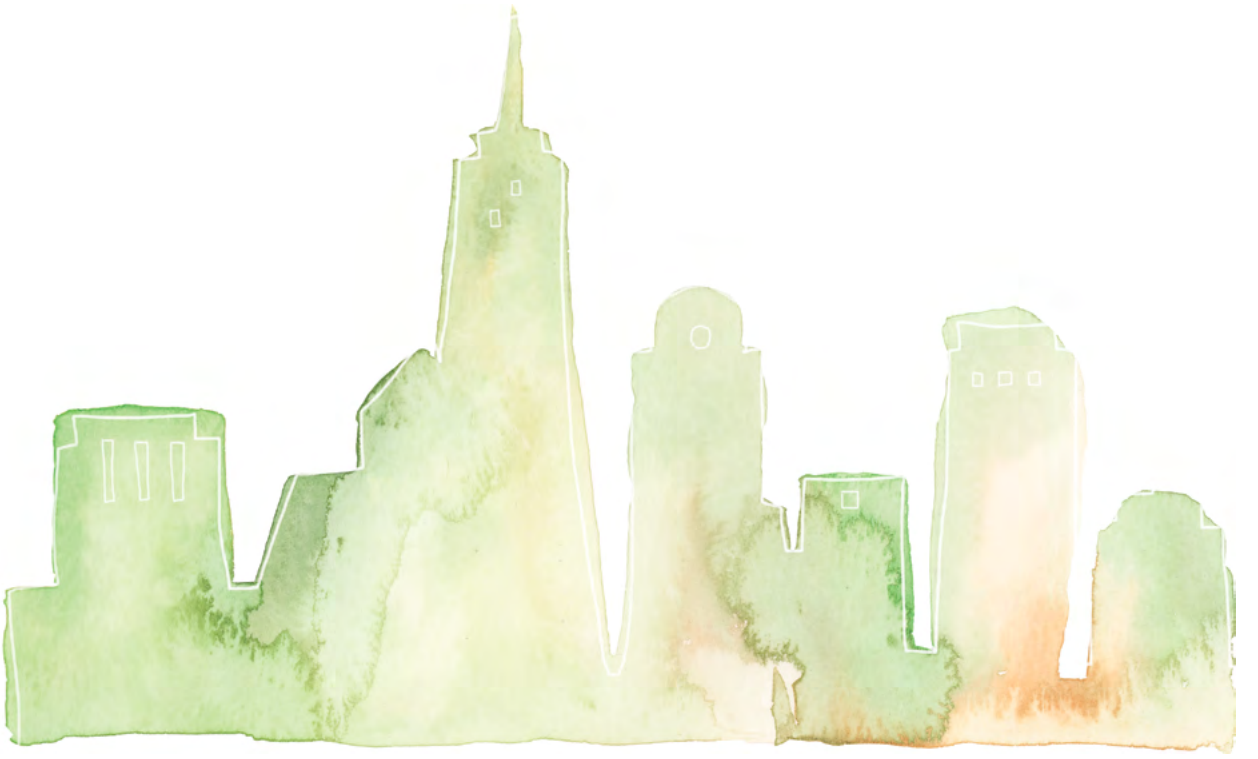
Figure 1. The study system. a. *Frankliniella occidantalis* in a micro titer two-choice leaf disc assay. **b.** *Arabidopsis thaliana* accessions in a two-choice thrips feeding damage assay. **c.** Origin of 349 accessions from the Arabidopsis HapMap population used in this thesis.

Scope and Thesis outline

This thesis is supported by The Netherlands Organisation for Scientific Research (NWO) through the Technology Foundation Perspective Program 'Learning from Nature' (STW10989). The 'Learning from Nature' program aimed to start collaborations among plant scientists with different fields of expertise. The Arabidopsis HapMap population is the center point of this collaboration, used by scientists in eight chair groups to study responses to eleven different individual stresses, and some combinations of these stresses. The main objectives that I address in this thesis are: (1) the development of a video-tracking platform to screen plants for resistance to *F. occidentalis*, (2) the identification and characterization of novel thrips-resistance genes and mechanisms in the Arabidopsis HapMap population.

Chapter 2 addresses the potential of genome-wide association (GWA) studies in discovering new genes that control host-plant resistance to insects. It discusses the prerequisites of attempting such studies, with a focus on the development of novel high-throughput phenotyping systems that can aid in the generation of reliable phenotypic information on large plant panels required for these studies. **Chapter 3** then demonstrates such a system, where we use EthoVision XT software to aid automated video tracking in 88-parallel two-choice leaf disc assays in microtiter plates, to assess host-plant resistance in Arabidopsis to *F. occidentalis*. This video-tracking system is used to screen the HapMap population for thrips preference. Two accessions from the HapMap population that are on opposite sides of the thrips susceptibility spectrum are used to optimize the phenotyping platform and to validate the platform with whole-plant and detached-leaf end-point assays. **Chapter 4** addresses an improved software package called EthoAnalysis that uses raw tracking data obtained from EthoVision XT to generate additional relevant behavioral analysis tools for investigating thrips behavior. Data from chapter 3 are re-analyzed with improved settings that accurately distinguish movement from non-movement events. The separate behavior events are further qualified based on velocity and duration of these events. The potential of this additional information is discussed in the light of quantitative genetic studies. **Chapter 5** explores stress resistance in the HapMap population on a much broader scale, including a total of 15 different biotic and abiotic stresses. Here we apply a multi trait genome wide association study to find shared patterns in stress tolerance between the different traits. **Chapter 6** uses the same 15 stresses in a comparison with a metabolomics dataset on this HapMap population. Here, we discover that levels of certain aliphatic glucosinolates correlate positively with the levels of resistance to thrips. This correlation is further investigated with the screening of a RIL (Recombinant Inbred Line) population for resistance to thrips, several knockout mutants and the analysis of co-localization

of mapping results between glucosinolates genes and thrips resistance. In **Chapter 7,** the general discussion, I describe prototypes of phenotyping platforms that can further aid screening for resistance to thrips in the future. Findings of Chapters 5 and 6 are discussed in a broader context, and some of the candidate genes are discussed in more detail for their potential role in resistance to thrips. Terpenoids and glucosinolates are given special attention in this chapter. The chapter concludes with suggestions for promising future research endeavors in the field of host-plant resistance to Western Flower Thrips.



Chapter two

Association mapping of plant resistance to insects

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Abstract

Association mapping is rapidly becoming an important method to explore the genetic architecture of complex traits in plants, and offers unique opportunities for studying resistance to insect herbivores. Recent studies indicate that there is a trade-off between resistance against generalist and specialist insects. Most studies, however, use a targeted approach that will easily miss important components of insect resistance. Genome-wide association mapping provides a comprehensive approach to explore the whole array of plant defense mechanisms in the context of the generalist–specialist paradigm. As association mapping involves the screening of large numbers of plant lines, specific and accurate High-Throughput Phenotyping (HTP) methods are needed. Here, we discuss the prospects of association mapping for insect resistance and HTP requirements.

Enhancing host–plant resistance against generalist and specialist insects

Host–plant resistance is one of the cornerstones of environmentally benign pest management systems (Panda and Khush 1995, Schoonhoven, van Loon et al. 2005). Devastating pests and diseases only rarely occur in nature, which is due to the tremendous degree of natural variation in plant defense mechanisms (Alonso-Blanco and Koornneef 2000, Anderson and Mitchell-Olds 2011). Only a relatively small degree of such variation is contained in cultivated crop populations (Gols, Bukovinszky et al. 2008), but wild populations provide ample opportunities for discovering novel mechanisms responsible for resistance to insects. A wide range of resistance mechanisms against herbivorous insects has been described (Panda and Khush 1995, Schoonhoven, van Loon et al. 2005), and the impact of mechanisms depends on the characteristics of the herbivore, such as insect diet breadth (Mewis, Tokuhisa et al. 2006, Rohr, Ulrichs et al. 2011). Although specialist insects, feeding on one or a few plant species within one family, are considered to be resistant to toxic compounds of their host (Karban and Agrawal 2002), generalist insects are thought to thrive on a wider range of hosts with relatively low levels of allelochemicals (Loxdale, Lushai et al. 2011, Price, Denno et al. 2011). Toxins, however, affect the performance of specialists as well (Vandenborre, Groten et al. 2010), and generalists can cope with variable levels of secondary metabolites (Loxdale, Lushai et al. 2011), implying a more complex relationship between insect host range and plant defense. More insight into plant defenses against specialist and generalist insects is needed to understand how plants deal with herbivorous insects that differ in the degree of specialization and to improve host–plant resistance of economically important crops against insect pests. Most studies have addressed this topic with a targeted approach, focusing on only one or a few types of secondary metabolites and a restricted amount of natural variation therein. In order to unravel the paradigm about resistance against specialists and generalists and to identify new plant defense mechanisms, comprehensive technologies are needed that can explore the apparent natural variation in multiple resistance mechanisms at the level of the genotype and phenotype.

Association mapping (see glossary) allows to screen many different wild and cultivated populations for genes involved in complex plant traits. Although association mapping has hardly been used in plant–insect studies thus far, it has the potential to allow new developments in eco-genomic studies of plant–insect interactions. One of the major prospects is the possibility to do genome wide association (GWA) mapping in order to retrieve functional genetic loci involved in plant defenses against herbivorous insects in an untargeted way. GWA mapping involves the screening of large

numbers of plant lines, which is currently a bottleneck because of the costs involved in this time- and labor-intensive methodology. The large number of plant lines to be screened in insect resistance studies will require High-Throughput Phenotyping (HTP) techniques that succeed in accurately identifying different resistance traits. Particularly in view of the high diversity in insect-resistance mechanisms and their degree of specificity towards their enemies, this will pose some challenges. In this review, we discuss the perspectives of GWA mapping and HTP techniques in the context of insect resistance, with special reference to strategies against specialist and generalist insects.

Glossary box

Association mapping: a population based method of mapping quantitative trait loci (QTLs) that takes advantage of historic linkage disequilibrium to link phenotypes to genotypes (also known as "linkage disequilibrium mapping").

Candidate gene: a gene, located in a chromosome region suspected of being involved in the expression of a trait of interest.

Confounding effect: an extraneous variable in a statistical model that correlates (positively or negatively) with both the dependent and independent variable.

Genome-wide association (GWA) mapping: comprehensive approach to systematically search the genome for causal genetic variation, using a large number of markers, by association between genotypes at each locus and a given phenotype.

High-Throughput Phenotyping (HTP): experimental set-up in which large amounts of specimens can be phenotypically screened, preferably automatic, fast, accurate, and with low costs.

Linkage disequilibrium: two loci that are in linkage disequilibrium (LD) are inherited together more often or less often than would be expected by chance.

QTL: Quantitative Trait Locus; a region in the genome that is responsible for variation in the quantitative trait of interest.

QTL mapping: a family based mapping method using well known pedigrees to generate F2 crosses in which the genetic architecture of traits can be explored (also known as traditional linkage mapping).

Quantitative genetics: the study of the heritability of quantitative traits, which are the products of two or more genes.

Association studies and linkage mapping

Understanding the genetic basis of phenotypic variation is one of the key goals in evolutionary biology. Family based QTL mapping (which uses well-characterized pedigrees (Balasubramanian, Schwartz et al. 2009, Brotman, Riewe et al. 2011, Dobón, Canet et al. 2011)) and association mapping (which uses linkage disequilibrium among numerous individuals of different populations (Atwell, Huang et al. 2010, Ingvarsson and Street 2011)) are the most commonly used tools for dissecting the genetic basis of phenotypic trait variation. In QTL mapping only a limited number of recombination events that have occurred within families and pedigrees can be studied, whereas with association mapping the recombination events that have accumulated over thousands of generations can be exploited (Zhu, Gore et al. 2008). Since the 1980's, QTL mapping has been used most frequently, but association mapping is a promising alternative method for dissecting complex traits (Chan, Rowe et al. 2010, Chan, Rowe et al. 2011). Increased mapping resolution, reduced research time, and larger allele numbers have been put forward as main advantages over traditional QTL mapping (Yu and Buckler 2006, Zhu, Gore et al. 2008). Association studies can be divided into two broad categories: (i) candidate-gene association mapping, in which variation in a gene of interest is tested for correlation with the phenotypic trait of interest, and (ii) Genome-Wide Association (GWA) mapping, where genetic variation is explored within the whole genome, aiming to find signals of association with the complex trait (Zhu, Gore et al. 2008) (see Table 1 for an overview). Because GWA mapping is less dependent on prior information about candidate genes than QTL mapping and candidate-gene association mapping, this is a promising method to identify novel loci involved in complex phenotypic traits. However, GWA mapping should not be regarded as a replacement of traditional QTL mapping. In fact, GWA mapping and QTL mapping have complementary advantages and disadvantages, that can lead to a better understanding of causal genetic polymorphism when these approaches are combined (Chan, Rowe et al. 2010, Mitchell-Olds 2010).

Table 1. Comparison of family based (QTL) and population based (association mapping) methods that aim to unravel the genetic basis of complex traits in plants

	QTL mapping	Candidate gene association mapping	Genome wide association mapping
Main advantages	- No population structure effects - Identification of rare alleles - Few genetic markers required	- Allows fine mapping - Relatively low costs	- Allows untargeted fine mapping (blind approach) - Detection of common alleles
Main disadvantages	- Limited genetic diversity - Not always possible to create crosses - Cannot distinguish between pleiotropic and physically close genes	- Detailed functional knowledge of trait is required - No novel traits will be found	- Confounding effects due to population structure - Will miss rare and weak effect alleles
General requirements	- Small 'original population size', low number of genetic markers, many replicates needed - Generated mapping material (eg. F2 population, (AI-)RILs, MAGIC lines, NILs, HIFs etc.)	- Large population size, small number of genetic markers, the bigger the population size, the less replicates needed - Prior genetic and biochemical knowledge on trait of interest - Prior knowledge on LD, nucleotide -polymorphism, breeding system and population structure	- Large population size, many genetic markers, the bigger the population size, the fewer replicates needed - Prior knowledge on LD, nucleotide -polymorphism, breeding system and population structure
Recent case study in *Arabidopsis*	QTL mapping with AI-RILs on flowering time (Balasubramanian, Schwartz et al. 2009) - 2 AI-RIL populationS (approximately 280 individuals each) - 181 and 224 markers - 12 to 70 replicates	Candidate gene approach on flowering time (Ehrenreich, Hanzawa et al. 2009) - 251 accessions - 51 SNPs - 10 replicates per accession	Whole genome approach on multiple phenotypic traits (Atwell, Huang et al. 2010) - 199 accessions in total - 216.150 SNPs - 4 replicates in general

QTL; Quantitative Trait Locus, RIL, AI-RIL, Advanced Intercross-Recombinant Inbred Line; MAGIC, Multiparent Advanced Generation InterCross; NIL, Near-Isogenic Line; HIFs, Heterogeneous Inbred Family; LD, Linkage Disequilibrium; SNPs, Single Nucleotide Polymorphisms. Combinations of these three approaches can allow the identification of false positives and negatives, but is much more laborious: a recent dual QTL mapping-GWA study (Brachi et al. 2010) involved phenotyping nearly 20,000 individual plants, including 184 worldwide natural accessions genotyped for 216,509 SNPs and 4,366 RILs derived from 13 independent crosses. See (Bergelson and Roux 2010) for an overview of different linkage mapping populations mentioned in this table.

Association mapping in plant sciences

In the last decade, GWA mapping has emerged as a tool for studying the genetics of natural variation and economically important traits in plants (Atwell, Huang et al. 2010). Flowering time, chemical composition, disease resistance, taste and many other economically and evolutionarily important traits have been studied in crop species (see (Zhu, Gore et al. 2008) for an overview). Apart from agriculturally relevant crops, the model plant Arabidopsis (*Arabidopsis thaliana*) is of great value for understanding complex traits using GWA mapping (Box 1).

The presence of recombination events that have accumulated in plants over thousands of generations, is both an advantage as well as a potential pitfall of GWA mapping, because functional QTLs that are correlated with population structure can result in many false positives (Mitchell-Olds 2010). Several statistical methods have been developed that use neutral genotypic information to account for confounding effects of population structure in GWA studies (Price, Patterson et al. 2006, Yu, Pressoir et al. 2006, Zhao, Aranzana et al. 2007). However, inadequate use of these models can lead to over-correction, resulting in false negatives which are equally problematic (Mitchell-Olds 2010). Studies that have combined GWA- and QTL mapping strategies (dual linkage-association mapping) pointed out a false-positive rate of 40% and a false-negative rate of 24% in assays that solely involved GWA mapping (Bergelson and Roux 2010). A major drawback of such a dual linkage-association mapping, however, is that it requires phenotyping of several thousands of individual plants, and the genesis of numerous linkage mapping populations (Brachi, Faure et al. 2010). GWA mapping in regional mapping populations (instead of GWA mapping at the species scale) is an alternative approach to reduce confounding due to population structure (Bergelson and Roux 2010).

Another major impediment in GWA studies is the phenomenon of missing heritability. Often, the associated QTLs can explain very little of the phenotypic variation, even after accounting for the effects of population structure. This phenomenon is attributed to several factors, including a scattered signal across numerous QTLs, each contributing to only a marginal proportion of the phenotype. Complex traits, such as insect resistance, are likely to encounter this problem (Visscher 2008, Myles, Peiffer et al. 2009). Integrating association mapping with transcriptional network analysis can decrease high false-positive rates and increase the resolution in scattered associations (Chan, Rowe et al. 2011). The scattering of genotype–phenotype associations can also be reduced by phenotyping multiple component traits instead of one multifactorial trait, as will be further discussed in the paragraph ‘Requirements for phenotyping’.

Box 1. *Arabidopsis*-insect interactions as a model for GWA studies

The model species, Arabidopsis (*Arabidopsis thaliana*), is often used in plant–insect studies for obvious reasons, such as the availability of extensive information about genetic variation and physiology, and numerous mutants. Even though Arabidopsis is not a crop, there are numerous devastating crop pest insects (such as the generalist insect herbivores *Frankliniella occidentalis* and *Myzus persicae* and the specialist insect herbivores *Pieris rapae, Plutella xylostella* and *Brevicoryne brassicae*) that readily feed on Arabidopsis (De Vos, Van Oosten et al. 2005, Abe, Ohnishi et al. 2008, De Vos and Jander 2009, Bruessow, Gouhier-Darimont et al. 2010, Bidart-Bouzat and Kliebenstein 2011). However, one disadvantage in the light of insect-plant biology is that many accessions of Arabidopsis are winter annuals, so the life cycle of Arabidopsis does not temporally overlap with the life cycle of many herbivorous insects. It is known that herbivore performance (quantified in terms of mortality and developmental time) is commonly better on plants with such a 'pausing' strategy, indicating that such plants may invest less in defense traits (Van Poecke 2007). This has likely influenced the evolution of signaling pathways in Arabidopsis, because the main biotic stresses likely comprise pathogens such as oomycetes, bacteria and fungi. Still, Arabidopsis is of great interest for studying insect resistance, since many insect defense mechanisms have been evolved within the Brassicaceae family, such as glucosinolates (Mewis, Appel et al. 2005, Mewis, Tokuhisa et al. 2006, Rohr, Ulrichs et al. 2011), and, many defense mechanisms against pathogens are also effective against herbivorous insects. Leaf toughness is for example effective against both microbial pathogens and insects (Schoonhoven, van Loon et al. 2005), and salicylic acid-, jasmonic acid- and ethylene-regulated defenses are involved in defenses against both pathogen and insect infestations (De Vos, Van Oosten et al. 2005, Mewis, Tokuhisa et al. 2006, Pieterse, Leon-Reyes et al. 2009, Verhage, Vlaardingerbroek et al. 2011).

Association mapping of plant–insect interactions

The complexity in the orchestration of insect resistance and its evolution in plants, makes it a difficult trait to study in a genomic context (Anderson and Mitchell-Olds 2011). So far, only few GWA studies have been reported that deal explicitly with plant-defense mechanisms against herbivorous insects (see (Atwell, Huang et al. 2010) for an example on aphids). One such study on glucosinolates (GSLs) - secondary defense metabolites within the Brassicaceae family involved in resistance

against herbivorous insects (Mewis, Appel et al. 2005, Mewis, Tokuhisa et al. 2006, Rohr, Ulrichs et al. 2011) was conducted using 96 Arabidopsis accessions exhibiting 43 distinct GSL phenotypes and 230.000 SNPs (Chan, Rowe et al. 2010). In this study, GWA analysis successfully identified two major polymorphic loci controlling GSL variation in natural populations, but variation in resistance to specialist and generalist insects remains to be investigated for these accessions. This would require an experimental setup in which GWA mapping and HTP of insect resistance are integrated (Figure 1).

GWA mapping of insect resistance will likely encounter similar obstacles as recognized in other GWA studies. Because insect resistance is generally under strong positive selection pressure, GWA mapping of insect resistance might, however, unlike GWA studies of human diseases (Myles, Peiffer et al. 2009, Ingvarsson and Street 2011), be less affected by rare alleles that are not included in the haplotype map. Nevertheless, a good representation of all (sub)populations is indispensable for detecting variation in host–plant resistance and preventing them from having a too low allele frequency in the experimental set up. Particularly, the confounding effects of population structure can have a large effect on the success of GWA studies of host–plant resistance, because resistance against specific insects could have evolved independently and be based on different mechanisms in different populations and habitats (Poelman, van Loon et al. 2008). Moreover, confounding effects due to strong population differences can be severe, when an intense evolutionary arms race between plant and herbivore has occurred as may be the case for specialist herbivorous insects and their host plants (Thompson 2005, Becerra 2007, Poelman, van Loon et al. 2008, Vermeer, Dicke et al. 2011). This will require statistical correction of population structure, which can enhance the chance of false negatives due to over-correction. This problem is expected to be less evident with generalists, because they lack a reciprocal evolutionary interaction with specific plants (Price, Denno et al. 2011).

Resistance against specialist versus generalist insect herbivores

Specialist and generalist insect herbivores have different ways to deal with the defensive mechanisms of their host plants, and this is expected to result in different associations. Besides morphological and structural aspects, chemical defenses involving secondary metabolites play a major role in plant defense against insects (Schoonhoven, van Loon et al. 2005). Secondary metabolites can be divided into two broad functional categories, based on their modes of actions: qualitative compounds, which can be interpreted as toxins, and quantitative defensive

compounds, with a dose-dependent effect, such as digestibility reducers (Price, Denno et al. 2011). Recent studies show that qualitative compounds (e.g. GSL and alkaloids) often fail to affect specialist insects, because specialist insects evolved ways to detoxify or tolerate these compounds (Price, Denno et al. 2011). In other words, if secondary metabolites play a role in defense against specialist insects, predominantly quantitative defensive compounds that reduce the digestibility are expected to be functional, whereas defense against polyphagous insects is mainly achieved by qualitative compounds. Toxins are even used by specialist insects to locate their host plants, or sequester these toxins for their own defense (Panda and Khush 1995, Schoonhoven, van Loon et al. 2005). Thus, plants have to 'choose' between investing in substantial concentrations of qualitative compounds to deter polyphagous insects, or marginal concentrations of the same compounds to decrease preference by specialist insects (Van der Meijden 1996, Poelman, Van Loon et al. 2010). The evolution of defensive traits against generalists could, therefore, lead to an increased host–plant preference by specialists and vice versa. This trade-off between resistance to specialists and generalists is expected to be reflected in genotype–phenotype associations of the host plant.

There are, however, many examples that do not support the qualitative–quantitative dichotomy. The generalist aphid *Myzus persicae* feeds on herbaceous plants in over 40 plant families, including families such as the Solanaceae that are well-known producers of toxic alkaloids (Blackman and Eastop 2006, Loxdale, Lushai et al. 2011). Moreover, specific toxins do affect specialist herbivores. For instance, silencing nicotine production in tobacco (*Nicotiana tabacum*) results in improved performance of the specialist herbivore *Manduca sexta* (Steppuhn, Gase et al. 2004) and overexpression of the lectin agglutinin in tobacco negatively affected the larval performance of *M. sexta* (Vandenborre, Groten et al. 2010). Isothiocyanates, breakdown products of GLS, negatively affect the performance of the specialist herbivore *Pieris rapae* (Agrawal and Kurashige 2003). The performance of *P. rapae* on the *coi1* mutants of Arabidopsis, that is compromised in the JA signal-transduction pathway, is significantly improved in comparison to wild-type plants, showing that even a specialist is affected by inducible plant defenses (Reymond, Bodenhausen et al. 2004). Interestingly, the effects of quantitative and qualitative defenses may interact: nicotine prevents a compensatory response of the generalist herbivore *Spodoptera exigua* to proteinase inhibitors and thus counters an insect adaptation to a qualitative defense (Steppuhn and Baldwin 2007).

The main deficiency in addressing the defense mechanisms of plants against specialist and generalist insect herbivores, is that most studies have used a targeted approach, focusing on only one or a few types of secondary metabolites in a limited number of plant lines. Because resistance and tolerance are likely to be phenotypic

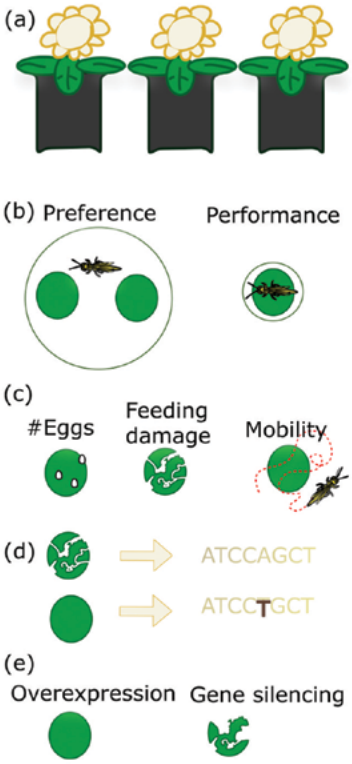

Figure 1. Screening plants for insect resistance through GWA mapping. This simplified overview shows how the genetic architecture underlying insect resistance can be determined in five steps, using GWA mapping. (a) Genotype SNPs for numerous accessions of the plant of interest; (b) Develop HTP choice and no-choice experiments to screen for insect preference and performance (using leaf discs in this example); (c) Screen for relevant insect-resistance parameters; (d) Find the genetic basis of phenotypic differences, using GWA mapping; (e) Validate candidate genes with reverse genetic tools, like overexpression and gene-silencing

traits that are composed of multiple factors, a targeted approach will easily miss important components. This is true for resistance to both generalists and specialists, but comparing the components and their relative strength of resistance to specialists and generalists may reveal how these traits are balanced.

A more comprehensive approach is, for example, taken in transcript profiling studies, where gene-expression signatures of infested plants and/or herbivorous insects are analyzed in different treatments (Reymond, Bodenhausen et al. 2004, Bidart-Bouzat and Kliebenstein 2011). Although several studies did not find a different plant response to specialist and generalist insects (Reymond, Bodenhausen et al. 2004, Bidart-Bouzat and Kliebenstein 2011), one study (Govind, Mittapalli et al. 2010) found a differential response in the insects that foraged on wild-type and mutant *Nicotiana attenuata*. The specialist *M. sexta* showed diet-specific alterations in gene expression, whereas the generalist *Heliothis virescens* regulated similar transcripts over different diets, indicating that the specialist is better adapted to both qualitative (nicotine), and quantitative (trypsin protease inhibitor) compounds of the host (Govind, Mittapalli et al. 2010). Another explorative approach is taken in a recent study, where metabolite fingerprints of *Plantago lanceolata* leaves differed

after they were attacked by specialist or generalist herbivores, and by insects belonging to different taxa (Sutter and Muller 2011). These examples show that untargeted approaches, such as transcript profiling, metabolic fingerprinting, and GWA mapping, allow to explore a large array of plant defense mechanisms in many plant lines.

Requirements for phenotyping

Phenotyping is a prime factor in GWA mapping of host plant resistance. Among vast numbers of genome-wide markers, the aim is to achieve significant statistical power for only those molecular markers that are located close to the genes that influence the phenotypic trait of interest. In reality, functional associations between phenotype and molecular markers are often confounded, both in association and QTL mapping studies (Aranzana, Kim et al. 2005, Chan, Rowe et al. 2010, Nemri, Atwell et al. 2010).

In the discussion about missing heritability of associations, where the identified genetic loci explain only little of the phenotypic variation, little attention has been paid to the role of phenotypes and phenotyping techniques. Some association studies of crop yield, for example, resulted in the characterization of numerous minor functional genes (Schon, Utz et al. 2004). This confirms the infinitesimal model of Fischer (Fischer 1918), which assumes a very large number of loci to be involved in quantitative genetics, each with a marginal effect on the phenotype. It is to be expected, however, that the number of functional (low-effect) QTLs involved is trait-specific. A complex trait is generally the result of numerous processes, which will result in a scattered association across multiple genetic loci: numerous QTLs are involved, that have a reduced statistical significance and each contribute to only a marginal proportion of the effect size of the phenotypic variation (Figure 2). Although a multifactorial character is inherent to complex traits, the efficiency of association mapping can be optimized by dissecting the phenotype into quantitative components with a minimum expected number of responsible mechanisms (Li, Yan et al. 2011). A genome-wide screening within the scope of only a few mechanisms attributing to the trait of interest will increase the success of finding novel functional genes. A drawback is that it narrows the scope of a genome-wide survey. Complex traits are generally based on gene networks; therefore the assessment of individual components will likely overlook interactions between components, and the network as a whole and its environment (Hammer, Cooper et al. 2006, Benfey and Mitchell-Olds 2008).

In insect resistance studies, typically multiple traits are phenotyped and reduced to one resistance variable, R. Most often, the total of life-history parameters of the insect

are summarized in the variable r_m, the intrinsic rate of population increase (Krips, A. et al. 1998, Awmack and Leather 2002). This summary statistic is an accurate parameter of the effect of resistance mechanisms on the herbivorous insects. However, insect performance is typically dependent on multiple plant traits (e.g. nutritional components of the host plant and multiple resistance mechanisms of the plant (Awmack and Leather 2002)). Hence, r_m may lack resolution in association studies and using this parameter may result in a high proportion of missing heritability due to scattered signals (Figure 2). We expect that dissecting the complex parameter in multiple specific phenotypic components, e.g. host preference, time interval before the insect starts feeding, reproduction, larval development time, and mortality, will contribute to solving the problem of missing heritability and will help to identify multiple underlying mechanisms (Figure 2). The combination of these individual mechanisms will ultimately allow plant breeding to achieve sustainable host-plant resistance in crops. Indeed, multi-parameter approaches, using a combination of phenotypic traits, for example both concentration of secondary metabolites and insect performance, have been postulated to deliver more significant relations to functional genetic data (Benfey and Mitchell-Olds 2008, Eberius and Lima-Guerra 2009). Apart from the parameterization of the phenotype(s), increasing the number of plant lines is of major importance for the statistical support of relevant associations (Myles, Peiffer et al. 2009, Ingvarsson and Street 2011). So far, most studies have used sample sizes of approximately 100 to 500 plant lines, but more genetic lines will increase the number and frequency of functional alleles and thereby improve the statistical power to detect them (Aranzana, Kim et al. 2005, Zhao, Aranzana et al. 2007, Kang, Zaitlen et al. 2008, Chan, Rowe et al. 2010, Li, Huang et al. 2010). Secondly, a larger number of replicates within plant lines will increase the accuracy of the phenotype and the statistical support of genotype-phenotype associations. Particularly phenotyping insect resistance, involving the interaction among two or more organisms and species, is sensitive to stochastic errors and could result in relatively high levels of missing heritability. Although there is an example of successful GWA mapping by assessing aphid offspring in four replicates on 96 Arabidopsis lines (Atwell, Huang et al. 2010), more replicates will reduce confounding effects. Moreover, the quality of phenotypic data can be improved by eliminating noise induced by the environment (Benfey and Mitchell-Olds 2008, Hall, Tegstrom et al. 2010). Many studies have shown that insect resistance is an adaptive response to several biotic and abiotic factors (Box 2) (Ballaré 2009, Holopainen and Gershenzon 2010, Poelman, Van Loon et al. 2010, Chan, Rowe et al. 2011). For example, it has been shown that the developmental stage of the plant altered the outcome of the GWA analysis, resulting in the identification of different functional genetic loci in different developmental stages (Chan, Rowe et al. 2011). This underlines the need for an experimental setup with uniform conditions among

the genetic lines (Figure 1). Some noise will be inevitable for plant species harbouring a high diversity of ecotypes that differ in optimal growth conditions and development time. On the other hand, 'uniform' laboratory assays can deliver functional associations different from field conditions (Atwell, Huang et al. 2010) due to genotype-by-environment interactions (Hammer, Cooper et al. 2006). Including several (a)biotic treatments or an additional field assay could yield more field-predictive outcomes.

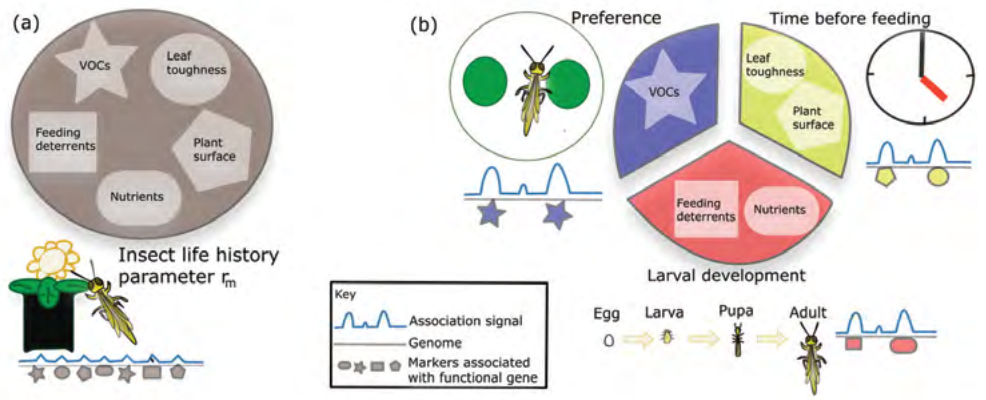

Figure 2. Dissecting insect resistance into component traits. Association mapping of a complex trait such as insect resistance can result in numerous associations with low statistical power. This is illustrated in (a) where the life history parameter r_m of the insect is associated with many genetic loci. One approach to improve resolution in genotype-phenotype associations, is to dissect the complex phenotype into component traits (b), e.g. insect preference (detection of repellent VOCs), time before the insect starts feeding (screening for the influence of leaf toughness and deterrent structures on the plant surface), and larval development (detection of e.g. feeding deterrents, toxins and nutrient content). Whereas the genetic architecture can overlap to some degree due to similar underlying processes, mapping these component traits will result in fewer genotype-phenotype associations with larger statistical power, and a higher proportion of functional associations. Genotype–phenotype associations can be further elucidated with, for example, metabolite fingerprints of VOCs, plant tissues or epicuticular waxes.

High-throughput phenotyping

For quantitative traits such as insect resistance, reliable phenotyping requires a substantial amount of space, time and manpower, and this will be increasingly so in the context of association studies that require large sample sizes. There is, thus, a need for HTP methods that are accurate and yet predictive of field performance. Particularly, in view of the differential impact of mechanisms to specialist and generalist insects as discussed earlier, insect and plant performance are not necessarily correlated with each other, as high levels of deterrent compounds do not always negatively affect the performance of herbivorous insects (Van der Meijden 1996, Poelman, Van Loon et al. 2010), and good insect performance does not always result in reduced plant performance (Box 2).

Box 2. Plant resistance to herbivorous insects

Host–plant resistance against herbivorous insects is generally defined as "the relative amount of heritable qualities possessed by the plant which influence the ultimate degree of damage done by the insect in the field" (Panda and Khush 1995). Herbivorous insects use host plants for oviposition, feeding and shelter. Plants can achieve protection against herbivorous insects by both indirect defense, i.e. the attraction and facilitation of natural enemies of the insect herbivore, and direct defense against the pest insect (Dicke 1999, Heil 2008, Dicke and Baldwin 2010). Three main categories of resistance against insect herbivores are (i) antixenosis, (ii) antibiosis, and (iii) tolerance (Panda and Khush 1995).

Antixenosis mechanisms deter the insect, or, after the insect has arrived on the plant, prevent it from settling. Generally, the insect 'decides' not to colonize the plant due to the absence or low availability of an attractant, or the presence or quantity of a deterrent. A wide range of components can act as attractants or deterrents: Volatile Organic Compounds (VOCs), color, topology of the plant, chemicals and morphology of the plant surface (e.g. trichomes, epicuticular waxes, substrate texture), and physical and chemical characteristics of internal plant tissues (e.g. secondary metabolites, nutrient content, toughness of the cell wall) (Schoonhoven, van Loon et al. 2005). Herbivorous insects use olfactory and visual cues in the pre-alighting stage, and assess olfactory, visual, tactile, and gustatory traits after arriving on the host plant. Plants that exhibit antixenosis have a reduced number of initial colonizers and a relatively small population of herbivorous insects.

After the insect has 'decided' to utilize the host, antibiosis mechanisms of the host can affect insect performance (e.g. growth, development, reproduction, and survival) by toxins released after tissue damaging, feeding deterrents (e.g. protease inhibitors), nutritional imbalance or tissue toughness. Antibiosis causes a decrease in the insect population size (Panda and Khush 1995). Plants can display antixenosis and antibiosis mechanisms constitutively, or after induction by e.g. herbivory or egg deposition (Dicke 1999, Hilker and Meiners 2006).

Tolerance represents the plant's ability to compensate insect damage by increased growth, reproduction or repair of the damage. In contrast to antixenosis and antibiosis, tolerance does not severely affect the insect herbivore, but rather minimizes the impact of herbivory on the performance of the plant itself (Panda and Khush 1995, Schoonhoven, van Loon et al. 2005).

Therefore, both plant and insect traits are relevant for assessing the underlying mechanisms of insect resistance. Because association mapping requires at least hundreds of plant lines to be screened, it poses some challenges to the phenotyping efforts. Below some potential HTP techniques for assessing insect resistance are discussed.

High-throughput phenotyping of plant defense

In the last decades, plant phenotyping techniques have gone through major developments (Montes, Melchinger et al. 2007, Fernie and Schauer 2009). Several of these methods can be applied to detect antixenosis, antibiosis or tolerance against insects and the benefits and costs involved for the plant (Box 2). Metabolite profiling techniques, like mass spectrometry and nuclear magnetic resonance, are the most obvious methods for screening primary and secondary proteins and metabolites in large-scale experiments (Fernie and Schauer 2009). However, image processing techniques are also highly suitable for HTP platforms (Montes, Melchinger et al. 2007, Fernie and Schauer 2009, Kokorian, Polder et al. 2010). These techniques translate changes in the spectral signature of a plant to quantify characteristics concerning plant growth, yield and (a)biotic stress. In the visible spectrum it is possible to detect damage caused by leaf chewing insects or for example silver damage due to thrips feeding (Abe, Ohnishi et al. 2008). Multi-colour fluorescence imaging has been used to assess feeding damage of mites and stylet penetrations of whiteflies (Buschmann and Lichtenthaler 1998). In the near-infrared spectrum, stress-related changes in plants and changes in organic compounds can be detected (Rutherford and vanStaden 1996, Chaerle and Van der Straeten 2000, Kramer, Morgan et al. 2000, Cozzolino 2009).

High-throughput phenotyping of insect performance and preference

Assessing insect performance rather than that of the host plant, delivers the opportunity to study the direct and indirect impacts of plant nutritional quality and defense mechanisms on the dynamics of the herbivore population (Awmack and Leather 2002). Although a wide variety of insect phenotyping techniques is available, only a marginal portion of these techniques is translated into high-throughput devices. This field in particular faces some challenges in developing methodologies that have low demands in terms of space, time and labor but are yet accurate and predictive of field performance.

Most insect studies have focused on insect performance; e.g. population density, insect growth, development rate, fecundity, survival and the intrinsic rate of population increase (r_m) (Awmack and Leather 2002). These parameters are correlated to both antixenosis and antibiosis. Assessing insect performance can be time consuming, depending on the generation time and life cycle of the insect, and is usually done in a non-automated way (Krips, A. et al. 1998, Poelman, Galiart et al. 2008). Image analysis of photographs or videos represents potential for automated indexing of insect performance parameters (e.g. the number of eggs, larvae and surviving adults).

A behavioral assay can, in contrast to just monitoring insect performance or plant traits, result in a detailed chronological dataset of the process of host selection and food uptake. An additional advantage is that a behavioral assay can potentially be much shorter than an end-point measurement of reproduction and survival (Hardie, Holyoak et al. 1992, Foster, Denholm et al. 2005). Food uptake is an important aspect of insect behavior, related to insect performance and host–plant resistance (Awmack and Leather 2002). Electronic monitoring of probing behavior in piercing-sucking insects has proven to be successful in finding feeding deterrents (Tjallingii and Hogen Esch 1993, Backus and Bennett 2009), but is hardly feasible in large-scale experiments necessary for association mapping. Alternatively, automated tracking of insect behavior allows to measure multiple factors involved in host selection: e.g. host preference, mobility of the insect, and the timing and duration of food uptake. An additional advantage is that it allows to screen the behavior of multiple individual insects and multiple arenas simultaneously (Allemand, Pompanon et al. 1994, Noldus, Spink et al. 2002, Reynolds and Riley 2002, Beeuwkes, Spitzen et al. 2008, Pistori, Viana Aguiar Odakura et al. 2010, Lacey and Carde 2011). The major challenge of high-throughput video-monitoring of insect behavior is to realize two- or three-dimensional arenas predictive of field performance (Prasifka, Hellmich et al. 2010). In large field trials a mark-release-recapture technique can be a cost-effective method to assess host preference and population growth of insects (Hagler and Jackson 2001). Ultimately, the choice of a phenotyping technique will largely depend on the study system and research focus.

Future perspectives

The development of accurate and field-predictive HTP will allow GWA mapping to increase insight into the genetic architecture of plant resistance to generalist versus specialist insects that will contribute to the development of host-plant resistance in crops. ‘Blind’ screening, unbiased by parental phenotypes and candidate genes, is the basis of this method and opens the opportunity to analyze the full scope

of existing natural variation in resistance mechanisms. Although current studies mainly focus on one or a few candidate mechanisms, the untargeted nature of GWA mapping will include multiple factors that contribute to resistance against generalist and specialist herbivores. We expect that the current assumptions about differential resistance mechanisms against specialists and generalists can be addressed more comprehensively using such an unbiased approach. A further step forward will be the integration of association mapping with transcriptomics, proteomics and metabolomics, to assess insect resistance at the levels of the genotype, gene expression, and metabolite and protein networks (Keurentjes, Fu et al. 2006, DellaPenna and Last 2008, Myles, Peiffer et al. 2009, Macel, van Dam et al. 2010, Chan, Rowe et al. 2011, Ingvarsson and Street 2011, Keurentjes, Angenent et al. 2011). However, a major determinant of finding phenotype–genotype associations is imposed by the plant species itself. At present, Next Generation Sequencing technologies result in an increasing amount of sequenced plant species and lines within a species, so that the scope of plant-insect association studies will be expanded to additional biological systems with a wider array of plant–insect interactions and resistance mechanisms. In the near future, also the genomes and genetic variation of an increasing number of insect herbivores will become available (Whiteman and Jander 2010). Comparing functional mechanisms in insect and plant populations at the genomic level, will allow the development of ecological insights in the evolution of plant-herbivore interactions and will take host-plant resistance studies to a next level.

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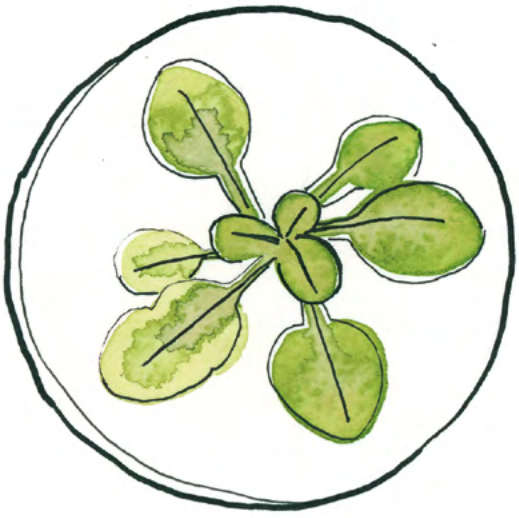

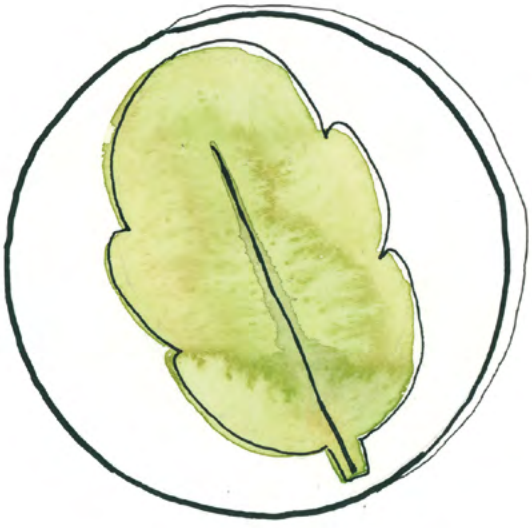

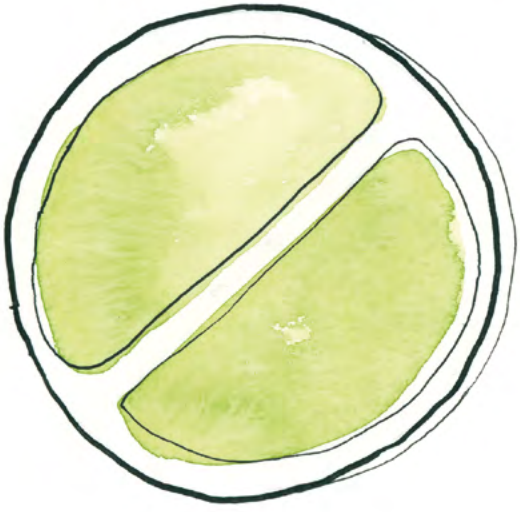

Chapter three

Automated video tracking of thrips behavior to assess host-plant resistance in multiple parallel two-choice setups

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Abstract

Piercing-sucking insects cause severe damage in crops. Breeding for host-plant resistance can significantly reduce the yield losses caused by these insects, but host-plant resistance is a complex trait that is difficult to phenotype quickly and reliably. Current phenotyping methods mainly focus on labor-intensive and time-consuming end-point measurements of plant fitness. Characterizing insect behavior as a proxy for host-plant resistance could be a promising time-saving alternative to end-point measurements. We present a phenotyping platform that allows screening for host-plant resistance against Western flower thrips (WFT, *Frankliniella occidentalis* (Pergande)) in a parallel two-choice setup using automated video tracking of thrips behavior. The platform was used to establish host-plant preference of WFT with a large plant population of 345 wild Arabidopsis accessions and the method was optimized with two extreme accessions from this population that differed in resistance towards WFT. To this end, the behavior of 88 WFT individuals was simultaneously tracked in 88 parallel two-choice arenas during 8 hours. Host-plant preference of WFT was established both by the time thrips spent on either accession and various behavioral parameters related to movement (searching) and non-movement (feeding) events. In comparison to 6-day end-point choice assays with whole plants or detached leaves, the automated video-tracking choice assay developed here delivered similar results, but with higher time- and resource efficiency. This method can therefore be a reliable and effective high throughput phenotyping tool to assess host-plant resistance to thrips in large plant populations.

Background

Next-generation sequencing provides genomic information on large plant populations at increasingly fast rates and with diminishing costs (Egan, Schlueter et al. 2012). This genomic information is of most value when linked to phenotypic traits of interest. In order to find genes underlying these traits of interest, efficient phenotyping platforms are urgently needed to reliably link genomic information to phenotypic information. Recently, major platforms have been established to phenotype plant populations with metabolomics (Fernie and Schauer 2009), proteomics (Altelaar, Munoz et al. 2013), transcriptomics (Crosetto, Bienko et al. 2015) and automated imaging techniques (Rousseau, Belin et al. 2013, Hairmansis, Berger et al. 2014, Guo, Fukatsu et al. 2015). High-throughput phenotyping is also key for future fundamental and applied research on plant-insect interactions (Kloth, Thoen et al. 2012, Goggin, Lorence et al. 2015). Some progress has recently been made in phenotyping plant resistance to pest insects (e.g. hemipterans (Kloth, ten Broeke et al. 2015) and lepidopterans (Green, Appel et al. 2012)). However, no efficient systems have been developed to study host-plant resistance to thrips.

Host-plant resistance is considered one of the cornerstones in integrated pest management (IPM) and can be defined as 'the relative amount of heritable qualities possessed by the plant which influence the ultimate degree of damage done by the insect in the field' (Panda and Khush 1995). Pinpointing these heritable qualities on the genomes of plants can greatly enhance the development of insect-resistant crops (Broekgaarden, Snoeren et al. 2011). However, identifying these traits is not a straightforward task, considering the wide range of components that contribute to host selection, host acceptance, growth, and reproductive success of herbivorous insects. Relevant factors include plant color, olfactory cues, plant topology and morphology, primary and secondary metabolites, and combinations of these factors (Schoonhoven, van Loon et al. 2005). Furthermore, the different components that underlie host-plant resistance are likely governed by multiple genetic loci, each marginally contributing in either positive or negative ways to the observed resistance. This complexity necessitates the development of reproducible high-throughput assays capable of dissecting the different components of host-plant resistance to insects (Kloth, Thoen et al. 2012)

Thrips are minute piercing-sucking insects and several species are major worldwide pests on vegetables and ornamental crops, especially due to their ability to act as vectors of tospoviruses (Lewis 1984). Breeding for host-plant resistance to thrips is important for sustainable pest management, and of special urgency with species like Western Flower Thrips (*Frankliniella occidentalis,* WFT) that have become resistant to many pesticides (Cloyd and Bethke 2011). Currently, methods to determine

host-plant resistance to thrips fall into two broad categories: **1)** 'end-point assays' monitoring plant damage and insect performance (reproduction and mortality) at the end of an experiment, and **2)** behavior assays monitoring insect preference throughout the course of an experiment. End-point assays establish the quality of a host plant days or weeks post inoculation. In these assays the area of feeding damage on the plant caused by thrips is quantified or estimated. Thrips damage can be assessed manually (Leiss, Cristofori et al. 2013), using imaging software (Abe, Ohnishi et al. 2008), or, as recently described in field trials, using hyperspectral imaging (Ranjitha, Srinivasan et al. 2014). In addition to damage assessments, insect performance (growth, survival and reproduction) can be recorded (Abe, Shimoda et al. 2009, Leiss, Cristofori et al. 2013). Thrips performance has also been assessed in end-point assays that use plant extracts (Leiss, Cristofori et al. 2013). Behavioral assays include Y-tube olfactometers (de Kogel, Koschier et al. 1999, Koschier, de Kogel et al. 2000) and flight tunnels (Davidson, Butler et al. 2006) to establish the role of plant volatiles. Choice assays that manually record thrips settlement over time have been used to assess the role of non-volatile dietary deterrents. Some constitutive plant defense traits like protease inhibitors can take 6 hours after ingestion before reaching their maximum effect on thrips behavior (Outchkourov, de Kogel et al. 2004).

None of the above methods have been automated yet to allow parallel, unattended screening of variation in resistance traits to thrips. Characterizing thrips behavior as a proxy for host-plant resistance is a promising yet challenging alternative approach. Promising, because it allows detailed determination of the degree of host-plant acceptance over time; challenging, in relation to technical constraints that have to be solved, like the small size of these insects, their thigmotactic behavior (thrips tend to crawl underneath surfaces for cover) and their low contrast against the complex backgrounds of plant tissues.

This study describes automated video tracking of thrips behavior as a method to assess host-plant resistance, which can complement end-point analysis. Automated video tracking of animal behavior was introduced in the early 1990s (Bakchine, Pham-Delegue et al. 1990, Noldus, Spink et al. 2002), but not applied until recently to the study of host-plant resistance to herbivorous insects (Kloth, ten Broeke et al. 2015). Previously, we demonstrated the value of video tracking aphid behavior in non-choice assays, assessing host-plant resistance in *Arabidopsis thaliana* and lettuce against *Myzus persicae* and *Nasonovia ribisnigri* respectively (Kloth, ten Broeke et al. 2015). Here, we present a behavior-based phenotyping approach using choice assays against a reference genotype. The method uses automated video tracking of thrips behavior in arrays of parallel two-choice arenas. No-choice assays may lead to traits

involved in antibiosis (traits with toxic or antinutritive effects like allelochemicals or proteins), whereas choice assays may better expose traits involved in host plant preference and antixenosis (deterrent or repellent traits like antifeedants, volatiles, surface waxes) (Goggin, Lorence et al. 2015). We applied this new phenotyping tool to screen 345 natural *Arabidopsis thaliana* accessions (the Arabidopsis HapMap population (Baxter, Brazelton et al. 2010)) for thrips resistance, as compared to one reference accession (Col-0). This led to the identification of both highly susceptible and resistant Arabidopsis accessions. The video-tracking method was subsequently validated and optimized with two 'extreme' accessions from the HapMap population (Cur-3 and Rmx-A180).

Results

Prescreening the Arabidopsis HapMap population to identify accessions resistant and susceptible to Western flower thrips

To identify Arabidopsis accessions that are resistant to WFT, thrips behavior was monitored on 345 accessions in a preliminary video-tracking setup with a moderate throughput of 20 parallel assays. The video-tracking platform consisted of a stationary camera mounted above a 96-well plate illuminated from below, with fans to regulate temperature to minimize condensation (Supplementary movie, can be viewed online: https://static-content.springer.com/esm/art%3A10.1186%2Fs13007-016-0102-1/MediaObjects/13007_2016_102_MOESM1_ESM.mov). Every well functioned as a two-choice arena by placing half a leaf-disc from a test accession and from a reference accession (Col-0) inside one well. Behavior of thrips on these two half leaf-discs was monitored to analyze host-plant resistance. In EthoVision XT, zone areas corresponding to the half leaf-discs of the control and test genotypes were assigned to quantify how much time thrips spent on either half in a recording period of 40 minutes. 345 accessions were screened in five separate rounds, using an incomplete block (alpha) design (see methods, statistics). Thrips resistance was measured as the proportion of time the thrips spent on the reference accession Col-0 compared to the test accession (Figure 1a). In the most susceptible lines thrips spent less than 20% of their time on the Col-0 reference accessions. In the presumably more resistant lines thrips spent on average more than 70% of their time on the Col-0 reference. We selected one resistant (Cur-3) and one moderately susceptible (Rmx-A180) accession to confirm the difference in resistance to thrips in end-point feeding assays with whole leaves and whole plants.

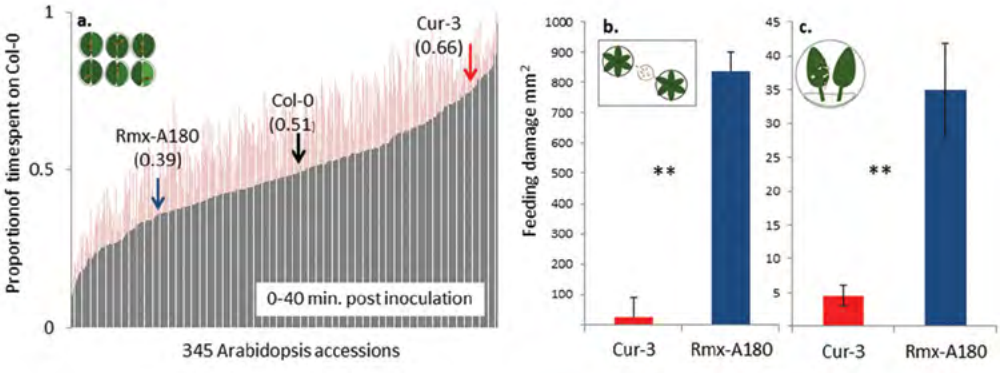

Figure 1. Phenotyping thrips resistance of *Arabidopsis* accessions with video tracking and damage assays. Thrips feeding preference was monitored with automated video tracking. Half leaf discs in a 96-well plate were used to screen the preference of thrips for 345 Arabidopsis accessions relative to reference accession Col-0. **(a)** The proportion of time thrips spent on Col-0 relative to the test accession is presented for 0-40 minutes post inoculation. Shown are genotypic means ± SE (N = 5). **(b)** Feeding damage after six days, in a two-choice whole plant assay. Mean ± SE; N = 9, P = 0.004 (Wilcoxon signed-rank test, two-tailed). **(c)** Feeding damage after six days, in a two-choice detached-leaf assay. Two adult females were released in a Petri dish that contained one leaf of both lines. Mean ± SE; N = 24, P = 0.004 (Wilcoxon signed-rank test, two-tailed)

Table 1. Results of three different dual-choice setups for testing thrips preference on two Arabidopsis accessions.

	Variable	Cur-3 Resistant	Rmx-A180 Susceptible
Video assay[1] (total 8 hrs)	Duration spent in zone (s)	5026 ± 470	8292 ± 631 **
	Duration not moving (s)	4122 ± 446	7494 ± 632 ***
	Duration moving(s)	895 ± 80	787 ± 73 *
	Activity ratio (mov/not mov) (%)	22 ± 2 %	11 ± 2% ***
	Distance moved (mm)	870 ± 70	926 ± 68
	Movement velocity (mm/s)	0.65 ± 0.02	0.68 ± 0.02
Leaf assay[2]	damage after 6 days (mm^2)	8.5 ± 2	45.6 ± 5 ***
Plant assay[3]	damage after 6 days (mm^2)	25 ± 5.5	837 ± 62.3 **
	# of nymphs after 7 days	2.2 ± 0.2	8.7 ± 1 ***

[1]Behavior of thrips was monitored for eight hours (one adult female thrips per arena, N = 68, details of parameter settings in the methods section).
[2]Feeding damage was estimated after six days (two thrips per arena, N = 24).
[3]Feeding damage after six days was scored. In addition, the number of emerged nymphs was scored from the original inoculation of 20 adult female thrips per arena, N = 9). All assays used female adults of approximately three weeks old.
[4] * P < 0.05, ** P < 0.01, *** P < 0.001, Wilcoxon signed-rank test

Comparing video tracking with end-point choice assays

To validate the resistant phenotype as selected by video tracking the HapMap population, the resistant Cur-3 and susceptible Rmx-A180 Arabidopsis accessions were screened as whole plants and detached leaves in two-choice assays for thrips resistance. For the whole plant assay, twenty adult female thrips were released in a closed container with one plant of either accession (N = 9). Feeding damage was assessed by counting the number of feeding spots. After six days, feeding damage was 33-fold higher on Rmx-A180 ($P = 0.004$, Wilcoxon signed rank test, Figure b). The susceptibility of this accession relative to Cur-3 was also demonstrated by the 4-fold higher number of thrips offspring recorded on Rmx-A180 after six days ($P < 0.0001$) (Table 1). Manually assessing feeding damage on whole plants requires a lot of space and time and, therefore, detached leaf assays can be more practical. We, therefore, also carried out an end-point assay with detached leaves of Arabidopsis accessions in Petri dishes. Two adult females were released per Petri dish containing a detached leaf of both accessions. After six days five times more feeding damage was found on the susceptible Rmx-A180 accession ($P < 0.001$) (Table 1, Figure 1c). Thus, the results of the automated video-tracking results were confirmed by these whole-plant and single-leaf endpoint assays.

Method optimization

The method used to screen the HapMap population was limited in throughput (20 arenas) and duration (only a 40 minute recording). The only parameter extracted in this initial thrips behavior screening was the proportion of time spent on the test accession compared to the reference accession Col-0. Our goals for optimizing the video-tracking platform were to **1)** increase throughput with hardware adjustments (more arenas, better camera); **2)** estimate phenotypic variance to accurately pinpoint required replicates needed to perform these assays **3)** evaluate additional behavioral parameters (movement and non-movement). The two extreme lines used for these optimization steps were the resistant Cur-3, and susceptible Rmx-A180 (Figure 1a, b). With a digital high-resolution camera we could track the behavior of thrips in 88 two-choice arenas simultaneously and recorded the behavior during eight hours with EthoVision XT. A demonstration movie of this setup with a sample of this recording can be viewed online (Supplementary Movie 1).

To validate the accuracy of the video-tracking method in annotating the correct location and behavior (movement and non-movement), we annotated the movement and location of thrips for 15 different arenas in this setup manually, using The Observer XT 10.5 software. This was done for 30 minutes of recording time and involved annotation of location (either Cur-3, Rmx-A180 leaf discs or

elsewhere - agar, arena wall or cover) and movement status (moving or non-moving). The data show that in this first half hour of the analysis thrips were not recorded on any of the two leaf discs for 697 seconds on average (Figure 2a).

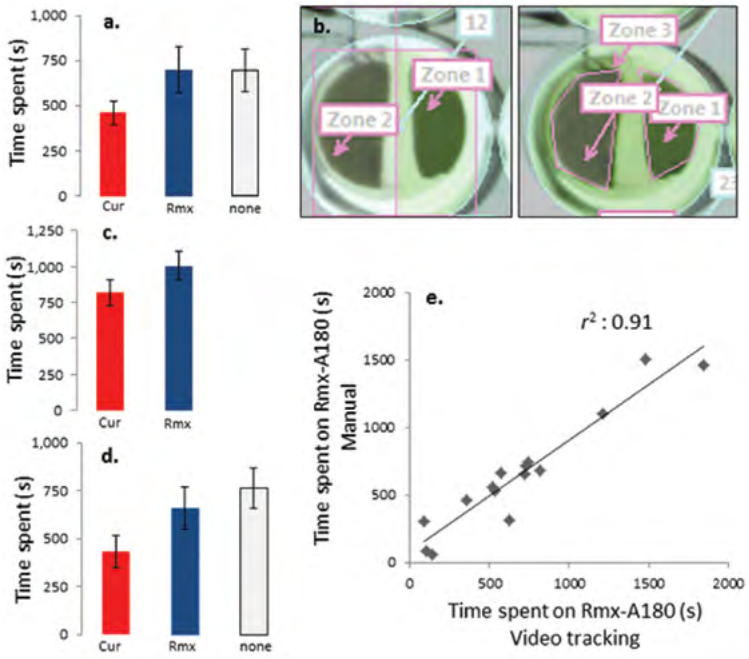

Figure 2. Validating arena settings with improved setup. Thrips behavior was assessed both visually and with automated video tracking in 15 arenas consisting of two-choice tests with Cur-3 versus Rmx-A180 Arabidopsis accessions. **a**. Time thrips spent on either leaf disc, or on none of them (circling around, sitting on agar), based on manual annotation. **b**. Arena settings used for initial HapMap population screening (left panel) and improved arena settings that manually highlight only the leaf discs, with a third zone referring to agar or boundary of the arena (right panel). **c.** Automated video-tracking data of the same 15 arenas with initial arena settings. **d**. Automated video tracking with improved arena settings. Mean ± SE; N = 15 **e.** Correlation of scoring of the total time spent by thrips on accession Rmx-A180 with automated video tracking using EthoVision XT (X axis) and manual annotation using The Observer XT software (Y axis).

This accounts for roughly 37% of the total recording, during which thrips either moved in circles in the upper part of the arena, or moved/rested on the agar. In the original arena settings used to screen the HapMap population, all arenas were divided in two zones. A zone referred to a leaf disc, as well as the surrounding area. To evaluate the effect of more accurate zone annotations, we applied zones that corresponded to the leaf outline exactly, and created a new zone that referred to all area that was not leaf (Figure 2b). With the former "two-zone" settings, the time that thrips spent on either leaf disc was overestimated in comparison to manual annotation (Figure

2c). By reshaping the zones individually to the leaf outline, we found distribution patterns that more accurately resembled the visual annotation (Figure 2d).

Manual annotations and video tracking correlated significantly, as exemplified for the annotated time thrips spent on the Rmx-A180 accession. ($P < 0.001$, Spearman Correlation test, Figure 2e). Using these new settings, the correlation was higher than in the old settings (from $r^2 = 0.81$ in old 'two-zone' settings to $r^2 = 0.91$ in the new 'three-zone' settings). To evaluate differences in the time spent on feeding with automated video tracking, we used not-moving events as a proxy. Movement behavior was difficult to accurately score manually due to the relatively low resolution of the recordings. We therefore tried several movement settings in EthoVision XT and determined the optimal settings by visual inspection. We defined the start of a movement event as the moment when a subject was moving with a speed of > 0.5 mm/s for at least 10 video frames (3 seconds), and this condition stopped when speed dropped to < 0.1 mm/s for 3 seconds (figure 3).

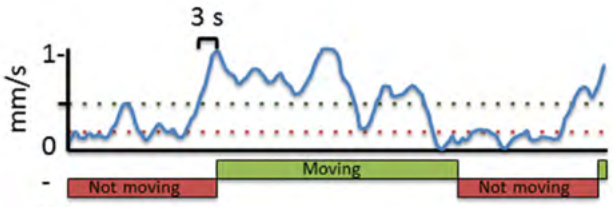

Figure 3. Movement determination in EthoVision XT. A movement event started when thrips obtained a speed above 0.5 mm/s averaged over 10 video frames (3 seconds) and stopped when speed dropped below 0.1 mm/s averaged over 10 video frames (3 seconds).

Thrips behavior on resistant versus susceptible accession

The entire Ethovision XT-recording of 88 arenas during 8 hours was subsequently analyzed using the accurate zone annotation method. The analysis confirmed the results from the earlier 40 min behavioral observation that thrips spent significantly more time on the susceptible accession Rmx-A180, but this preference proved consistent now for the entire eight hours of recording (on average 61% of time spent on any leaf was spent on accession Rmx-A180, $P = 0.0012$, Wilcoxon signed-rank test) (Table 1). Behavior can also be tracked over time in pre-defined time bins to study potential induced defenses, for instance. In assessing thrips behavior in time bins of one hour, we found a significant preference for the susceptible Rmx-A180 accession in all time bins, but 6 and 7 hours post inoculation the largest difference was observed (Figure 4a). Based on the movement settings described in figure 3, the total time spent not moving was found to be significantly longer on the susceptible Rmx-A180

accession, compared to the resistant Cur-3 accession (Table 1). The proportion of the total detected time spent moving differed significantly between the two accessions in all time bins, except at 4 and 8 hours post inoculation (Figure 4b).

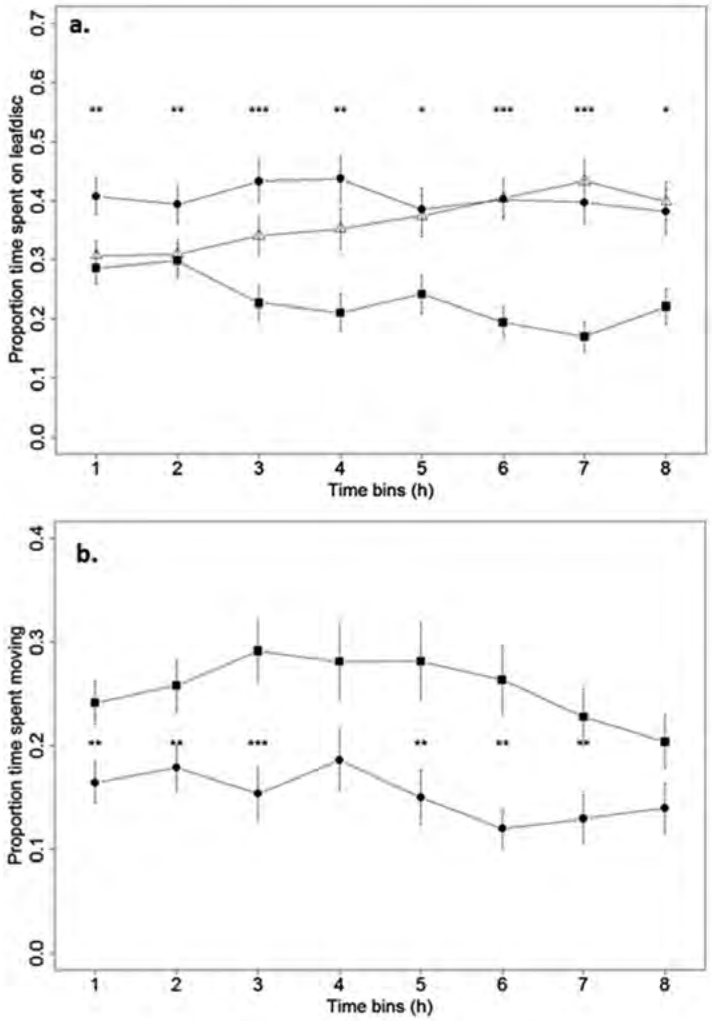

Figure 4. Thrips preference over time in two-choice test with Arabidopsis accessions Cur-3 versus Rmx-A180. a. Two-choice assay with leaf discs of accessions Cur-3 versus Rmx-A180 showing the proportion of time spent by thrips on the resistant Cur-3 (closed squares), the susceptible Rmx-A180 (closed circles) and off leaf (open triangles). **b.** Proportion of time spent moving on accessions Cur-3 (squares) and Rmx-A180 (circles). Mean ±SE, N = 68. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Wilcoxon signed-rank test based on difference between the two accessions).

Throughput

The screening throughput for plant resistance to thrips strongly depends on the required replication level to achieve statistically significant results. In order to know the minimum number of replications for the genotype contrast of Cur-3 and Rmx-A180 we simulated experiments with different levels of replications and tested for significance. Simulated data sets ($n = 10.000$) were generated for video tracking and end-point assays, based on experimental means and standard deviations derived from this study. The generated data sets were subsampled with 1000 iterations without replacement for several replicate levels ($n = 5, 10, 15, 20, 25, 30$). Paired t-tests were executed for each iteration and the percentage of significant p-values ($P < 0.05$) per replicate level was calculated (Table S1). The results simulate the efficiency to detect a degree of resistance as recorded for accession Cur-3 relative to a highly susceptible accession such as Rmx-A180 in the screening of large panels of different genotypes. Permutation tests on the video-tracking data set over 8 hours showed that 15 replicates will lead to a majority of significant outcomes. Five replicates were enough to find significant differences among more than 50% of the simulations in both end-point assays.

Discussion

Quantifying thrips behavior as a proxy for plant resistance

In this study, two-choice assays were used to assess host-plant resistance to thrips. With active insects like thrips, these two-choice assays generate activity distribution patterns on a test versus reference accession that are easy to obtain by means of automated video tracking. We used the proportion of time spent on a reference accession in our pre-screening of the Arabidopsis HapMap population to find resistant and susceptible accessions relative to Col-0. Our assumption was that leaf discs of accessions on which thrips spend less time are more resistant. We validated this assumption by screening two extreme accessions from the HapMap population in several two-choice assays. The assumed resistant (Cur-3) and susceptible (Rmx-A180) accessions were confirmed to be resistant and susceptible respectively when screened against each other using the more detailed automated video tracking method on leaf discs and in two-choice end-point feeding damage assays on whole plants and detached leaves. The method of automated video tracking insect behavior in two-choice leaf disc assays was subsequently optimized using these two extreme Arabidopsis accessions from this population. For optimization, parameters relating to movement were quantified and analyzed. Movement time, distance and speed are

parameters assumed to be associated mostly with searching for (better/more) food, whereas time spent not moving is assumed to be mostly associated with probing and feeding. Other confounding behavior activities like searching for shelter, grooming and resting will occur, but are assumed in the homogenous arena environment to represent systematic errors that are not genotype dependent. When thrips feed on plant cells, they thrust their stylets through the plant epidermis. This thrusting coincides with typical head nodding and could be an informative parameter to score as well (Stafford, Walker et al. 2011). However, the setup with a large number of 88 parallel arenas as used in this study was aimed at increasing throughput at the expense of resolution to monitor such detailed behavioral parameters. The recordings with Ethovision XT were done at the maximum available video resolution for live tracking (1280x960 resolution for a 100x75 mm area (1 mm^2 = 164 pixels, pixel width 78 μm). A female adult WFT is approximately 1.4 mm long and 0.3 mm wide (0.42 mm^2), which translates into approximately 69 pixels for one thrips in full view. Plant cell size is 10- 100 μm and thrips may move only such distances between probes in neighboring groups of cells. Our movement threshold was set to <0.5 mm during three seconds. Consequently, one long non-movement event may refer to several feeding events in close proximity. Previous studies on cucumber plants showed that feeding scars from WFT on susceptible plants were grouped together, whereas the feedings scars on resistant plants were less numerous and more scattered. This correlated with more restless behavior and a bigger proportion of time spent walking (Parker, Skinner et al. 1995) which would be picked up by our method of studying thrips behavior. Previous studies on *Thrips tabaci* behavior showed that, in general, feeding was the predominant behavior recorded on leek and cucumber plants. *T. tabaci* were shown to spend roughly eightfold more time on feeding, than on inactivity (Riefler and Koschier 2009). The use of adult females that had been starved overnight before the experiment, was shown to make it even more likely that the 'not moving' state represents a feeding event. Starvation periods of at least four hours were shown to increase the response of thrips to visual and olfactory cues associated with a food source (Davidson, Butler et al. 2006). In our experiments using overnight-starved insects, we took the entire period of not moving as a proxy of time spent feeding assuming all non-feeding activities not to be genotype specific.

WFT were found to consistently spend 40% less time on the resistant Cur-3 Arabidopsis accession, during the entire 8-hour recording ($P = 0.001$, Wilcoxon signed-rank test). In addition, WFT spent significantly more time moving on the resistant Cur-3. Choice (genotype x/y) and activity (movement versus non-movement time per genotype) are independent behavioral parameters and both showed that thrips preferred the susceptible accession Rmx-A180, presumably spending more time feeding during the time of non-movement. Having several independent parameters to assess host-plant

resistance to thrips is valuable in quantitative genetics. Different genes in plants might influence different aspects of thrips behavior. Although the duration of time spent in a specific zone showed a significant difference (choice parameter) for the two accessions tested, the highest levels of significance were found in the parameters "duration not moving" and "proportion of time moving", indicating that the actual behavior exhibited on a specific leaf disc harbors variables that can be used to phenotype plants for thrips resistance more accurately. A potentially interesting next step would be to also discriminate between short and long non-movement events (Kloth, ten Broeke et al. 2015). Short non-movement events could refer to test probes, non-movement events that last longer than 10 seconds are potential food uptake events. The Ethovision software version used could not yet produce statistics on individual events however.

Advantages and limitations of automated video tracking

To evaluate advantages and limitations of the end-point assays and automated video-tracking assays used in this study, we listed the different variables that can be determined with these assays, the number of insects used per assay, the duration of one experiment in each assay, and the actual labor involved in performing these assays (Table 2). There are five main advantages of automated video tracking over end-point measurements:

1) More detailed choice and movement parameters relating to a specific developmental stage of the insects (adult, nymphs) on a specific developmental stage and tissue of a plant are obtained and these are made relevant using a direct within-assay comparison to a reference plant genotype and followed for as long as 8 hours. These behavioral parameters on specific tissue samples can be the result of component traits such as leaf volatiles, leaf toughness, constitutive and induced chemical defenses that add up positively or negatively to the overall susceptibility/resistance of plants as measured by endpoint assessment. Dissecting overall plant defense into component traits, as done in video tracking, is expected to lead to stronger genetic signals in quantitative genetic studies (Kloth, Thoen et al. 2012).

2) The automated video-tracking method is faster and more objective than the current rating systems that visually score feeding damage, which often do not allow precise quantification and are sensitive to subjectivity and inconsistency of the human observer (Goggin, Lorence et al. 2015). The automated process in which video-tracked thrips behavior is dissected into components of choice, movement and speed is not subject to human measurement and annotation errors and the data are much more quickly obtained and statistically analyzed. Permutation tests on the video-tracking data set over 8 hours showed that 15 replicates will lead to a

majority of significant outcomes. However, this is based on the experimental means of two accessions (Cur-3 and Rmx-A180) that are found at opposite extremes of the host-plant resistance spectrum. More replicates are likely necessary to pick up more subtle differences in resistance or tolerance.

3) Controlled conditions. The use of leaf discs with uniform plant biomass of a chosen tissue type and developmental stage in closed arenas immediately after harvesting for just a few hours removes a lot of plant and environmental variability accumulating during prolonged multiday experiments on whole plants or detached leaves. The validity of comparisons between independent experiments will be improved in this way. In addition, plant samples can be taken from their normal optimal production site like an open field which normally would not allow a proper insect resistance test to be performed.

4) In genetic studies there is often only one plant per genotype (e.g. from crossing populations, outcrossing species) which makes it far more difficult to efficiently identify genetic markers linked to insect resistance if whole plants or leaves are required for replication. In video tracking, multiple leaf discs can be generated from a single plant or leaf to obtain a practically reliable estimate of the resistance level for a particular plant genotype but not accounting for interplant and environmental variation.

5) The space and resource efficiency of the use of leaf discs is much greater. The 88 parallel experiments required an experimental space of only 100 cm^2, requiring fewer insects than whole plant damage assays and only $1/6^{th}$ or less of the time. In plant breeding, insect assays are mostly carried out in greenhouse compartments requiring thousands of insects with a lot of containment measures to avoid the spread of insects to other parts of the greenhouse. Here, the plants may be evaluated for other traits in parallel, can be grown in the open field, and only need to sacrifice a few leaves for tests done in the laboratory.

There are also downsides to the use of leaf discs. The generation of leaf discs introduces mechanical damage that may induce or inhibit physiological processes unrelated to thrips infestation and potential resistance under natural circumstances. It also offers only a narrow window on the total insect-plant interaction during plant and insect development. Yet, some of the issues raised against the use of leaf discs with phloem-feeding insects like whiteflies and aphids (ten Broeke 2013), are of less concern when working with epidermal cell-feeding insects like thrips which do not depend on phloem turgor for normal behavior. Our leaf-disc assays showed the same pronounced differences as the end-point assays with intact plants and detached leaves. In general, video tracking methods offer major advantages for prescreening a plant population, but resistance characteristics in selected genotypes should always be validated under field or greenhouse conditions to validate their relevance.

Conclusion

End-point measurements and detailed initial behavioral screenings are essentially complementary approaches for measuring different aspects of insect resistance and potentially generate different outcomes. Combining these approaches will, therefore, be the most robust approach to efficiently identify the factors responsible for thrips preference and performance. The two-choice video-tracking platform presented here may proof to be a valuable high-throughput alternative to the classical damage assays to assess host-plant resistance to thrips. This method in its optimized form can screen hundreds of plant samples per set up per day. This will likely benefit selection and breeding of cultivars that are resistant to piercing-sucking insects.

Table 2. Comparison of three different two-choice assays to acquire data on plant resistance to thrips.

	Whole plants	**Detached leaves**	**Video tracking**
			
Main advantages	• Non-invasive • reproduction and survival data	• limited space and number of insects required • more standardized setup (allows automated imaging)	• detailed behavioral parameters • quick and objective • controlled conditions
Main disadvantages	• space consuming • large numbers of thrips required • time consuming analysis • large environmental variation	• limited mechanical damage at petiole • senescence of material • time consuming analysis	• mechanical damage at edge of leaf disc • relationship to endpoint values unknown
Variables obtained	• Feeding damage • Reproduction	• Feeding damage	• Duration spent in zone • Duration not moving • Duration moving • Ratio moving/not moving • Distance moved • Velocity
Inoculation	30 minutes	60 minutes	30 minutes
Duration	6 days	6 days	8 hours
Analysis	2-4 hours	1 hour	10 minutes
Minimum number of replicates[1]	5	5	15
# thrips required	100 (5x20)	10 (5x2)	15 (15x1)

[1] The minimum number of replications is based on the criterion that >50% of experiments should be significantly different among genotypes ($P < 0.05$ (Table S1)).

Methods

Insects

The Western flower thrips (*Frankliniella occidentalis* (Pergande)) used in this study were originally collected from chrysanthemum flowers and reared on green common bean pods (*Phaseolus vulgaris*) in glass bottles placed in a climate chamber (25 ± 1 ˚C, L:D 8:16). Twice per week, 200 adult females were transferred to fresh bottles with bean pods to synchronize the offspring production. In the experiments adult females (20 days after emergence of larvae from the eggs) were used, that were starved overnight in Perspex tubular cages closed on one side with gauze and on the other side with two layers of stretched sheets of Parafilm containing a droplet of water to enable drinking. Thrips were anesthetized with CO_2 and placed on ice just prior to experiments.

Plants

We used *Arabidopsis thaliana* as host plant species. Initial screening of resistance to thrips was done for the HapMap population, consisting of Arabidopsis accessions collected globally (Baxter, Brazelton et al. 2010). We obtained phenotypic information on 345 out of the 360 available accessions. Rmx-A180 (CS76220, collected by J. Bergelson, Latitude 42.036, Longitude -86.511, Michigan, USA) and Cur-3 (CS76115, collected by F. Roux, Latitude 45.000, Longitude 1.75, France) were used for follow up experiments. For insect assays, plants were grown from seeds in small plastic pots (5 cm diameter) on pasteurized soil (4h at 80˚C; Lentse potgrond, Lent, The Netherlands) in a climate room (21 ± 1 ˚C, 50 – 70% relative humidity; 8h:16h L:D photperiod; light intensity 200 μmol $m^{-2}s^{-1}$). For all experiments, five-week-old plants were used.

Video-tracking setup

Thrips behavior in the HapMap population screen was recorded with a monochrome camera (Ikegami, model: I CD-49E, type: REV, 768 x 576 pixels (PAL), analog output) with a varifocal lens (Computar H3Z4512 CS-IR, 4.5-12.5 mm F1.2) for the HapMap population screening. This allowed the screening of 20 two-choice arenas simultaneously. In the optimization step with two extreme Arabidopsis accessions, we used a digital camera (GigE Basler acA2040-25gc). In both cases, a backlight unit (FL tubes, 5000 K) was used to illuminate the arenas. Ca. 1 cm above the backlight unit, 96-wells microtiter plates (flat bottom suspension cells from Sarstedt, product number 831835500) that contained the two-choice arenas were placed on a custom made platform. A fan blew air between the backlight unit and microtiter plate to prevent condensation. Room temperature was kept constant at 21-22˚C.

Video-tracking software settings

We tracked thrips behavior with EthoVision® XT 10.0 (Noldus Information Technology B.V., Wageningen, The Netherlands) video tracking and analysis software. Due to the large number of arenas screened simultaneously (88), and the method to detect insects (live tracking), a maximum resolution of 1280x960 pixels with 3.5 video frames per second was used. Dynamic subtraction and center-point detection were used as detection methods, with a dark contrast of 8-255. Subject size detection was limited to the range of 10-160 pixels. Pixel smoothing was set to medium. Moving thresholds were set to start when thrips velocity reached above 0.5 mm/s averaged over 10 video frames (3 seconds) and stopped below 0.1 mm/s (figure 3).

Arabidopsis HapMap population screening

Thrips preference was phenotyped in two-choice arenas using 96-well plates, consisting of two half leaf discs from Col-0 and one of the HapMap accessions. Arabidopsis leaf discs (6 mm in diameter) were punched with a cork borer, cut in half and placed into the wells with soft tweezers on a layer of 1% technical agar that filled the wells for ¾ of the volume. Position bias was corrected for, by alternating the Col-0 leaf disc position (left or right) in every row. Female adult thrips (starved overnight) were anesthetized and kept on ice prior to the recordings. A soft brush was used to place thrips in the individual wells. Optical adhesive film (Micro Amp, Biosystems) was used to seal of the 96 well plates to prevent thrips from escaping. Plants were screened in 5 rounds of 360 accessions. Plants were randomly allocated to blocks (20 accessions per block, 18 blocks per round). One sampling day consisted of 5 blocks (100 accessions), except for the last day (3 blocks, 60 accessions). Manual quality checks on all recordings detected some arenas with non-moving thrips. These non-moving thrips were considered dead, and discarded from the analyses. Thrips position was monitored for 40 minutes with an analog monochrome camera mounted 50 cm above the two-choice arena plate. The proportion of time spent on accession Col-0 was assessed with EthoVision XT software and used as a proxy for host-plant preference.

Video tracking of two extreme Arabidopsis accessions

Arabidopsis accessions Cur-3 (resistant) and Rmx-A180 (susceptible) were used for optimizing the video tracking setup. A digital camera (GigE Basler acA2040-25gc) allowed the screening of 88 arenas simultaneously. Arenas were set up the same way as the HapMap screening, except that in this assay a half leaf disc of each of the two extreme accessions were placed in one well and an additional neutral zone was created. Thrips behavior was monitored for 8 consecutive hours. In addition

to the proportion of total time thrips spent on one accession (parameter used to assess resistance in the HapMap screening), additional parameters were "duration of time not moving (s)", "duration of time moving (s)", "movement proportion per genotype", "distance moved (mm)" and "movement velocity (mm/s)". A movement event started when thrips obtained a speed above 0.5 mm/s averaged over 10 video frames (3 seconds) and stopped when speed dropped below 0.1 mm/s averaged over 10 video frames (3 seconds). The Observer XT 10.5 Software (Noldus IT, Wageningen, The Netherlands) was used for visually assessing thrips position in 15 selected arenas for the first half hour of recording, to validate detection and arena settings in automated video tracking.

Whole-plant assay

Plastic containers (length: 17 cm, width: 11.5 cm, height: 6.5 cm) functioned as two-choice whole plant arenas. The transparent lids of the containers had a circular piece of mesh in the center for ventilation. Thrips preference was screened in nine replicates and evaluated by placing both plants in opposite corners with a perspex tubular cage closed on one side with gauze in the middle that contained 20 adult female thrips (starved overnight) per treatment. The containers were then placed in a climate chamber (25 ± 1 °C, L:D 8:16). Feeding damage was estimated in mm^2 after six days by counting the number of small feeding spots on the entire plants. One small spot accounted for approximately 3 mm^2 damage (bigger spots were counted as 2-5 small spots). The number of adult and juvenile thrips on both plants was determined by submerging and shaking the aboveground tissues in a flask of 70% ethanol and filtering it through a mesh. The residue of thrips was flushed from the filter into a Petri dish to count adult and juvenile thrips separately using a stereomicroscope.

Detached-leaf assay

'Thrips proof' Petri dishes with a diameter of 5 cm (BD falcon, Product Number: 351006) were used for two-choice detached-leaf assay (N = 24). One ml of 1% technical agar was poured into a Petri dish, that was left to solidify in a 20° slope. One leaf per plant was harvested with a pair of scissors, and the leaf petiole was inserted into the layer of 1% technical agar, alternating left-right, to compensate for potential position effects. Two adult female thrips (3 weeks after egg hatching, starved overnight at 25 °C) were used. After six days a climate chamber (25 ± 1 °C, L:D 8:16), feeding damage was assessed as described above for the whole-plant assay.

Statistics

Data distribution and homogeneity of variances of all two-choice assays were tested with a Shapiro test and a Levene's test. Normally distributed data were tested with a paired Student's t-test, data that were not normally distributed were tested with Wilcoxon-signed rank tests using IBM SPSS Statistics 19 software. Correlation between manually annotated behavior of insects, and behavior annotated with automated video tracking was tested with a Pearson correlation test. For the screening of the HapMap population, plants were screened in 5 rounds (complete replicates) using an incomplete (alpha) block design for 360 accessions (phenotypic data were obtained only for 345 accessions). Within each round plants were randomly allocated to 18 blocks of 20 accessions, the blocks representing plants being screened in one recording. One sampling day consisted of 5 blocks (100 accessions), with the exception of the last day (3 blocks, 60 accessions). Genotypic means (BLUEs) were calculated using the following linear mixed model:

$$Y = \mu + REP + GEN + REP{:}BLOCK + E,$$

where REP denotes complete replicate and REP:BLOCK is a random term for blocks nested within replicate. From the 360 accessions in the block design, we obtained phenotypic information for 345 accessions. Simulation to assess the number of required replicates were done in R, using the rnorm command. Simulated datasets were created ($n = 10.000$) for video tracking and end-point data, using the mean and STDEV derived from the experiments described in this study. The mean and STDEV values for Cur-3 and Rmx-A180 to generate the simulated datasets were respectively 7926 ± 5252 and 12159 ± 5610 (seconds spent on each leaf disc in 8 hours of recording), 1352 ± 783 and 1890 ± 912 (seconds spent on each leaf disc in the first hour of recording), 8.5 ± 9.9 and 45.6 ± 24.4 (mm^2 feeding damage detached leaf assay) and $837.7 \pm 187{,}2$ and 25.7 ± 16.5 (mm^2 feeding damage whole plant assay). Figures 1 and 2 were created in Windows Excel 2010, Figure 3 in PPT and Figure 4 in R (R_core_team 2014).

Competing interests

Software packages used to assess insect behavior (EthoVision XT and The Observer XT) were developed by Noldus Information Technology B.V., the affiliation of authors OK and LN. All authors confirm that the contents of this manuscript have not been affected by any competing interest.

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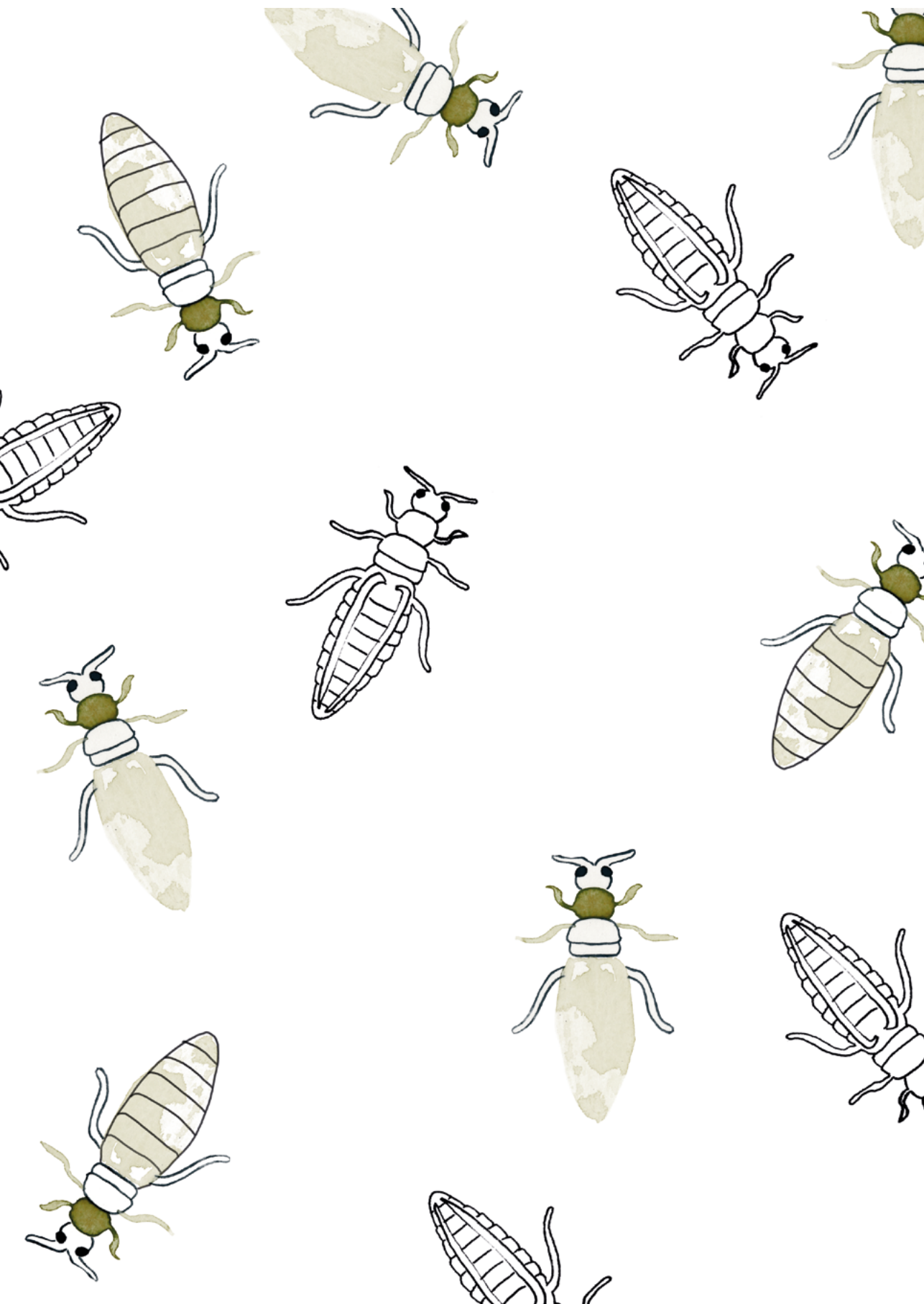


Chapter four

Evaluation of EthoAnalysis software for the analysis of thrips behavior recorded by videotracking

Manus P.M. Thoen, Johannes Kruisselbrink, Leo Poleij, Gerrie L. Wiegers, Marcel Dicke and Maarten A. Jongsma

Abstract

Thrips are minute polyphagous herbivorous pest insects. Host-plant resistance to thrips is a complex trait that is difficult to phenotype quickly and reliably. Current phenotyping methods mainly focus on labor-intensive and time-consuming end-point measurements of feeding damage or insect performance. Traditional visual rating systems that score feeding damage often do not allow precise quantification and might be sensitive to subjectivity and inconsistency of the scoring process. An automated process to phenotype host-plant resistance to thrips would greatly aid the breeding process for thrips-resistant cultivars. Previously, we reported on a new phenotyping platform that allows screening for host-plant resistance against thrips in a parallel two-choice setup using automated video tracking of thrips behavior with EthoVision software, followed by a manual analysis of the recorded behavior. Here, we re-analyze the same video tracking data obtained with EthoVision software, now with a novel software package, EthoAnalysis, that allows for automated extraction of more detailed behavioral parameters from the raw tracking data, and automated statistical analysis. The method is validated with two Arabidopsis accessions that differ in susceptibility to western flower thrips. Through 22 parameter iterations we arrived at optimized settings for 54 variables. Most of these 54 variables could be further divided in time bins of one hour, leading to 214 different variables that describe different behavioral characteristics in time, frequency, duration, distance and speed. The three overarching behavioral categories, i.e. settlement (choice), movement, and pausing, were automatically corrected for percentage of time thrips were detected. The value of these variables is discussed in the light of potential, underlying mechanisms of plant defense.

Introduction

Thrips are tiny piercing-sucking insects, represented by over 5000 species. The order Thysanoptera is notorious for the many pest species it contains. *Thrips tabaci, Thrips palmi* and *Frankliniella occidentalis* are a few examples of thrips species with devastating effects in agriculture (Lewis 1984). The loss in yield could be greatly reduced if crops would be better equipped with defenses against these piercing, cell-feeding insects. Breeding for host-plant resistance to thrips has gained much interest in recent years. A crucial element in the breeding process is the accurate estimation of the resistance level of large populations of plant accessions (Eigenbrode and Trumble 1993). This requires robust phenotyping systems that can accurately screen many different plant lines in a high-throughput manner (Kloth, Thoen et al. 2012, Goggin, Lorence et al. 2015). We have recently demonstrated the value of automated video tracking of the western flower thrips (*F. occidentalis*) to establish host-plant resistance levels in *Arabidopsis thaliana* (Thoen, Kloth et al. 2016). That study, however, focused primarily on settlement of the insect in a two-choice setting. The additional parameters reported in that study, related to movement, were all sensitive to differences in detection percentages (tracking efficiency), and no detailed behavioral categories were reported that could distinguish short and long or fast and slow moving or pausing events. Combining all pausing events, independent of duration in a summary value labeled as 'total time spent feeding', neglects important details of animal behavior (Benjamini, Lipkind et al. 2010). An understanding of thrips biology is required to assess the biological relevance of a distinction between, for instance, short and long pausing events. A single feeding event of thrips can be divided in five consecutive steps: 1) Placing the tip of the mouth cone on the cell surface; 2) Thrusting the mandible through the plant surface layers; 3) Inserting the maxillary stylets into the cavity created by the mandible intrusion; 4) Sucking of the contents of punctured cells; 5) Retracting stylets and lifting the mouth cone. Step 3, in which maxillary stylets are inserted into the created cavity is considered the start of a probing event, where cell contents are evaluated by the thrips. Only if this test probe is satisfactory, step 4 (the sustained sucking of contents) will follow. Feeding events where this last step does not follow, are thus not real feeding events, but just probing events. These 'test probes' can occur more frequently when plant material is of suboptimal quality for thrips. Given that these probes were reported to generally take less than 10 seconds (Lewis 1984), we argue that the relative number of short probes could serve as a proxy for host plant suitability. The goal of the present study was to evaluate the utility of a novel software package, EthoAnalysis, to provide more detailed insight in the behavior of thrips on different plant accessions. To accomplish this, the following workflow was used: **1. Controlled experiments with video tracking**. Leaf discs of two accessions were placed into each of 88 arenas. In

each arena, one insect was placed and video recorded over a time span of 8 hours. **2. Video tracking data generation.** EthoVision video-tracking software was used to determine the position, zone and velocity of each insect in each videoframe during the complete run of the experiment. **3. Video tracking data analysis.** A new software package EthoAnalysis was applied to convert raw tracking data exported from EthoVision into zone-specific movement and pausing events, and to generate higher level behavioral variables. These behavioral variables were less sensitive to differences between genotypes in tracking efficiency (detection). The variables included zone preference, average velocity, total time moving/pausing, short/medium/long lasting moving and pausing and slow/medium/fast moving events, mostly also per timebin of an hour. For the distinct combinations of genotypes, which form the analysis groups of interest, the behavioral statistics were aggregated using appropriate default statistical models to derive general estimates of these behavioral statistics for the analysis groups. Using this approach, differences in behavior can be identified. Here, we evaluate the utility of EthoAnalysis to study host-plant resistance to thrips. We used a dataset from a previous study (Thoen, Kloth et al. 2016) where the behavior of western flower thrips (*F. occidentalis*) was recorded in 88 parallel two-choice arenas. The two wild *A. thaliana* accessions Cur-3 and Rmx-A180 of that study were shown to be highly resistant and susceptible to thrips, respectively. EthoAnalysis provides a number of filters to remove entire records or specific events based on various quality criteria. Setting these filters and tuning their parameters generally allows navigating between data quality and data quantity. Additionally, for some behavioral statistics, EthoAnalysis requires insect specific parameters to be set, similar to the velocity threshold. For instance, defining the categories of short/medium/long movement/halting events. After a sensitivity analyses of the various parameter settings we compared the EthoAnalysis outcome with previously obtained results obtained with EthoVision, and discuss its additive value in the field of plant-insect science.

Results

EthoAnalysis methodology

The video tracking software EthoVision produces series of track samples (videoframes at ca 3-4/sec) for all insects/arenas. Each track sample contains an (x,y)-coordinate, a velocity, and an indication of the current zone in which the insect resides unless the tracking is not successful because EthoVision could not detect the insect, in which case the sample is recorded as not detected. Based on the setting of a velocity

threshold and a look-ahead window EthoAnalysis software translates these raw data into series of zone-specific events of three types: pausing, moving, and non-detected (events that do not contribute to the calculation of behavior statistics). For details see the M&M. Each event has a starting time and a duration, and from these series of events, the various behavioral statistics are extracted. The series of behavioral events are constructed by iterating over the track samples and determining for each sample whether the current state is moving, halting, or unknown (non-detected). Each consecutive sequence of track samples with the same movement state and occurring in the same zone forms a behavioral event of the movement state of that series. In the given case we had three zones (Z). Z0 contained the susceptible Rmx-A180 leaf disc, Z1 the resistant Cur-3 leaf disc, and Z2 represented a neutral area that did not contain any leaf material (Figure 1a). For every single insect a velocity histogram was generated providing feedback on the effect of the chosen threshold setting (Figure 1b). Distinctive patterns and durations of movement and pausing may reveal behavior of thrips related to acceptance (pausing, thus probing or feeding) and rejection (movement, thus searching or escaping). To determine the movement state of a track sample at time t, the software adopts the procedure illustrated in Figure 1c. The look-ahead window exists to ignore minor drops below the velocity threshold within a movement event, or single spikes above the velocity threshold while pausing. The optimal velocity threshold and look-ahead window can be highly hardware-, species- and host-plant specific as demonstrated previously for two different aphid species on lettuce and Arabidopsis (Kloth, ten Broeke et al. 2015). Initial parameter optimization with the insect of interest is, therefore, required to accurately define variables related to movement and pausing events. Parameter optimization is accomplished by defining behavior categories (based on certain intervals of velocity and duration), filtering of tracks (based on detection percentage per insect over the eight hours of recording) and filtering of events (based on consecutive movement or pausing events due to zone transitions). For the Rmx-A180 vs Cur3 dataset we first performed a sensitivity analyses on various parameter settings in EthoAnalysis to optimize the comparison of behavioral parameters calculated for Z0 (Rmx-A180) and Z1 (Cur-3) (Table 1), the two zones of interest.

Optimizing EthoAnalysis input parameters

Identifying pause and movement events

The look-ahead window and the velocity thresholds of the insects determine how EthoAnalysis identifies the behavior as a moving or pausing event. EthoAnalysis provides frequency histograms of the velocity per individual insect to give the user an idea of what might be a good velocity threshold to start with (Figure 1b). The frequency

maximum of velocity (the most common speed) differs between individual insects (see Suppl. Figure 1 for some examples), but we most commonly observed a velocity around 0.2 mm/s. Based on visual observation this velocity represents the velocity between small feeding events. We propose that movements with a velocity higher than 0.8 mm/s represent thrips actively walking around in the arena. To evaluate this we compared the differences between RMX-A80 and Cur-3 with five different velocity thresholds (0.75, 0.50 , 0.25, 0.10 and 0.075 mm/s) with the 50% detection threshold dataset. Among these five velocity threshold settings, the thresholds of 0.25 mm/s and 0.10 mm/s were most in line with our manual observation on moving behavior when we analyzed this with Observer XT Software in our previous study, where we opted for a 0.1 mm/s velocity threshold with a 10 frames window (Table 1, Thoen et al., 2016). With these two settings we tested three different look-ahead windows (3 frames (1s), 5 frames (2s) and 10 frames (3 s)). We found that a look-ahead window of 5 frames with a pausing velocity threshold of 0.10 mm/s resulted in data that were best in line with data obtained by manual observations (Table 1, Suppl. Figures 1 and 2). Look ahead windows of 3 frames often resulted in truncated events that should be considered single long events (Suppl. Figure 2a), look ahead windows of 5 frames displayed the optimal event determination (Suppl. Figure 2b), whereas a look ahead window of 10 frames fused independent events that are likely several consecutive changes in the moving/pausing stage (Suppl. Figure 2c). With these settings we explored further modification of parameters.

Filtering records based on detection percentage

In our set-up, the behavior of 88 thrips individuals in 88 arenas was recorded in parallel with relatively low contrast of the insects on leaves. As a result, the small insect subjects were sometimes poorly detected. Filtering of arena records based on a subject detection percentage threshold is then useful to automatically select the most reliable records. However, there is a cost of statistical power due to the drop in replicates. Comparing outcomes without filtering any tracks ($n = 88$), to filtering of records with $>25\%$ detection ($n = 71$), $>50\%$ ($n = 60$) and $>75\%$ ($n = 30$), we found that the $>75\%$ filter was not capable of distinguishing behavior of thrips between the two accessions for most parameters. This can be explained in part because EthoAnalysis corrects all parameters for duration detected and includes a number of robust event specific statistics (average speed, movement and pausing duration) that are less sensitive to detection duration. The high detection ($>75\%$) subset of data then does not compensate for the loss of replicates, and consequently statistical power, in the majority of the statistics. The 50% cutoff allowed us to work with a high-enough number of records (60 vs 68 in Thoen et al. (2016)) while still maintaining a relatively high quality of data samples, and was therefore chosen as the preferred setting for further exploration of the data.

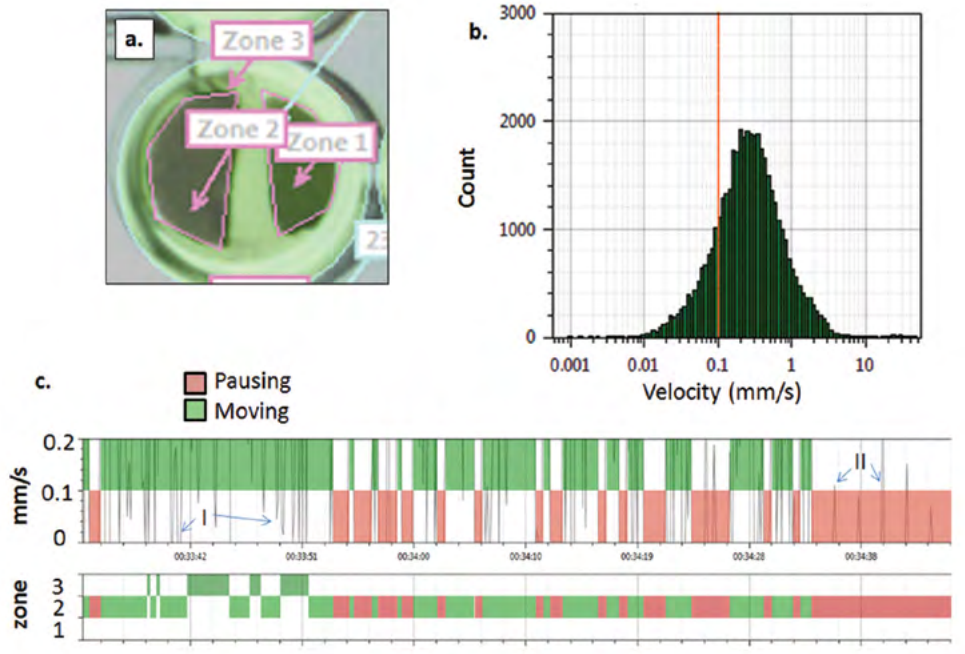


Figure 1. Determining behavior state of thrips. **a.** One arena from the arena setup consisting of 88 parallel two-choice assays with halved leaf discs from Arabidopsis accessions Cur-3 and Rmx-A180. **b.** Velocity distribution of one thrips to aid in selecting proper thresholds for the determination of movement and pausing events. **c.** Thrips velocity over time: thrips per arena are assigned to either a movement or pausing state based on the velocity threshold and a look-ahead window. I: drops in velocity that do not stop the movement state due to the look ahead window. II: spikes in velocity that do not stop the pausing state due to the look ahead window.

Filtering events based on zone transitions

Due to the strict assignment of all events to specific zones in two-choice assays, every moving/pausing event terminates when the subject leaves a designated zone. This means that five short movement events can actually be one long movement event during which an insect walks through several zones without stopping. This also affects pausing events that occur on zone boundaries due to jitter of the tracking centerpoint. To omit these zone transition artefacts it is possible to filter out all trans-zone movement and pausing events. Applying this filter removed up to 70% of all movement events and approximately 5% of all pause events. Filtering these events created a dataset that only included distinct events that started and ended in the same zone and thus reflected zone-specific differences and omit zone-transition artefacts (Suppl. Figure 3).

Setting short, medium and long pausing events

The duration of a pausing event contains information on actual feeding behavior of thrips, and may allow to distinguish between test probing and sustained localized

feeding behavior (Lewis 1984). To find differences in this parameter, the correct time setting is crucial. We have compared three different settings that distinguish between the three different duration intervals of pausing events. The difference between short, medium and long pausing events was set at different thresholds. We tested parameter settings for medium length of pausing events as [>5s, <10s], [>5s, <30s] and [>10s, <30s] per event. We chose to work with a medium pausing duration threshold of [>5, <10] because we observed that 30s pausing events were very rare in this setup (Suppl. Figure 4).

Setting short, medium and long movement events

Thrips are very active insects that, even on suitable host plants, tend to move a lot (Riefler and Koschier 2009). We postulate that many short movement events might refer to the continuous moving to neighboring cells of previous feeding locations; thus, this behavior might be a proxy for a suitable host plant. Long movement events might then be an indication of a host plant being of low quality. We have compared three different settings to distinguish between the duration of these events, where the medium movement events had a duration of [>1, <5], [>1, <10], or [>5, <10] seconds. The distribution of movement events shows that the short medium movement setting of [>1,<5] harbors a more equal distribution of events (Suppl. Figure 5). We, therefore, opted to work with the movement duration setting of [>1,<5] seconds.

Setting slow, medium and fast movement events

In addition to the duration of movement events, one can distinguish between the velocities of movement events. To distinguish between slow, medium and fast movement events, we compared three different quality settings where the medium velocity of movement events was; [>0.1,<0.25], [>0.1,<0.35], [>0.15, <0.35] mm/s. The frequency distribution of speed event categories which could best separate slow and fast movements was obtained when using a medium velocity of movement setting of [>0.15,<0.25] mm/s (Suppl. Figure 6). In these settings, all movement with a velocity lower than 0.15 mm/s could be considered slow movement that occurs in between feeding events. Movements with a velocity higher than 0.25 mm/s can be considered fast movement events indicative of searching for other food sources. The medium speed category is than a gray zone from which no biological conclusions can be drawn.

Table 1. Optimized EthoAnalysis settings for studying behavior of Western Flower Thrips in two-choice assays.

Parameter	Setting
Record filters	
Exclude records with pauses > x seconds	3600 seconds
Exclude records with a detection < x%	50%
Threshold settings	
Look ahead window	5 frames (1.43 seconds[1])
Velocity threshold[2]	0.1 mms/s
Event filters	
Ignore consecutive pausing events due to zone change	
Ignore consecutive moving events due to zone change	
Category intervals	
Short pausing events	<5 seconds
Medium pausing events	>5 seconds / <10 seconds
Long pausing events	>10 seconds
Short movement events	<1 second
Medium movement events	>1 second / <5 seconds
Long movement events	<5 seconds
Slow movement events	<0.15 mm/s
Medium speed movement events	>0.15 mm/s / <0.25 mm/s
Fast movement events	>0.25 mm/s

[1]Based on 3.5 video frames per second recording.
[2] In Thoen et al. (2016) Ethovision these parameters were 0.5mm/s for 10 continuous frames triggered moving, < 0.1 mm/sec was pausing

Differences in thrips behavior on Cur-3 versus Rmx-A180 accessions.

The first overarching behavioral category settlement (choice) was already studied in the previous analysis of this dataset (Thoen, Kloth et al. 2016). During 8 hours of recording, thrips spent significantly more time on the susceptible Rmx-A180 accession than on Cur-3 (paired t-test, P = 1.7E-05), in agreement with the previous report, but more significant (Thoen et al. 2016). This trend was visible in all 8 time bins, but the relative time thrips spent in either zone differed only significantly in the first, second and fifth hour (Suppl. Table 1, Duration detected per zone per hour). The second overarching behavioral category to look at is pausing behavior. Thrips spent significantly more time pausing on Rmx-A180 (Table 2), and this difference was also significant in most individual time bins (Figure 2a, Suppl. Table 1, estimated duration pausing per zone per hour). This variable was also available in EthoVision, but EthoAnalysis allows the analysis of individual events in addition.

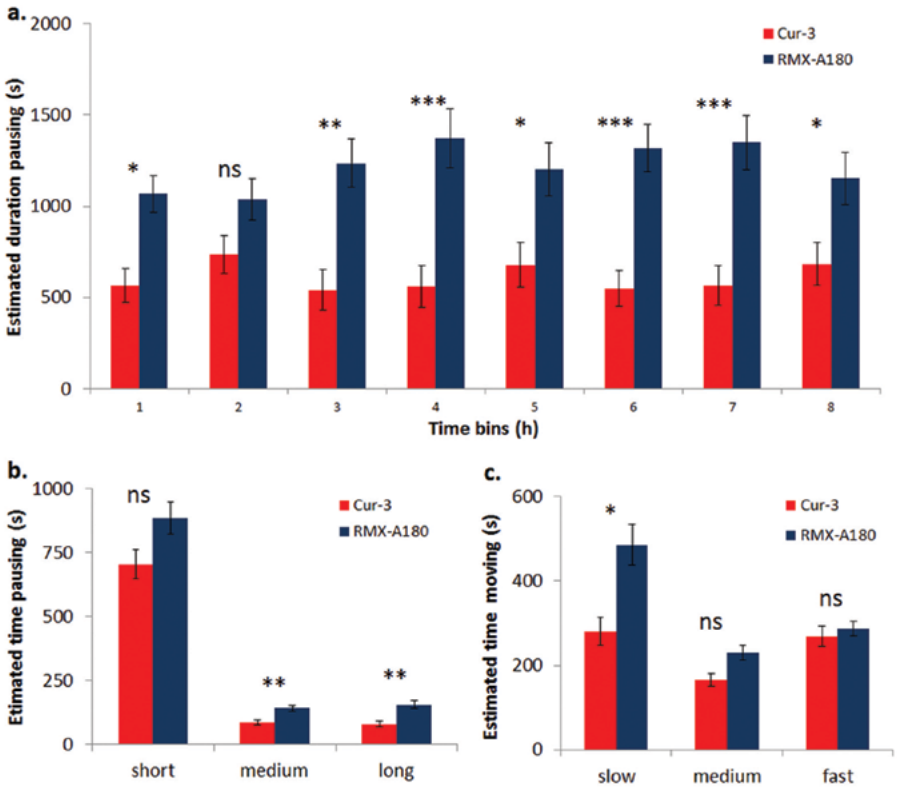


Figure 2. Differences in thrips pausing behavior on Cur-3 and Rmx-A180. a. Differences in total time spent halting in eight time bins. b. Time spent with short pauses (less than 5 s), medium pauses (more than 5, less than 10 s) and long (more than 10 s) pauses over the course of eight hours. c. Time spent with slow (less than 0.15 mm/s), medium (in between 0.15 and 0.25 mm/s) and fast (faster than 0.25 mm/s) movement events over the course of eight hours. Means ±SE, n = 60. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ns = not significant, based on difference between Cur-3 and Rmx-A180, two-sided t-test on the difference between the transformed behavioral statistics).

We can, for instance, see that an average pausing event lasts almost twice as long on Rmx-A180 compared to Cur-3 (Table 2), and with the exception of the second hour this difference was significant in all time bins (Suppl. Table 1, Average pausing duration per zone per hour). Although the number of pausing events per zone did not differ between the two accessions over the total duration of the recording, we do observe differences in individual time bins where more pause events occur on Rmx-A180. When analyzing these events in distinct duration events, we observe that the number of short pause events did not differ between the two accessions, but the number of medium and long pause events did differ (Figure 2b, Table 2).

The third overarching category is moving behavior. There is no difference in the estimated distance moved per zone (Table 2). However, the average velocity per zone does differ ($P = 0.002$). Although the total amount of time spent moving

by thrips is greater on the susceptible Rmx-A180 accession ,the average time a movement event takes is longer on the resistant Cur-3 accession (Table 2). This difference is most pronounced when looking at the average movement duration of medium length (P =0.0009, Suppl. Table 1). The most significant difference

Table 2. Comparison of behavior variables derived from EthoVision and EthoAnalysis

	EthoVision[1]			EthoAnalysis[2]		
Variable	Cur-3 Resistant Means ±SE, n= 68	Rmx-A180 Susceptible Means ±SE, n= 68	Significance	Cur-3 Resistant Means ±SE, n= 60	Rmx-A180 Susceptible Means ±SE, n= 60	Significance
Duration spent in zone (s)	5026±470	8292±631	**	5218±532	8622±696	****
Duration pausing (s)	4122±446	7494±632	***	4571±566	8797±815	**
Duration moving(s)	895±80	787±73	*	1085±91	1350±101	NS
Activity ratio (mov/pausing) (%)	22±2 %	11±2%	***	35±2	20±2	****
Distance moved (mm)	870±70	926±68	NS	328±27	350±23	NS
Movement velocity (mm/s)	0.65±0.02	0.68±0.02	NS	0.31±0.01	0.27±0.01	**
Average pausing duration (s)	-	-	-	4.8±0.4	7.8±0.8	***
Average moving duration (s)	-	-	-	1.6±0.05	1.3±0.04	****
Detection percentage (%)	-	-	-	18.1±1.8	30±2.4	**
Number of pauses	-	-	-	631.9±56.3	850.6±58.1	NS
Pause frequency[3]	-	-	-	869.5±74	1182.2±83.9	NS
Short pause frequency	-	-	-	703.6±56.8	884.6±63	NS
Medium pause frequency	-	-	-	85.3±10.9	141.9±12.4	**
Long pause frequency	-	-	-	80.6±12.7	155.8±15.8	**
Movement frequency	-	-	-	713.5±67.5	1003.4±78.2	NS
Short movement frequency (<1 s)	-	-	-	379.9±41.5	582.5±48.1	NS
Medium movement frequency (>1s <5s)	-	-	-	299.8±26.4	387.7±29.3	NS
Long movement frequency (>5s)	-	-	-	33.7±3.2	33.2±3.1	NS
Slow movement frequency (<0.15mm/s)	-	-	-	280±33.8	485.9±48.1	*
Midspeed movement frequency (>0.15<0.25mm/s)	-	-	-	165±15.9	230.1±18.1	NS
Fast movement frequency (>0.25mm/s)	-	-	-	268.6±24.7	287.4±17.4	NS

[1] Based on data exported and analyzed in Thoen *et al.* 2016, Wilcoxon signed rank test
[2] Based on settings described in table 1, two-sided t-test on the difference between the transformed behavioral statistics
[3] Frequencies are corrected for tracking efficiency

between the two accessions (P = 2E-05) is observed when comparing the ratio of movement to pausing duration per zone (Suppl Table 1 Ratio movement to pausing duration per zone). When distinguishing between short, medium and long movement events, we see that the time spent on short movement events is the only category where thrips spent significantly more time moving on Rmx-A180 over Cur-3 (Figure 3d). In addition, the distinction between slow, medium and fast movements shows that only the slow movement events take up more time on Rmx-A180 (Figue 2c). When the analysis from EthoAnalysis is compared to the analysis from EthoVision similar trends are observed, mutually validating the results, although some relevant differences are also clearly visible (Table 2). The most notable difference is the average movement velocity of thrips, which differed in EthoAnalysis between the two accessions, but EthoVision did not make this distinction.

Correlation of behavioral statistics

Many of the EthoAnalysis behavioral statistics are likely dependent on each other to some degree. Both dependence and independence may have causes in genes (both plant and insect) or environment (assay quality). To test this, we have applied a Spearman correlation test on statistics obtained for Z1 (Rmx-A180) (Figure 3). This diagram of the correlation between the statistics of the most robust traits for the entire 8 hours of the recording shows how specific thrips behaviors correlate neutrally, negatively (blue boxes) or positively (yellow boxes) with each other. It indicates that most of the statistics show strong correlations with each other. For instance, insects that perform many slow movement events will have high average duration of short movement events and a high duration of medium pausing events. The statistics long pausing frequency, estimated pausing duration, duration spent in the zone and duration of long pauses form another cluster of statistics that correlate positively with each other. The number of short pauses on the other hand are not the inverse of number of long pauses. Insects that perform many short pauses (indication of test probes on sub-optimal food sources) also perform many long pauses (indications of feeding events on suitable host-plants).

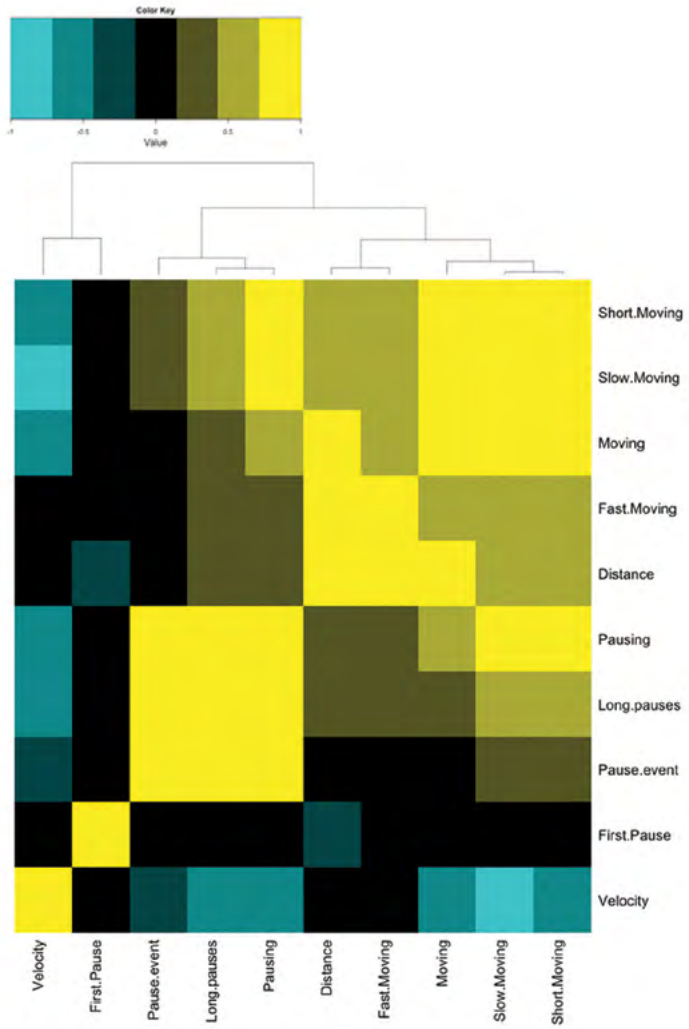


Figure 3. Correlation diagram of behavioral variables. Colors indicate spearman *Rho* correlation values, where blue boxes indicate negative correlations and yellow boxes positive correlations between traits. Traits were clustered hierarchically according to their raw phenotypic output from behavioral parameters obtained for each arena on the susceptible Rmx-A180 accession, using Ward's minimum variance method. Short moving = time spent on movement events with a duration shorter than 1 second. Slow Moving: time spent on movement events with a average velocity lower than 0.15 mm/s. Moving: total time spent moving. Fast moving: time spent moving with a velocity higher than 0.25 mm/s. Distance: total distance moved. Pausing: total time spent pausing. Long pauses: Time spent on pauses that took longer than 10 s. Pause event: the average duration of one pause event. First pause: latency to the first pausing event. Velocity: average velocity.

Discussion

We have evaluated a novel software package, EthoAnalysis, for the automated analysis of the behavior of thrips in a two-choice arena setting on two Arabidopsis accessions, relative to manual analysis of EthoVision outputs. The tracking data used and manual analysis were published in a previous study (Thoen et al. 2016). EthoAnalysis is a novel software tool to analyze in-depth behavioral statistics that are not available in the previously used EthoVision software. It can be used to combine data of multiple experiments, extract statistics and report summary statistics per accession comparison to aid in the identification of differences in insect behavior that may be due to potential differences in resistance.

Settings that optimize visualization of the differences in behavior for western flower thrips

We conclude from varying the different parameters in EthoAnalysis, that most adjustments can strongly influence the output of your experiment. Selecting unrealistic parameter settings has the potential of creating data without biological relevance. EthoAnalysis creates a data report after analysis that indicates for all variables to what extent they are normally distributed. This helps users in detecting irregularities in their data that can skew the outcome of the analyses. It is important to manually check if the selected speed thresholds indicate a solid distinction between moving and pausing behavior. This can be checked for example in the frequency histograms (EthoAnalysis, Suppl. Figure 1-6) and video recorded behavior with visual acquisition (EthoVision). When using kinematic variables to describe the behavior of insects, it is essential to create robust outlier resistant data. EthoAnalysis allows filtering to calculate, for instance, average velocity for an insect only when it passes specific thresholds. The settings of these thresholds have been extensively adjusted in the sensitivity analyses performed in this study to most accurately describe relevant thrips behavior. Although these settings (Table 1) are both species- and experimental-design dependent, they can still function as a starting point for future experiments in this field. With these settings we could extract 214 variables in three overarching behavioral categories related to host-plant acceptance, most of them comprised of several dependent and independent variables. These three broad categories are settlement, pausing behavior and movement behavior. With these settings and variables, the differences were characterized between the Arabidopsis accessions Cur-3 and Rmx-A180. Ideally, experiments are repeated to confirm that significant differences observed in variables are true positives.

Settlement

The most basic information one can obtain when screening thrips behavior in a two-choice arena, is insect settlement over time. Preference for one genotype over the other can be deduced from distribution ratios across test and reference accessions (Thoen, Kloth et al. 2016). Long recordings can give additional information on changes in thrips preference over time, due to resistance mechanisms that might take a few hours to establish their effects on thrips, or resistance mechanisms that require certain induction by physical damage or herbivory, before defense pathways are activated. An example of 'slow acting defense compounds' are protease inhibitors, which can take at least 4 hours to result in a significant effect on thrips choice behavior (Outchkourov, de Kogel et al. 2004). In addition, thrips activate the jasmonic acid (JA)-pathway upon herbivory in Arabidopsis and other Brassicaceae species (De Vos, Van Oosten et al. 2005, Abe, Ohnishi et al. 2008, Abe, Shimoda et al. 2009) leading to a defense which can take even longer to be fully mounted. In Arabidopsis, a mitogen-activated protein (MPK4) is activated 2 to 5 minutes after wounding. Upon wounding a complex cascade of regulation and crosstalk of several transcription factors (e.g. MYC and ERF) takes place leading to defensive responses. These induced defenses up-regulate genes that trigger the metabolism of a wide array of defensive compounds. The timing of the activation of downstream transcription factors in these phytohormone-regulated pathways has been studied with qPCR or transcriptomics (Pieterse, Van der Does et al. 2012). Studying herbivore behavior over time can pinpoint the exact moment such induced defensive mechanisms influence actual herbivory. In addition to settlement over time, the first choice an insect makes for probing can represent biologically meaningful information. The first choice can be regarded as a choice without prior knowledge on nutritional value, nonvolatile toxins and other morphological and chemical aspects of plant defense. Many experiments on thrips preference have solely focused on choice behavior based on olfactory cues, mostly done in Y-tube olfactometer settings (de Kogel, Koschier et al. 1999, Koschier, de Kogel et al. 2000). We did not observe differences in initial preference between Cur-3 and Rmx-A180 in this study.

Interpretation of pausing events

In addition to the host-choice over time, the actual behavior (pausing and movement) on plant material where thrips settle and feed holds relevant information on the nutritional quality and physical accessibility of the host-plant. Although previous studies on *Thrips tabaci* demonstrated that most of the pausing behavior is in fact feeding behavior (Riefler and Koschier 2009), the additional parameters reported by the EthoAnalysis software may allow disentangling feeding from non-feeding within pausing events. Feeding events that take 10 seconds or more can be considered to

refer to actual food intake but shorter ones are unlikely candidates (Lewis 1984). Thus this parameter can function as a proxy for host-plant acceptance. Probing events that take longer than 10 seconds might indicate several consecutive probes in close succession which do not cross the movement threshold. In our study we do not observe differences in the duration of short pausing events between the two accessions. However, the medium and long pausing events are significantly longer in the susceptible Rmx-A180 accession. This indicates that the short test probes are of similar duration on resistant and susceptible accessions, but the longer actual feeding events are much longer on the susceptible accession. Furthermore, the medium and longer pausing events are significantly more frequent in Rmx-A180, wherein short pausing events are just as frequent on both accessions. The latency in which these short, medium and long probes take place on the different genotypes can be of additional value. We know, for instance, for aphids that the time it takes before they reach the phloem for the first time (an actual feeding event) is cell-wall dependent, and differs among genotypes (Kloth, ten Broeke et al. 2015). Aphids and thrips differ in their feeding mode of action, thus this latency variable might not be as relevant in a non-phloem feeding insect like cell-content feeding thrips. However, this has not been studied before, and using this variable could establish the relevance of latency to test probes, real feeding events, and long feeding events. In the study presented here, we did not observe differences in the latency to first pauses between the two tested accessions.

Interpretation of movement events

Movement can refer to searching behavior, for instance for food or shelter. Movement velocity refers to the average velocity of thrips across all movement events, by dividing total distance moved over the total time moved. It can be considered as a measurement of thrips vitality. However, some compounds can have a paralyzing effect, making thrips more sluggish. For instance, upon contact with pyrethrum, sodium channels on the thrips nerve cells are opened. This causes repetitive firing of neurons, leading to rapid paralysis and death (Bradberry, Cage et al. 2005). Furthermore, primary metabolites like sugar, proteins, carbohydrates as well as nitrogen content influence life-history parameters of thrips (Brodbeck, Stavisky et al. 2001). Unsuitable plant material due to low nutritional value could lead to starvation, with an increasingly slower locomotion as consequence. This variable is especially of interest when studied over time, to observe how speed alters due to the presence or absence of certain compounds. In assays that use diluted compounds instead of leaf material, the toxicity is often inferred 24 hours post inoculation of the pesticide (Espinosa, Contreras et al. 2005). These activity parameters may provide information on activity patterns of potential pesticides, and what their

mode of activity is on thrips behavior. In our analyses comparing thrips behavior between Rmx-A180 and Cur-3, the average velocity per zone differed. The total amount of time spent moving by thrips is greater on the susceptible Rmx-A180 accession, but the average time a movement event takes is higher on the resistant Cur-3 accession. We did not observe differences in average velocity in our previous study with the same dataset (Thoen, Kloth et al. 2016). This is because velocity in EthoAnalysis is calculated over movement events that start and stop within one zone. Repetitive circling behavior of thrips in the 96-well setup we used, will have a high velocity, but will also generally cross three zones in one circle. The consecutive event filter with zone boundary violations will also filter out detection artefacts that can have unnatural velocity values, where highly skewed data without this filter can show a normal distribution when the filter is applied (Suppl. Figure 7). These relatively fast movement events are thus filtered in EthoAnalysis, leaving a more reliable velocity parameter that is host (zone) specific. The average velocity did not correlate with the estimated distance moved and the frequency of fast movement events, indicating that these are variables independent of each other in thrips on this genotype. This implies that these variables could lead to different candidate genes when these variables are used in quantitative genetic studies of these two Arabidopsis accessions. It seems that long movement (>5 s), and fast movement (>0.25 mm/s) events were not genotype dependent, as these variables did not differ between Rmx-A180 and Cur-3. These variables might be relevant in other setups, but our setup with halved leaf discs (2mm wide) made it improbable for thrips to move longer than 5 seconds without crossing zone barriers. However, thrips tend to have more slow movement events on susceptible plant material. This behavior is in agreement with the feeding behavior of thrips, which continuously make relatively slow movements to nearby cells when they feed on a suitable host plant.

Conclusion

Here we have presented an evaluation of a novel software package, EthoAnalysis, that can automatically establish host-plant preference for the generalist insect *F. occidentalis* (Western Flower Thrips) using raw video tracking export files of EthoVision software (Thoen *et al.* 2016). There are several benefits from using EthoAnalysis to analyze EthoVision data (Table 3). The analysis is performed a lot quicker in EthoAnalysis. The statistical rapport that is produced by EthoAnalysis gives researchers a quick overview of relevant differences between two or more tested accessions. Filters automatically removing entire records or events with poor data can be applied in EthoAnalysis, so that more reliable records remain. In comparison, EthoVision only allows manual filtering of entire tracks based on visual

Table 3 Comparison of EthoAnalysis and EthoVision for analyzing insect behavior

	EthoVision	EthoAnalysis
Event determination	Pre-analysis (adjusting event thresholds requires re-running the recorded video through analyses, this takes 5-20 minutes)	Post-analysis (adjusting event thresholds is done on raw track data, this takes 10-30 seconds)
Traits	• settlement (choice) • average velocity • duration moving and non-moving • distance moved	• settlement (choice) (per hour) • average velocity (per hour) • duration moving and pausing (per hour) • distance moved (per hour) • average distance moved per event (per hour) • average duration pausing or moving event (per hour) • frequency of moving and pausing (per hour) • movement event sub-categories for duration, velocity, and frequency • pausing event sub-categories for duration and frequency • ratio duration movement/pausing (per hour) • ratio duration movement/detection (per hour) • ratio duration pausing/detection (per hour) • time to first pausing
Data filter	Manual record filtering on detection percentage	Automatic record filtering on detection percentage threshold, pause duration (detected but dead). Automatic event filtering on a duration threshold of events, Automatic event filtering of interrupted events due to zone transitions.
Detection dependency	No correction of data for the percentage of detection per subject	Robust traits that are corrected for the percentage of detection per subject
Statistics	No statistical analyses in software package	Built-in statistical package and default transformations on data
Graphical visualization of data	Table of means per subject. No graphs of experimental results	Summary tables, graphs per trait, visualization of statistical models used, export of pdf report with all data per arena
Post-recording video interaction	Visual acquisition enables you to check manually if input settings (zones, moving thresholds etc.) are in line with what's observed in the video. Heatmaps (EthoVision XT10 and higher) allow you to see the positional distribution of the insects for the entirety of the recording	No direct interaction with the video

inspection of deviant records. EthoAnalysis can redefine input thresholds without the need to re-run the entire recording, as with EthoVision. This saves much time when many different settings must be adjusted to find the optimal settings to describe relevant behavioral variables. Both the quantity and quality of variables obtained from EthoAnalysis are surpassing what can be obtained from EthoVision.

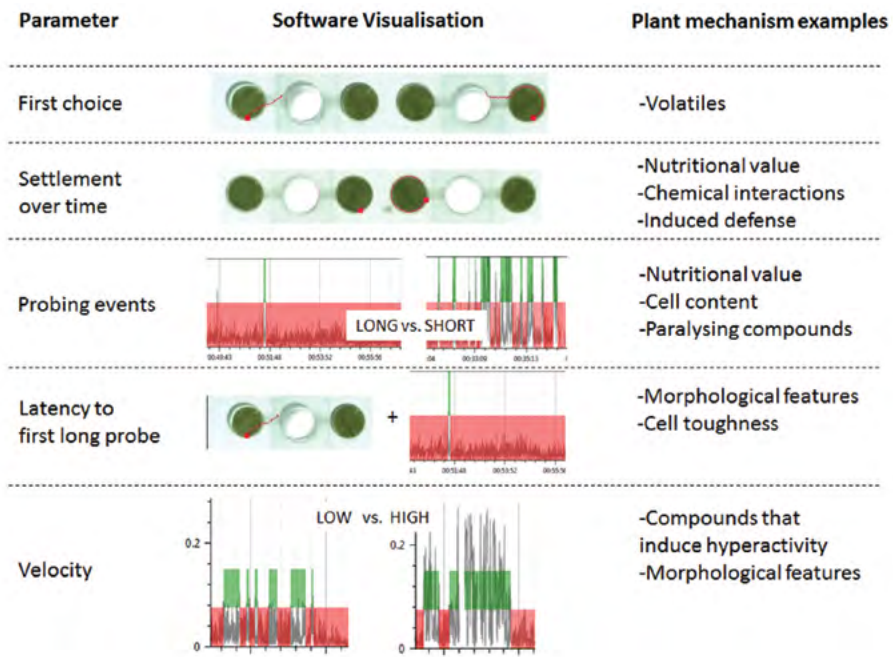


Figure 4. Behavioral parameters and plant defense mechanisms. Depicted are several behavioral parameters that can be studied with automated video tracking (left), their visualization in tracking and analysis software (middle), and the hypothesized plant defensive mechanisms that may be discovered in quantitative genetic studies.

This is mainly due to appropriate filtering of tracks and events and the possibility to distinguish between trait categories like short/medium/long and slow/medium/fast moving and halting events. EthoAnalysis also includes a built-in software package based on R, that properly transforms data and applies the correct statistical models to evaluate behavioral parameters, which are visualized in graphs in an extensive pdf report. The recording and arena settings must still be done in EthoVision, and post-recording visualizations like 'visual acquisition' are useful when manually checking if specific variable settings are in line with the observed behavior in the recorded movie. EthoAnalysis is a valuable add-on software package optimized for extracting relevant behavioral variables of insects.

We have validated the utility of EthoAnalysis with a resistant and susceptible *Arabidopsis thaliana* accession in two-choice assays. New behavioral variables were introduced that may reveal different aspects of the behavior of insects. Additional and independent behavioral parameters can point to different defensive strategies of plants that can be discovered in quantitative genetic studies (Figure 5). For instance, first choice in quantitative genetic studies might discover candidate genes involved in the production of volatile cues that attract or deter thrips. Settlement over time relates to induction and time-dependent chemical interactions. Plant genes related to nutritional value and chemical compounds that directly act on the insect's sensory organs might be detected in quantitative studies using this variable. Probing events relate to plant genes involved in nutritional value and secondary metabolism at the cell-content level. Latency to first pause may give insight in how long it takes before specific probing behavior occurs. If defensive mechanisms are parallel to that of phloem feeders like aphids and whiteflies, we might discover genes related to cell walls, epidermal waxes and trichome density for instance, that somehow influence the time it takes for thrips to start a successful feeding event. Studies that address thrips behavior outside the context of host-plant resistance, looking for instance at the influence of acting as a vector of tospoviruses (Stafford, Walker et al. 2011), attraction to volatiles (Koschier, de Kogel et al. 2000) or pesticide activity on thrips (Espinosa, Contreras et al. 2005), can also greatly benefit from this method.

Methods

Experimental materials

An eight-hour recording of 88 parallel two-choice assays from a previous study (Thoen, Kloth et al. 2016) was used for the present study. In brief: 88 five-week-old *Arabidopsis thaliana* plants (accessions Cur 3 and Rmx-A180) were used as source of leaf discs that were used to fill a 96-well plate with a halved leaf disc from either accession (Cur-3 and Rmx-A180) on a layer of agar. We used a digital camera mounted on top of a backlight unit that illuminated the arenas. Ca. 1 cm above the backlight unit, a 96-well plate containing 88 arenas with 88 unique combinations of the halved leaf discs from Cur-3 and Rmx-A180. The Western flower thrips (*Frankliniella occidentalis* (Pergande)) used were adult females that had been starved overnight in Perspex tubular cages. Thrips were anesthetized with CO_2 and placed on ice just prior to the recording. The EthoVision XT 10.0 settings used are described in Thoen, Kloth et al. (2016). Both leaf discs were assigned to a specific zone (Z0 and Z1); in addition, there was a neutral zone that did not contain leaf

material (Z2). Raw data files with genotypes in a 'Genotype' column separated by a '$' sign were exported per subject for analysis in EthoAnalysis. Data were analyzed in EthoAnalysis with the settings described in Table 1.

Settings determining event state and duration

The video tracking software, EthoVision XT, produces series of track samples for all insects/arenas. Each track sample contains an (x,y)-coordinate, a velocity, and an indication of the current zone in which the insect resides unless the insect was not detected. EthoAnalysis translates these series into series of zone-specific events of three types; *pausing*, *moving*, and *not-detected*. Each event had a starting time and a duration, and from these series of events, the various behavior statistics are extracted.

The series of behavior events are constructed by iterating over the track samples and determining for each sample whether the current state is moving, pausing, or unknown. Each consecutive sequence of track samples with the same movement state and occurring in the same zone forms a behavior event of the movement state of that series.

The following procedure is followed to convert the track samples into series of events:

- Start at the first track sample at the beginning of the trial with a *movement state* of *unknown* and process the track file sample by sample in time.
- Iterate over the sample records using the following decision rules:
  - Determine the *movement state* of the current sample based on these rules:
    1. Current state is *moving* if either of the following two conditions is met:
       1. **Start moving:** The previous state is not *moving* **and** the current velocity is greater than or equal to the velocity threshold **and** any of the next *n* samples (the look-ahead window) has a velocity greater than or equal to the velocity threshold. (This protects both the moving and pausing states from short pause or movement spikes)
       2. **Remain moving:** The previous state is *moving* **and** any velocity in the current or the next *n* samples is greater than or equal to the velocity threshold.
    2. Else, if the current state is not recognized as *moving*, the current state is *pausing* if either of the following two conditions is met:
       1. **Start pausing:** The previous state is not *pausing* **and** the current sample has a positive detection.
       2. **Remain pausing:** The previous state is *pausing* **and** any sample in the current or the next *n* samples has a positive detection.

3. Otherwise, if the current state is not recognized as *moving* or *pausing*, the current state is set to *not-detected*.

- If the current *movement state* is not equal to the previous *movement state* **or** the current zone is not equal to the previous zone, then add a new event for the previous state with its start time, end-time, and, if moving, the distance moved during this event.

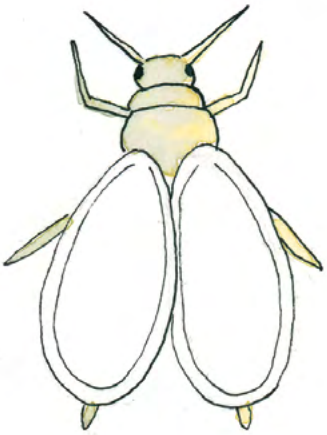
Statistics

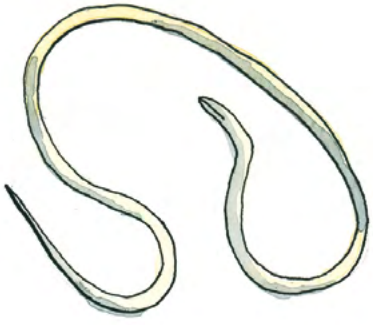
Every arena consisted of a unique combination of plant material from either Cur-3 or Rmx-A180, thus no pseudo replicates were present to account for. The analysis of two-choice assays in EthoAnalysis comprises a two-sided t-test on the difference between the transformed behavioral statistics of the two zones containing the genotypes (i.e., Z0 – Z1). For each statistic, a default transformation is chosen that is most suitable for the type of data of the statistic. For count data, a $\log(m+1)$.

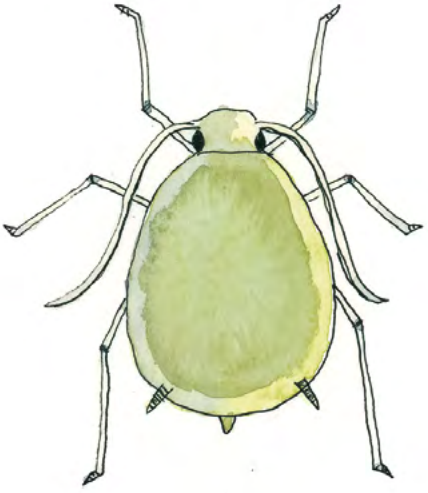
Procedure: per record, transform the extracted behavioral statistics of zone Z0 and Z1 using the default transformation T(·). Compute the difference $\Delta = T(Z_0) - T(Z_1)$ for all records. Test, for the sample mean $\overline{\Delta}$ the null hypothesis $H_0 : \overline{\Delta} = 0$ against $H_a : \overline{\Delta} \neq 0$ and compute the 95% confidence intervals of the difference on the transformed scale $\mathrm{CI} = \overline{\Delta} \pm t_{1-\frac{\alpha}{2},\, N-1} \frac{s}{\sqrt{N}}$, with $\alpha = 0.1$. Backtransform the confidence intervals using the inverse of the transformation $T^{-1}(\cdot)$. For the log-transformation, the back-transformed confidence interval $[T^{-1}(CI_{lower}), T^{-1}(CI_{upper})]$ reflects the difference in terms of ratios. E.g., if the back-transformed confidence interval is [2,4], then we can say that with 95% confidence that the statistic of zone 0 is between two, and four times as high as zone 1. When no transformation is used, the confidence intervals are defined as the absolute differences of the statistic between the two zones. The analysis is based on the assumption that the records are independent and that the differences between the (transformed) statistics of the two zones follow a normal distribution.

Spearman correlations on raw data variables (extracted with settings from table 2) from Z1 (accession Rmx-A180) were performed in R with the 'Hmisc' package. Heatmap of correlations was created with the 'gplots' package.

Supplementary files can be found online: http://dx.doi.org/10.18174/387710.







Chapter five

Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping

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**These authors contributed equally to this work*

under review

Abstract

Plants are exposed to combinations of various biotic and abiotic stresses, but stress responses are usually investigated for single stresses only. Here we investigated the genetic architecture underlying plant responses to 11 single stresses and several of their combinations by phenotyping 350 *Arabidopsis thaliana* accessions. A set of 214k SNPs was screened for marker-trait associations in Genome-Wide Association analyses using tailored multi-trait mixed models. Stress responses that share phytohormonal signaling pathways also share genetic architecture underlying these responses. For the 30 most significant SNPs average QTL-effect-sizes were larger for dual stresses than single stresses. Plants appear to deploy broad-spectrum defensive mechanisms influencing multiple traits in response to combined stresses. Association analyses identified QTLs with contrasting and with similar responses to (a) biotic versus abiotic stresses and (b) belowground versus aboveground stresses. Our approach allowed for an unprecedented comprehensive genetic analysis of how plants deal with a wide spectrum of stress conditions.

Introduction

In nature, plants face variable environments that impose a wide range of biotic and abiotic stresses. These include e.g. belowground and aboveground stresses, stresses imposed by unicellular and multicellular organisms, short and long-lasting stresses. Under natural conditions, these stresses do not occur in isolation but are commonly present simultaneously (Rizhsky, Liang et al. 2004, Bergelson and Roux 2010, Mittler and Blumwald 2010, Vile, Pervent et al. 2012, Prasch and Sonnewald 2013, Rasmussen, Barah et al. 2013, Kissoudis, van de Wiel et al. 2014, Rivero, Mestre et al. 2014, Sewelam, Oshima et al. 2014, Suzuki, Rivero et al. 2014). Thus, plants are under strong selection to adapt to local conditions and have evolved sophisticated mechanisms to withstand multiple adverse environmental conditions (Howe and Jander 2008, Bergelson and Roux 2010, Pieterse, Van der Does et al. 2012, Stam, Kroes et al. 2014, Brachi, Meyer et al. 2015, Julkowska and Testerink 2015, Kerwin, Feusier et al. 2015). Yet, investigating this in a targeted experimental way is a major challenge due to the complexity of multiple stress exposure. To gain insight into the adaptation of plants to the wide variety of stress-inducing conditions they face, genetic variation and mechanisms underlying stress resistance should be studied (Alonso-Blanco, Aarts et al. 2009, Brachi, Meyer et al. 2015, Kerwin, Feusier et al. 2015). The responses of plants to stresses have traditionally been investigated for individual stresses (Howe and Jander 2008), but research focus is currently shifting towards plant responses to combinations of stresses (Holopainen and Gershenzon 2010, Pierik and Testerink 2014, Stam, Kroes et al. 2014, Suzuki, Rivero et al. 2014, Kissoudis, Chowdhury et al. 2015). The emerging picture is that responses to stress combinations cannot be predicted reliably from the responses to individual stresses (De Vos, Van Zaanen et al. 2006, Makumburage, Richbourg et al. 2013). For instance, the majority of transcriptional responses of Arabidopsis to combinations of two abiotic stresses could not be predicted from responses to the individual stresses (Rasmussen, Barah et al. 2013). Moreover, phenotype expression in response to two biotic stresses could not be predicted on the basis of existing information on interactions between underlying signaling pathways (De Vos, Van Zaanen et al. 2006). Phytohormones are major players in a signaling network, mediating responses to both biotic and abiotic stresses (Pieterse, Leon-Reyes et al. 2009). For instance, chewing insect herbivores elicit especially the jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET) signaling pathways, phloem-sucking insects and biotrophic microbial pathogens elicit especially the salicylic acid (SA) pathway, and drought elicits the abscisic acid (ABA) pathway (Pieterse, Leon-Reyes et al. 2009). The phytohormonal responses exhibit extensive crosstalk, resulting in specific changes in plant phenotype in response to individual stresses (De Vos, Van Oosten et al. 2005, Pieterse, Van der Does et al. 2012).

In plant breeding, resistance and tolerance to multiple stresses is a common selection target (Braun, Rajaram et al. 1996). A well-known strategy to achieve resistance and tolerance is by evaluation of candidate varieties in multi-environment trials, i.e., field trials at multiple locations during several years (van Eeuwijk, Bink et al. 2010, Malosetti, Ribaut et al. 2013). In such trials, multiple stresses can occur, but their occurrence and the intensity with which they occur is not guaranteed and, therefore, plant breeders developed the concept of managed stress trials in which specific and well-defined stress conditions are imposed for a single or a small number of stresses (Cooper and Hammer, 1996; Cooper et al. 2014). Recently, the urge to manage environmental factors even more precisely has led to the development of phenotyping platforms, where, again, mainly single stresses are investigated (Fiorani and Schurr, 2013; Granier and Vile, 2014; Kloth et al. 2015).

Most studies, outside plant breeding, that examined plant responses to multiple stresses included only one or a few genotypes (Holopainen and Gershenzon 2010, Rasmussen, Barah et al. 2013, Pierik and Testerink 2014, Stam, Kroes et al. 2014, Suzuki, Rivero et al. 2014, Kissoudis, Chowdhury et al. 2015). To obtain a further understanding of the genetic architecture of complex traits such as plant adaptation to a diversity of stresses, extensive study of the natural genetic variation within a species is instrumental. Genome-wide association (GWA) analysis is an important tool for this, requiring a large number of well-genotyped plant accessions. Yet, although the interest in natural variation and GWA mapping is rapidly increasing (Wijnen and Keurentjes 2014, Ogura and Busch 2015), a large-scale evaluation of natural genetic variation for resistance of plants to the full diversity of stresses that they are exposed to, including pathogens, herbivores and abiotic stresses and their interactions, has not been made to date. To elucidate the genetic architecture of plant stress resistance, an integrated approach is needed that models the genetics of responses to a range of single and combined stresses, including the interaction between those responses. Here, we have taken a comprehensive and integrated approach to investigate the genetics underlying plant responses to 15 carefully standardized single stresses or stress combinations (Table 1), making use of a global population of 350 Arabidopsis accessions that have been genotyped for 214k SNPs (Baxter, Brazelton et al. 2010, Li, Huang et al. 2010). The standardization of these 15 stress conditions is an important element of the study because it allows for phenotyping of well-defined stress responses. We developed a tailored multi-trait GWA analysis that allowed the identification of candidate genes associated with adaptive plant responses to multiple stresses that were validated by gene expression and mutant analyses.

Table 1. Phenotypes assessed.
The dataset contains three plant stress categories; abiotic stress, biotic stress and combinations of both abiotic and biotic stress. Phenotype assessments that were performed under similar environmental conditions have similar background shading (light and dark grey). 'Phenotype' refers to different phenotypic assessments (in some cases the first principal component of a group of phenotypes). 'Treatment' refers to the sort of stress that was applied. Additional information on traits can be found in Supplementary Methods.

	Stress	Trait name	Trait phenotype	Treatment
Abiotic stresses	Salt	Salt_1	Main root length, number of lateral roots and straightness	75 mM NaCl
		Salt_2	Main root length	125 mM NaCl
		Salt_3	Number of lateral roots	125 mM NaCl
		Salt_4	Main root angle	125 mM NaCl
		Salt_5	Biomass	25 mM NaCl
	Drought	Drought_1	Biomass	Drought
		Drought_2	Biomass	Drought
	Osmotic	Osmotic	Biomass	PEG8000
	Heat	Heat	Number of siliques	35 ˚C
Biotic stresses	Parasitic plant	Parasitic plant	Attachments	*Phelipanche ramosa*
	Nematode	Nematode	Offspring, eggmass	*Meloidogyne incognita*
	Whitefly	Whitefly_1	Survival, whiteflies	*Aleyrodes proletella*
		Whitefly_2	Reproduction, eggs	*A. proletella*
	Aphid	Aphid_1	Behavior T1, probing	*Myzus persicae*
		Aphid_2	Behavior T2, probing	*M. persicae*
		Aphid_3	Offspring, aphids	*M. persicae*
	Thrips	Thrips_1	Feeding damage	*Frankliniella occidentalis*
		Thrips_2	Behavior T1	*F. occidentalis*
		Thrips_3	Behavior T2	*F. occidentalis*
	Caterpillar	Caterpillar_1	Leaf area consumed	*Pieris rapae*
		Caterpillar_2	Biomass	*P. rapae*
		Caterpillar_3	Number of damaged leaves and feeding sites	*P. rapae*
	Fungus	Fungus	Number of spreading lesions	*Botrytis cinerea*
	Double stress	Fungus and caterpillar_1	Biomass	*B. cinerea* and *P. rapae*
		Fungus and caterpillar_2	Number of damaged leaves and feeding sites	*B. cinerea* and *P. rapae*
		Caterpillar and fungus	Number of spreading lesions	*P. rapae* and *B. cinerea*
Abiotic and biotic stress	Double stress	Drought and fungus	Number of spreading lesions	Drought and *B. cinerea*
		Drought and caterpillar	Number of damaged leaves and feeding sites	Drought and *P. rapae*
		Caterpillar and osmotic_1	Projected leaf area	*P. rapae* and PEG8000
		Caterpillar and osmotic_2	Biomass	*P. rapae and* PEG8000

Results

The phenotypic response of a population of 350 Arabidopsis accessions to an extensive set of stress-inducing conditions was quantified *relative to* the respective control treatments. Correcting for the respective control means that in the residual signal for a trait, effects of earliness, flowering time, general robustness, vigour, etc., have been removed already. Therefore, the traits as analysed represent a kind of stress *per se* response from which all kinds of disturbances have already been eliminated. Thirty traits, including e.g. root length, number of damaged leaves, or number of pathogen-inflicted spreading lesions (Table 1) were quantified when the plants were exposed to 15 different stresses, i.e. four abiotic stresses (drought, salt stress, osmotic stress and heat), seven biotic stresses (parasitic plant, phloem-feeding aphid, phloem-feeding whitefly, cell-content feeding thrips, leaf-chewing caterpillar, root-feeding nematode, and necrotrophic fungus) and four stress combinations (fungus and caterpillar, drought and fungus, drought and caterpillar, caterpillar and osmotic stress). For detailed information on the carefully standardized stress treatments, the trait definitions and phenotyping, see Supplementary Methods.

Heritability of responses to biotic and abiotic stresses

The phenotypic analysis resulted in a wide range of marker-based narrow sense heritability (Kruijer, Boer et al. 2015) estimates with 15 traits of low ($h^2 < 0.2$), 10 of moderate ($0.2 < h^2 < 0.5$) and 5 of high ($h^2 > 0.5$) heritability (Figure S1). The number of abiotic stress traits per heritability category was similar, while the number of traits related to biotic and combined stresses decreased with increasing heritability class. The most heritable traits were responses to feeding damage by thrips (Thrips_1; $h^2 = 0.8$), and nematodes ($h^2 = 0.7$), and responses to salt (Salt_1 and Salt_3; resp. $h^2 = 0.6$ and $h^2 = 0.7$) and heat (Heat; $h^2 = 0.6$) (Table S1). The traits related to combined stresses have predominantly low heritabilities; however, it should be emphasized that the combined stresses especially relate to combinations involving fungal and caterpillar stress.

Genetic commonality underlying responses to different stresses

To analyze the phenotypic variation between Arabidopsis accessions as a function of molecular marker variation, we used various mixed model approaches (see Methods section). We estimated marker-based genetic correlations, i.e. correlations based on the genome-wide commonality of SNP effects underlying pairs of traits (see Methods), to investigate the magnitude of genetic commonality underlying resistance mechanisms in response to a range of biotic and abiotic stresses. For brevity, we will refer to these marker-based genetic correlations as genetic

correlations. Such genetic correlations can be interpreted as upper boundaries to the joint determination of pairs of traits by genetic factors. Genetic correlation analysis revealed a strong connection between the responses to parasitic plants and to aphids (r = 0.8), which were both negatively associated with other stress responses (Figure 1). Parasitic plants and aphids have in common that they target phloem and xylem tissue (Tjallingii and Hogen Esch 1993, Dorr and Kollmann 1995), and induce the

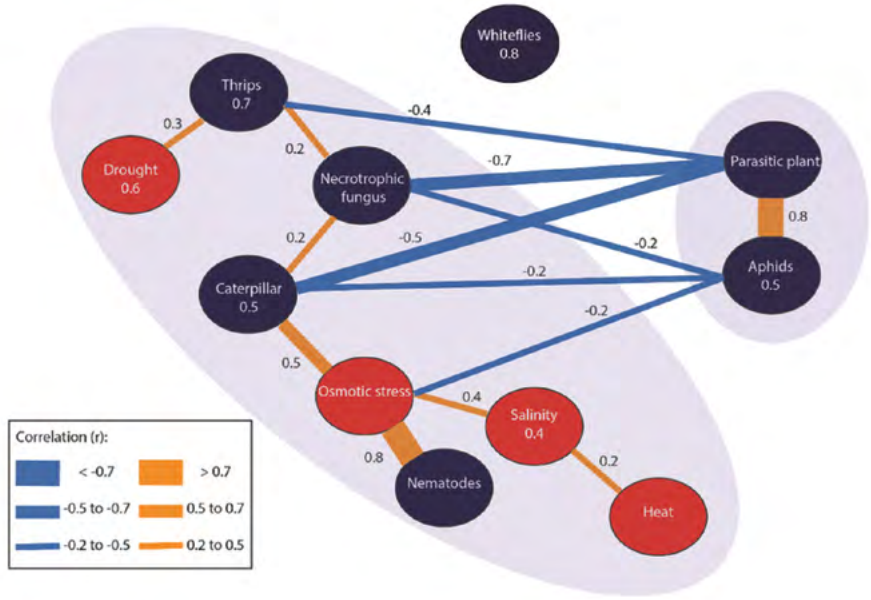


Figure 1. Mean genetic correlations between responses to abiotic (red) and biotic (dark blue) plant stresses. Thickness of lines represents the strength of mean genome-wide correlations, annotated with r values (orange = positive, blue = negative correlation). The more shared genetic associations between stresses, the higher the absolute genetic correlation. Correlations are negative when alleles have opposite effects, i.e. resulting in increased resistance to one stress, but decreased resistance to the other stress. Values in balloons represent mean within-group correlation (not shown for groups consisting of a single trait). Mean between-group correlations are not shown if they are below an absolute value of r = 0.2. Two clusters can be distinguished: (1) parasitic plants and aphids and (2) the other stresses, except whiteflies.

SA phytohormonal pathway (De Vos, Van Oosten et al. 2005, Runyon, Mescher et al. 2008). In contrast, the biotic stress responses that were negatively associated with the responses to parasitic plants and aphids, i.e. responses to necrotrophic fungi, caterpillars, and thrips, represent JA-inducing stresses (De Vos, Van Oosten et al. 2005, Pieterse, Leon-Reyes et al. 2009, Pieterse, Van der Does et al. 2012). Because the SA and JA pathways predominantly interact through negative crosstalk (Pieterse, Leon-Reyes et al. 2009), the two main clusters resulting from the genetic correlation analysis represent different phytohormonal signaling response mechanisms. We also observed a strong genetic correlation between plant responses to osmotic stress and

root-feeding nematodes. This supports the notion that root-knot nematodes trigger a differentiation of root cells to multinucleate giant cells with severely altered water potential and osmotic pressure (Baldacci-Cresp, Maucourt et al. 2015). While the correlations between traits at the phenotypic level were generally rather low, the genetic correlation analysis revealed a common genetic basis underlying the responses to sets of single and combined stresses (Figure S2).

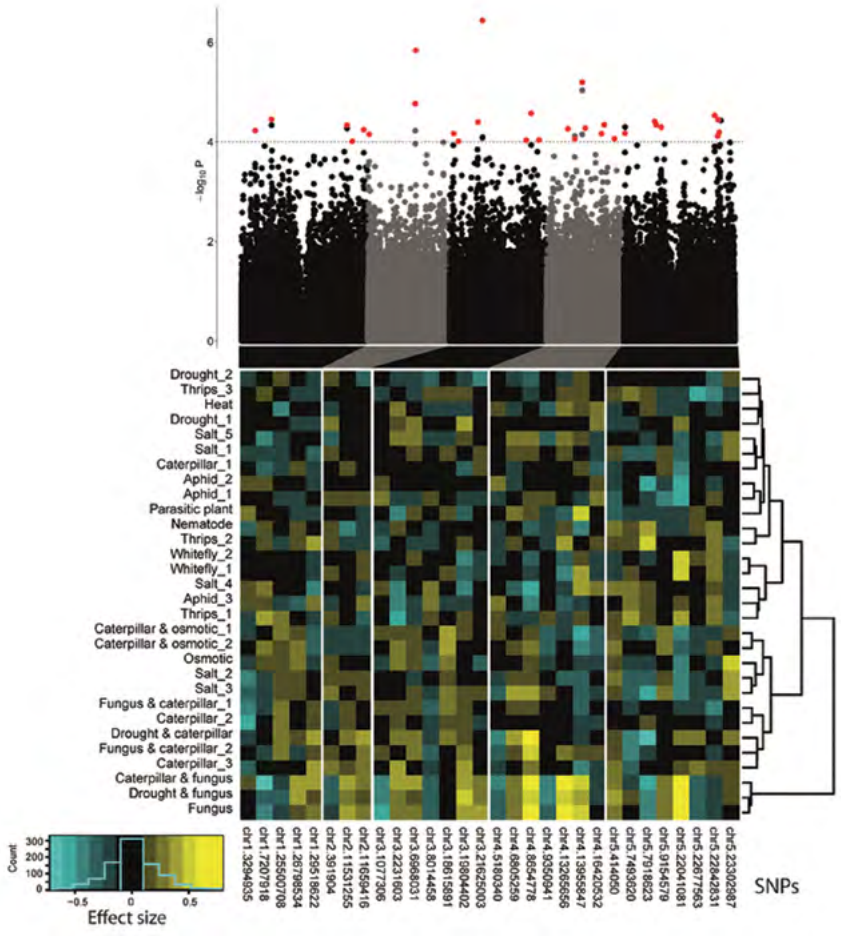


Figure 2. Multi-trait mixed-model (MTMM) GWA mapping with 30 different stress responses of *Arabidopsis*. The top panel shows the 214k SNPs with their corresponding -$\log_{10}(P)$ values for the five chromosomes. The lower panel depicts the trait-specific effect sizes of the rare alleles for significant SNPs ($P < 0.0001$) as estimated by the full MTMM. When several SNPs were located within the 20 kb linkage disequilibrium half-windows around the most significant SNP in a region, the effects for the SNP with the on average strongest absolute effects are shown (red-flagged in the Manhattan plot). SNPs are named by chromosome number and position on the chromosome. Negative effect sizes (blue) correspond to reduced plant resistance due to the rare allele, positive effect sizes (yellow) to increased resistance due to the rare allele. Stress responses were clustered hierarchically according to their effect, using Ward's minimum variance method. The key shows the frequency distribution for the effect sizes of the SNPs.

Candidate genes underlying responses to stresses

To identify individual candidate genes that contributed most to the pattern of genetic correlations, we fitted multi-trait QTL mixed models (MTMMs) to the total set of 30 traits, using a 214k SNP set that is commonly used for GWA studies in Arabidopsis (Kim, Plagnol et al. 2007, Atwell, Huang et al. 2010, Li, Huang et al. 2010, Horton, Hancock et al. 2012, Bac-Molenaar, Fradin et al. 2015). Our multi-trait GWA approach closely follows the modeling framework developed by Zhou and Stephens (2014) and generalizes the use of MTMMs as described previously (Boer, Wright et al. 2007, Malosetti, Ribaut et al. 2008, Alimi, Bink et al. 2013) for classical biparental offspring populations to association panels. This GWA analysis identified 30 chromosome regions with multiple, significant SNP-trait associations. From each of those regions, the significant SNP with the strongest effect was chosen to represent the locus (Figure 2; Table S2). Clustering of stresses by estimated SNP-effect profiles (Figure 2) indicates that multiple SNPs were associated with response to more than one stress. Stress combinations induced large QTL allele substitution effects in the MTMM mapping (Figure 2 and Table S2), indicating that combinations of stresses trigger broad-spectrum defensive mechanisms. A total of 125 genes were in linkage disequilibrium (LD) with the 30 most significant SNPs from the GWA analysis. Twenty of these genes were stress-related according to gene ontology (GO) annotation data (Table S3). Of these 20 genes, six have been functionally characterized by at least one study (Table 2a). For these six genes, we explored expression data to evaluate the biological relevance of these genes in stress-responsive mechanisms of Arabidopsis (Figure S3). Of special interest were SNPs chr5.7493620, chr5.22041081 and chr4.6805259, that were in LD with *WRKY38* (encoding a WRKY transcription factor involved in SA-dependent disease resistance) (Kim, Lai et al. 2008), *AtCNGC4* (involved in pathogen resistance) (Chin, DeFalco et al. 2013) and *RMG1* (coding for disease resistance protein) (Yu, Lepere et al. 2013) respectively.

Table 2. Candidate genes resulting from (a) MTMM analysis of all 30 stress responses as presented in Figure 2 and (b) contrast-specific analysis with MTMM for contrasting effects of biotic and abiotic stresses as presented in Figure 3.

Table2a

Marker	Gene in LD	Gene name	Gene description*	Responsiveness	References
chr2.11659416	*AT2G27250*	*CLV3*	One of the three *CLAVATA* genes controlling the s ize of the shoot apical meristem (SAM) in Arabidopsis	unknown	(Clark, Jacobsen et al. 1996, Fletcher, Brand et al. 1999, Shinohara and Matsubayashi 2010)
chr3.19804402	*AT3G53420*	*PIP2*	A member of the plasma membrane intrinsic protein subfamily PIP2.	Heat, salt & heat, heat & Silwet	(Martiniere, Li et al. 2012, Peret, Li et al. 2012, Rasmussen, Barah et al. 2013, Sanchez-Romera, Ruiz-Lozano et al. 2014)
chr4.6805259	*AT4G11170*	*RMG1*	Encodes RMG1 (Resistance Methylated Gene 1), an NB-LRR disease resistance protein with a Toll/interleukin-1 receptor (TIR) domain at its N terminus.	flagellin	(Yu, Lepere et al. 2013)
chr5.7493620	*AT5G22570*	*WRKY38*	Member of WRKY Transcription Factor; Group III	SA, *Pseudomonas*	(Mare, Mazzucotelli et al. 2004, Kim, Lai et al. 2008)
chr5.22041081	*AT5G54250*	*CNGC4*	Member of Cyclic Nucleotide Gated Channel family, a downstream component of the signaling pathways leading to hypersensitive response (HR) resistance. Mutant plants exhibit gene-for-gene disease resistance against avirulent *Pseudomonas syringae* despite the near-complete absence of the HR. Salicylic acid accumulation in *dnd2* mutants is completely *PAD4*-independent.	Cold, flagellin	(Jurkowski, Smith et al. 2004, Keisa, Kanberga-Silina et al. 2011, Chin, DeFalco et al. 2013, Rasmussen, Barah et al. 2013)
chr5.23302987	*AT5G57560*	*TCH4*	Encodes a cell wall modifying enzyme, rapidly upregulated in response to environmental stimuli	Heat, heat & silwet, heat & salt, heat & high light, high light, high light & cold, high light & salt	(Braam and Davis 1990, Xu, Campbell et al. 1996, Purugganan, Braam et al. 1997, Iliev, Xu et al. 2002, Rasmussen, Barah et al. 2013)

Table 2b

Marker	Gene in LD	Gene name	Gene description*	Responsiveness	Reference
chr1.30381439	*AT1G80820*	*CCR2*	*CINNAMOYL COA REDUCTASE*. Encodes a cinnamoyl CoA reductase isoform. Involved in lignin biosynthesis.	Cold & flagellin & silwet	(Luderitz and Grisebach 1981, Lauvergeat, Lacomme et al. 2001, Zhou, Jackson et al. 2010, Rasmussen, Barah et al. 2013)
chr1.30381439	*AT1G80840*	*WRKY40*	Pathogen-induced transcription factor. Binds W-box sequences in vitro. Forms protein complexes with itself and with WRKY60. Co-expression with *WRKY18* or *WRKY60* made plants more susceptible to both *P. syringae* and *Botrytis*.	Cold & flagellin & silwet	(Chen, Lai et al. 2010, Pandey, Roccaro et al. 2010, Liu, Yan et al. 2012, Rasmussen, Barah et al. 2013)
chr1.6038270	*AT1G17610*	*CHS1*	*CHILLING SENSITIVE 1*, mutant accumulates steryl-esters at low temperature.	Cold & high light	(Rasmussen, Barah et al. 2013, Wang, Zhang et al. 2013, Zbierzak, Porfirova et al. 2013)
chr5.171177	*AT5G17640*	*ASG1*	*ABIOTIC STRESS GENE 1*; Expression of this gene is induced by abscisic acid and salt stress.	ABA, salt	(Coste, Ramsdale et al. 2008, Batelli, Massarelli et al. 2012)
chr5.23247572	*AT5G57380*	*VIN3*	Encodes a plant homeodomain protein VERNALIZATION INSENSITIVE 3 (VIN3). In planta VIN3 and VRN2, VERNALIZATION 2, are part of a large protein complex that can include the polycomb group (PcG) proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE), CURLY LEAF (CLF), and SWINGER (SWN or EZA1). The complex has a role in establishing FLC (FLOWERING LOCUS C) repression during vernalization.	Cold	(Sung, Schmitz et al. 2007, Bond, Wilson et al. 2009, Finnegan, Bond et al. 2011)
chr5.23293119	*AT5G57560*	*TCH 4*	Encodes a cell wall-modifying enzyme	Heat, heat & silwet, heat & salt, heat & high light, high light, high light & cold, high light & salt	(Braam and Davis 1990, Xu, Campbell et al. 1996, Purugganan, Braam et al. 1997, Iliev, Xu et al. 2002, Rasmussen, Barah et al. 2013)

chr5.23293870	*AT5G57490*	*VDAC4*	Encodes a voltage-dependent anion channel (VDAC: AT3G01280/ VDAC1)	*Pseudomonas*	(Lee, Hoang et al. 2009, Tateda, Watanabe et al. 2011)
chr5.23366252	*AT5G57685*	*GDU3*	Encodes a member of the GDU (glutamine dumper) family proteins involved in amino acid export: At4g31730 (GDU1)	unknown	(Chen, Zhang et al. 2010)

* based on information on http://www.arabidopsis.org/tools/bulk/go/index.jsp_

Phytohormonal signaling underlying contrasts in stress responses

The MTMM framework allowed imposing constraints on the values of the estimated QTL effects, thereby providing a powerful testing framework for QTLs that have a common effect for the stresses belonging to one particular group of stresses as contrasted to the effect for another group of stresses (see Materials and Methods section 3.6). We investigated whether polymorphisms for genes involved in SA and JA biosynthesis or genes responsive to signals from these pathways were the cause of the negative genetic correlations between the groups of traits sharing one or the other phytohormonal signaling pathway. To this end, we performed a multi-trait GWA mapping to test the contrast between: (1) parasitic plant and aphid response, versus (2) the most negatively correlated traits, i.e. fungus, caterpillar, thrips and drought response (Figure 1). Fifteen SNPs were significantly associated with contrasting effects between the two trait clusters (Figure S4). Seven of these SNPs, were in LD with one or more genes known to be involved in JA-, SA- or resistance-related signal transduction (Table S4). Among these genes are *LOX5*, whose product is involved in facilitating aphid feeding (Nalam, Keereetaweep et al. 2012, Nalam, Keeretaweep et al. 2012), *MYB107* encoding a transcription factor responsive to SA (Stracke, Werber et al. 2001, Yanhui, Xiaoyuan et al. 2006), the JA-inducible genes *TPS02* and *TPS03* encoding terpene synthases (Huang, Abel et al. 2010) and *MES16*, encoding a methyl jasmonate esterase (Christ, Schelbert et al. 2012). Using TAIR10 annotations, we found that in total there are 371 genes that have an annotation related to JA and SA signaling (JA-SA genes). Our GWA analysis identified significant SNPs inside or in a 20 kb neighbourhood of five of those. In the remainder of the genome, i.e. non JA-SA, we identified 162 genes close to or with significant SNPs. So, in candidate regions for JA-SA, we had a ratio of 5/371 = 1.35% significant genes, while in non-candidate regions, we found 162/27863 = 0.58%. This is an enrichment of 2.33 times, significant at $\alpha = 0.05$ (Fisher exact probability test, mid-P value < 0.046;Rivals et al., 2007). Following Atwell et al. (2010), an upper bound for the false discovery rate (FDR) is then 1 / 2.33 = 0.43.

In addition to screening for SNPs with contrasting effects, we screened for SNPs with a similar effect across the above-mentioned trait clusters (Figure S5) and found candidate genes involved in oxidative stress and plant responses to salinity and pathogens (Table S5).

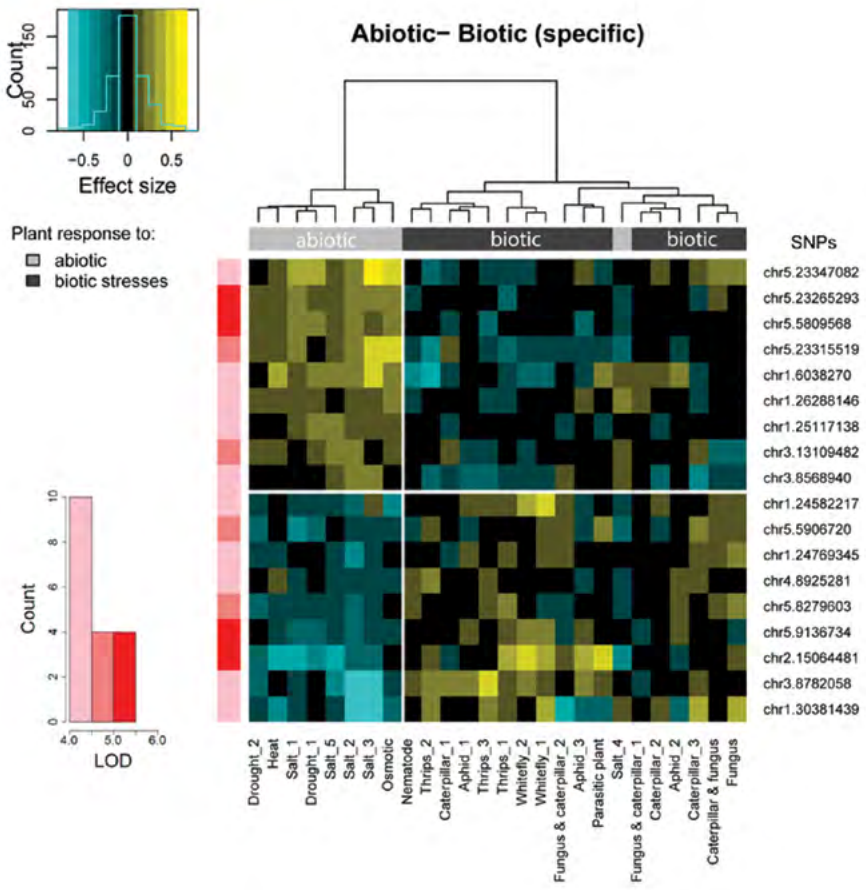


Figure 3. Genetic associations specific for contrasting plant responses to abiotic and biotic stresses. Genetic associations (in red) were estimated with a contrast-specific GWA analysis using MTMM. For exploratory purposes, significant SNPs ($P \leq 10^{-4}$) for the biotic-abiotic contrast were clustered on their trait-specific effect sizes as estimated in the full MTMM, that is, without imposing a contrast restriction on the SNP effects. If there was another SNP in LD that had a higher effect size, this SNP was used a representative for the LD block. Negative effects (blue) were cases where the rare allele was associated with a detrimental effect on the plants, positive effects (yellow) were cases where the rare allele was associated with increased resistance to the stress. The rare alleles of the top 9 SNPs are associated with enhanced resistance to abiotic stresses and reduced resistance to biotic stresses; the bottom 9 SNPs show the inverse. Stresses were clustered on the basis of SNP effects using Ward's minimum variance method. The key shows the frequency distribution of SNPs across effect sizes.

QTLs underlying contrasts in responses to biotic and abiotic stresses

We expected a negative correlation between the responses to abiotic and biotic stresses due to antagonistic interactions between ABA and the SA and JA/ET pathways (Anderson, Badruzsaufari et al. 2004, Fujita, Fujita et al. 2006, De Torres Zabala, Bennett et al. 2009, Kissoudis, Chowdhury et al. 2015). Testing for this contrast within the GWA analysis using our MTMM approach significantly identified 43 SNPs with a QTL effect that changed sign between biotic and abiotic conditions. For presentation purposes, traits were grouped by a cluster analysis across SNPs, while SNPs were grouped by clustering across traits. Figure 3 shows the SNPs with the strongest overall effects, identified in 18 LD intervals. The minor alleles of nine of these SNPs displayed a positive effect on biotic stress response traits and a negative effect on abiotic response traits. The remaining nine SNPs displayed the opposite effect (Figure 3). Several candidate genes were identified in LD with the SNPs that are specific for plant responses to either abiotic or biotic stresses (Table 2b), such as *TCH4* (encoding a cell-wall modifying enzyme), *AtCCR2* (involvement in lignin biosynthesis) and *ASG1* (a gene induced by ABA and salt stress), were identified. Transcription data (Figure S6) support the notion that these genes play a contrasting role in responses to abiotic and biotic stresses and reveal an antagonistic responsiveness between ABA and JA treatment (*TCH4*) or a specific responsiveness to either ABA (*AtCCR2*, *ASG1*, *ATVDAC4*) or JA (*ATWRKY40*). This is in line with the hypothesis that there are antagonistic effects between abiotic stress responses, predominantly involving the ABA pathway, and wound and biotic stress responses involving the JA-ET or SA pathways (Kissoudis, Chowdhury et al. 2015). Previous studies have, however, also revealed an overlap in abiotic and biotic plant responses, such as similar transcriptomic perturbations after salinity and pathogens stress (Ma, Gong et al. 2006). A screen for QTLs with similar effects on resistance to biotic and abiotic stress (Figure S7) identified three genes annotated to be responsive to stress stimuli (Table S6). Transcriptional data show that these genes respond differentially to different (a)biotic stresses and phytohormones (Figure S8). *ARGAH2*, encoding an arginase enzyme with a role in the metabolism of polyamines and nitric oxide, is involved in both SA- and JA-mediated resistance to both biotrophic and necrotrophic pathogens, and is also responsive to abiotic stimuli such as temperature, salt and light intensity (Figure S8) (Jubault, Hamon et al. 2008, Gravot, Deleu et al. 2012, Rasmussen, Barah et al. 2013). *PKS1* is known to be involved in adaptation in plant growth in response to light (Fankhauser, Yeh et al. 1999, Molas and Kiss 2008), but also seems to be responsive to *Botrytis* (Figure S8). These genes are promising candidates for consistent effects across biotic and abiotic stresses.

QTLs underlying contrasts in responses to below- and aboveground stresses

We expected a negative correlation between responses to below- and aboveground stresses. A strong QTL signal was found on chromosome 1 for this contrasting response (Figure S9). The associated marker (chr1. 13729757) had 12 genes in LD with it, of which 11 are annotated as pseudogenes. Transcriptional data on abiotic stresses for the only protein coding gene (*AT1G36510*) shows an upregulation in above tissues, yet a downregulation in the root tissues (Winter, Vinegar et al. 2007). Marker chr5.16012837 showed the strongest signal for similar effects on responses to below- and aboveground stresses (Figure S10) for which the *pathogenesis-related thaumatin superfamily protein* (*AT5G40020*) is the most promising candidate gene.

Validation of identified QTLs

To obtain experimental support for the most interesting QTLs resulting from the MTMM, we tested homozygous T-DNA insertion lines for candidate genes *RMG1* and *WRKY38* (both resulting from the MTMM analysis), and *TCH4* (from MTMM analysis on biotic versus abiotic contrast) for several of the stresses addressed in this study. Two independent *rmg1* T-DNA insertion lines showed a phenotype that was different from the wild type (Col-0) for some of the stress conditions (Figure 4, Supplementary Methods Section SM.11), being more resistant to caterpillar feeding and osmotic stress (Figure 4). *RMG1* (AT4G11170) encodes an NB-LRR disease resistance protein, which acts as a pattern-recognition receptor (PRR) that recognize evolutionarily conserved pathogen-derived signatures, and transcription is induced by the bacterial peptide flg22 (Yu, Lepere et al. 2013). The rare allele of the corresponding marker chr4.6805259 is associated with enhanced resistance to salt stress and the combined stresses 'caterpillar and drought' and 'caterpillar and fungus' and with enhanced susceptibility to drought stress. Gene expression data show that *RMG1* is upregulated by several abiotic and biotic stresses (Figure 4). In addition, gene ontology enrichment analysis of the co-expression network of *RMG1* shows an overrepresentation of genes involved in immune responses and maintenance of ion homeostasis. The latter is based upon co-expression with five genes encoding glutamate receptors (*GLR1.2*, *GLR1.3*, *GLR2.5*, *GLR2.8*, and *GLR2.9*), putatively involved in ion-influx-mediated long-distance signaling of wound, pathogen and salt stress (Ma, Gong et al. 2006, Mousavi, Chauvin et al. 2013, Choi, Toyota et al. 2014, Kissoudis, Chowdhury et al. 2015). T-DNA insertion lines for *TCH4* and *WRKY38* did not show a phenotype different from the wild type (Col-0) for any of the tested stress conditions. Whether this is dependent on the genetic background used, remains to be investigated.

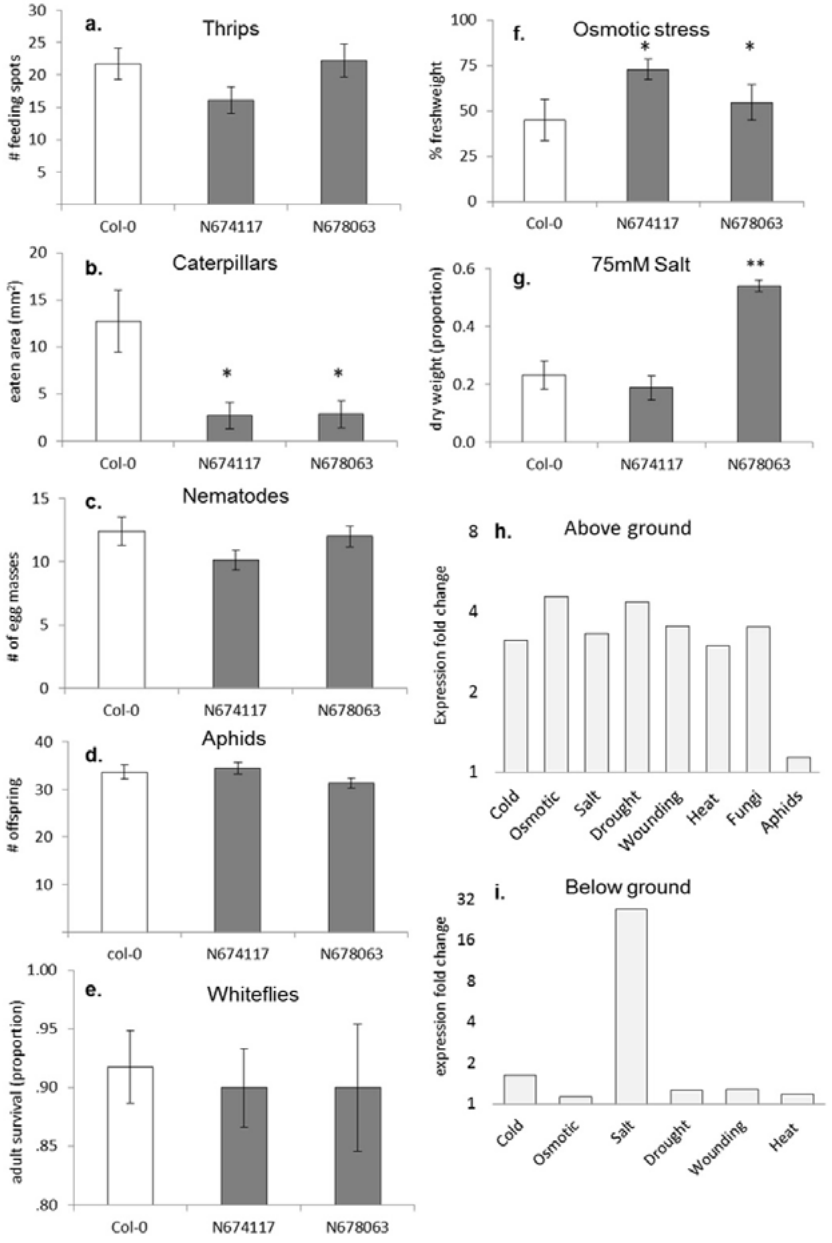


Figure 4. Phenotypes of *RMG1* T-DNA mutant screenings. Phenotypes are given for two T-DNA lines in the *RMG1* gene and for Col-0 as control. a. Number of thrips feeding spots on a detached leaf, 6 days post infestation (N = 24); b. Leaf area consumed by *P. rapae* caterpillars (N = 6); c. Number of nematode egg masses (N = 23); d. Number of *M. persicae* aphid offspring (N = 10-17); e. Percent survival of adult whiteflies (*A. proletella*) (N = 10); f. Plant fresh weight after osmotic treatment in comparison to control (% relative to control) (N = 4); g. Plant dry weight after 75mM salt treatment in comparison to control (ratio)(N = 7-10); Mean ± SE, +: $P < 0.10$, *: $P < 0.05$, **: $P < 0.01$, difference in comparison to Col-0. Relative expression fold change for *RMG1* compared to untreated control plants in aboveground (h) and belowground (i) tissue. Expression data from Arabidopsis eFP browser (http://bbc.botany.utoronto.ca).

Summarizing, our multi-trait GWA methodology facilitated a detailed analysis of the genetic architecture of resistance in Arabidopsis to a wide diversity of biotic and abiotic stresses. Application of this methodology revealed novel candidate genes associated with multiple stress responses, where specific contrasts were identified with some genes positively associated with the resistance to one set of stresses while being negatively associated with another set of stresses. In plant breeding (Brady, Kruckeberg et al. 2005, Ballesteros, Mason et al. 2015), such genes are classified as adaptive. Alternatively, other genes were identified with consistent effects across a wide spectrum of stress conditions. Such genes are labelled as constitutive in the plant breeding literature (Brady, Kruckeberg et al. 2005, Ballesteros, Mason et al. 2015). Both adaptive and constitutive QTLs are important factors to contribute to improved stress resistance and tolerance in commercial crop species (Brady, Kruckeberg et al. 2005, Ballesteros, Mason et al. 2015).

Discussion

We developed a novel mixed-model approach to multi-trait GWA mapping with a special feature for testing contrasts between groups of stresses to identify the genetic architecture underlying a total of 30 stress response traits in Arabidopsis. The strength of our statistical approach was that our multi-trait mixed model accounted simultaneously for dependencies between genotypes and between traits, providing a natural and appropriate correction for multiple testing, while maximizing power for the detection of QTLs for the stress contrast under study. As we addressed a large number of stresses, our phenotyping experiments were distributed across a series of laboratories and were not performed simultaneously. To mitigate as much as possible the occurrence of QTLs induced purely by experiment-specific differences in plant management and environmental control, our phenotypic responses were defined in terms of control-corrected responses. This type of correction will emphasize QTLs for resistance and tolerance per se and will decrease detection power for QTLs related to development and viability.

The extensive phenotyping executed in this study was done under carefully controlled conditions in climate chambers. Ideally, phenotyping should be done in nature because that is where genetic variation is exposed to natural selection (Bergelson and Roux 2010, Brachi, Faure et al. 2010, Brachi, Faure et al. 2013). Here, we have phenotyped the plant population to 15 different stresses under laboratory conditions and our data show an interesting pattern based on genetic correlations that matches with phytohormonal signalling underlying stress responses (Figure 1). This indicates that the genetic architecture recorded here is biologically relevant. Drought and salt stress responses share signal-transduction mechanisms (Zhu 2002)

which is represented by the genetic correlations recorded (Figure 1). Insect damage is commonly associated with drought or osmotic stress and this is also clear from overlap in underlying phytohormonal signalling (Pieterse et al. 2012). Figure 1 shows that drought stress and osmotic stress correlate with insect stresses. Extending studies of genetic variation and the genetic architecture underlying responses to multiple stresses to natural conditions will be an important next step (Bergelson and Roux 2010).

Through the approach developed here, candidate genes for adaptive stress responses were identified that are involved in contrasting responses when comparing biotic and abiotic stresses, above- and belowground stresses, and attack by phloem feeders compared with other biotic stresses. Among these genes many are involved in phytohormone-mediated processes, supporting the notion that the phytohormonal regulatory network plays an important role in plant stress responses (Pieterse, Van der Does et al. 2012). The MTMM approach further showed that certain SNPs were associated to multiple stress responses and that transcriptional patterns of genes to which the SNPs were linked, as well as the phenotype expressed upon knocking out one of these genes, matched with the observed stress responses of the plants. The *RMG1* gene that was identified through this procedure has relevant effects on plant phenotype in the context of responses to individual stresses. *RMG1* is a bacterium-inducible resistance gene whose activity is modulated by the plant through RNA-directed DNA methylation (RdDM) (Yu, Lepere et al. 2013). *RMG1* expression activates the SA pathway (Yu, Lepere et al. 2013). Thus, the increased resistance against caterpillars in *rmg1* mutants may be the result of elimination of SA-mediated interference with JA-induced resistance to caterpillars (Pieterse, Van der Does et al. 2012). *RMG1* appears to be inducible by several stresses and deserves further in-depth analysis for its role in plant response to multiple stresses.

Our data show that for the 30 most significant SNPs resulting from the MTMM analysis, the average absolute effect size for double stresses is on average higher than that for single stresses ($P < 0.007$, Table S2). This suggests that resistance mechanisms involved in countering dual stresses are of a more general nature, in contrast to the rather specific resistance mechanisms involved in single stress responses. However, the combined stresses included in this study especially involve fungal and caterpillar stresses. Future studies including other combined stresses are needed to further investigate the suggested pattern.

The MTMM framework that we used for GWA mapping provides unbiased estimates for QTL allele substitution effects together with correct standard errors for these effects. Within the same framework we developed unique facilities to test hypotheses on QTL-by-stress interactions in multi-trait models, which are

not available in competing meta-analysis approaches (Zhu, Feng et al. 2015). The variance-covariance structure that we used for the polygenic term protects against inflated type I error, i.e. too many false positive SNP-trait associations, as a consequence of population structure and kinship on the genotypic side and genetic correlations between traits on the trait side. The inclusion of trait correlations will, for most QTLs, improve the power of detection in comparison to single-trait GWA mapping (Korte, Vilhjalmsson et al. 2012, Zhou and Stephens 2014); section 3.6). Our choice for the variance-covariance structure of the polygenic term as a Kronecker product of a compressed kinship on the genotypes with an approximated unstructured variance-covariance model on the environments is sometimes used in plant breeding for genomic prediction models (Burgueno, de los Campos et al. 2012). However, implementation of such models in GWA mapping and especially on the scale that we present here, with 30 traits, is unprecedented and is practically far from straightforward. It required substantial work on preparatory phenotypic analyses as well as fine-tuning of the genotypic and trait variance-covariance structures to achieve convergence of the mixed models.

The MTMM analyses identified candidate genes associated with contrasting responses to biotic and abiotic stresses. Stress combinations appeared to have a strong influence on the MTMM outcome, indicative for significant interactions between different stresses when occurring simultaneously, and underlining the importance of studying the resistance of plants to combinations of stress. Transcriptional data and phenotyping of mutants provide initial support for the role of several of the candidate genes identified. Studies of plant responses to a diverse set of biotic stresses show that the transcriptional pattern is stress-specific and that phytohormonal signaling pathways can explain up to 70% of the induced gene regulation (De Vos, Van Oosten et al. 2005). Taking the outcome of the MTMM analyses to investigate the involvement of identified candidate genes in the resistance of plants to several stresses, not only in Arabidopsis but also in related crop species such as e.g. *Brassica* species will be valuable in the breeding by design of future crops to protect them against combinations of stresses, including biotic and abiotic stresses. This will be of great value for next generation crops.

Materials and Methods

1. Arabidopsis thaliana population

In this study we included 350 *Arabidopsis thaliana* (L.) Heynh. accessions from the Hapmap population (http://bergelson.uchicago.edu/wp-content/uploads/

2015/04/Justins-360-lines.xls). The Hapmap population has been genotyped for 250K bi-allelic SNPs (Baxter, Brazelton et al. 2010, Platt, Horton et al. 2010, Chao, Silva et al. 2012) and after quality control and imputation this SNP-set was reduced to a set of 214,051 SNPs.

2. Definition of the target traits

For every experiment, the target traits were derived from the individual plant data using the following strategy. First, when residuals deviated from normality, a logarithmic, arcsine or square root transformation was applied to the original observations. Second, genotypic (accession) means for each treatment were calculated using a mixed model to account for design effects. Different mixed models were used in the experiments, reflecting the different designs. In all cases, accession effects were modelled as fixed, and the accession means were the best linear unbiased estimator (BLUE) of these effects. Third, for traits measured in treatment and control conditions, differences or residuals (when regressing treatment on control values) were defined, in order to obtain a measure of stress tolerance that was corrected for the expression of the same trait under control conditions. Finally, within each experiment, the traits were replaced by the first principal component if the latter explained more than half of the variation in all traits in this experiment; in all other cases the original traits were retained. An overview of final traits and their corresponding sections in the Supplementary Methods can be found in Table 1. In case of replacement by the first principal component, original traits and the variance explained by the first principal component are listed (Methods Tables M1-M5). In total, phenotypic data for 73 individual traits were obtained by 10 different groups. All calculation were performed in R, unless stated otherwise. Mixed model analysis was performed with the R-package asreml (Butler, Cullis et al. 2009). In all equations the term E denotes residual error. All other terms represent fixed effects unless stated otherwise. A colon (:) is used to define interactions between terms.

3. Statistics

3.1 Genetic correlation networks and heritability

Pairwise genetic correlations between traits were estimated using a multi-trait mixed model (MTMM)(Korte, Vilhjalmsson et al. 2012). Residuals were assumed uncorrelated for traits that were measured on different plants. For some pairs of traits the likelihood was monotone, which can also occur in single-trait mixed models(Kruijer, Boer et al. 2015). In this case, the genetic correlation was estimated by the (Pearson) correlation between the univariate G-BLUPs (De los Campos, Hickey et al. 2013) estimated for these traits. A network between predefined groups

of traits was constructed by connecting groups whose average genetic correlation across pairs of traits was above 0.2.

Narrow sense heritability (Table S1) was estimated using the mixed model $Y_i = \mu + A_i + E_i$ where Y_i represents the phenotypic means of accessions ($i = 1,...,350$), and A_i and E_i random genetic and residual effects. The vector of additive genetic effects follows a multivariate normal distribution with covariance $\sigma_A^2 K$, K being a marker-based relatedness matrix. The residual errors are independent, with variance σ_E^2. We obtained REML-estimates of σ_A^2 and σ_E^2 , and estimated heritability as $h^2 = \sigma_A^2 / (\sigma_A^2 + \sigma_E^2)$. This is an estimate of narrow-sense heritability, since the model for the genetic effects only captures additive effects, and σ_E^2 is the sum of environmental and non-additive genetic effects (see e.g. Kruijer et al. (2015)).

3.2 Multi-trait mixed models

Following(Zhou and Stephens 2014), we assume the MTMM $Y = XB + G + E$, with Y being the genotypes by traits ($n \times p$) matrix of phenotypic observations. The terms XB, G and E stand for respectively the fixed effects (including trait specific intercepts and SNP-effects) and the random genetic and environmental effects. G follows a zero mean matrix-variate normal distribution with row-covariance (marker-based kinship) matrix and column (trait) covariance matrix $V_g \cdot V_g$ is a $p \times p$ matrix modeling the genetic correlations between traits. This is equivalent with $g = vec(G)$ (the vector containing the columns of being multivariate normal with a covariance matrix defined by the Kronecker product $V_g \otimes K$ (Zhou and Stephens 2014). Similarly, $vec(E)$ follows a zero mean normal distribution with covariance $V_e \otimes I_n$, where V_e accounts for the non-genetic correlations between traits.

3.3 Factor-analytic models

Since V_g and V_e contain a total of $p(p + 1)$ parameters, the MTMM above becomes difficult to fit for more than 10 traits(Zhou and Stephens 2014). For V_g we therefore assumed a factor analytic model, which is well known in the context of QTL-mapping for experimental populations with limited numbers of markers (Boer et al., 2007), but has not been used in the context of multivariate GWAS. As almost all traits were derived from measurements on different plants, a diagonal model $V_e = diag(\sigma^2_{e,1},...,\sigma^2_{e,p})$ was chosen for the environmental covariances. For V_g a second order factor analytic structure was chosen $V_g = \sigma^2_g(\lambda\lambda^t + diag\left(\tau^2_1,\ldots,\tau^2_p\right))$, where σ^2_g represents a scale parameter, the magnitude of genetic effects, the $p \; x \; 2$ matrix λ contains the trait specific scores belonging to the factor analytic part of the model that provides a rank one variance-covariance structure between traits, and $diag\left(\tau^2_1,\ldots,\tau^2_p\right)$ provides trait specific residual genetic variances(Piepho 1997, Meijer 2009). The model was fitted with the R-package ASRreml (Butler, Cullis et al. 2009).

3.4 Compressed kinship

Factor analytic models have been successfully applied to experimental populations with a simple genetic relatedness structure (Boer, Wright et al. 2007, Malosetti, Ribaut et al. 2008, Alimi, Bink et al. 2013), but currently available software could not perform REML-estimation for the Hapmap-population. The kinship matrix was therefore replaced by a compressed kinship matrix (Bradbury, Zhang et al. 2007, Zhang, Ersoz et al. 2010), modeling the genetic relatedness between a number of internally homogeneous groups. Assuming there are m such groups, containing n_1, ..., n_m accessions each, the original kinship matrix K is replaced by $\mathrm{Z}K_C Z^t$, where K_C is the kinship matrix for the groups, and Z is the $n \times m$ incidence matrix assigning each of the n accessions to one of the m groups. The groups were created by a procedure that restricted the marker data to be linear combinations of environmental covariates representing the conditions at the place of origin of the accessions, as explained below.

Compressed kinship was calculated as the average kinship within genetic groups. Genotypes were assigned to k genetic groups by performing Ward clustering based on the squared Euclidean distance along the first $k - 1$ principal components calculated from a matrix of standardized SNP scores, followed by cutting the resulting dendrogram into k distinct clusters (van Heerwaarden, Hufford et al. 2012, Odong, van Heerwaarden et al. 2013, van Heerwaarden, Odong et al. 2013).

The use of a compressed kinship matrix requires a choice of the level of compression, as determined by the number of genetic groups over which the individual kinship is averaged. This choice needs to balance the gain in computational efficiency with model fit (Zhang, Ersoz et al. 2010) and the ability of the compressed matrix to capture the correlation between genetic dissimilarity and phenotypic differences, which is ultimately the reason for including a kinship matrix in the association model. There are currently no standard methods to determine the optimum level of compression, at least not when used in a multi-trait setting. We determined the appropriate level of compression for each association model based on the model likelihood, convergence and correspondence between kinship and phenotypic and geographical similarity. The latter was quantified as the Frobenius norm of the difference between the complement of the compressed kinship matrix, expanded to a block matrix of full rank, and the Euclidean distance matrix of phenotypic traits or geographic coordinates. We considered a range of 4 to 100 groups. Correspondence with phenotypic and geographical dissimilarity increased steeply from 4 to around 35 groups, after which correspondence with geographic distance increased more slowly and the correspondence with phenotypic distance showing a local decrease until 58 groups. Model likelihood was relatively stable above 4 groups but convergence was erratic depending on the modeled contrasts. For each

model the number of groups was therefore chosen to be the minimum number of groups needed to achieve a level of correspondence approximating that found at 35 groups, under condition of model convergence.

3.5 Multi-trait GWAS

Traits (columns of Y) were standardized. Along the genome, MTMMs of the type $Y = XB + G + E$ were fitted with initially for each marker trait-specific QTL effects $\beta_1,\ldots,\beta_p$ (contained in *B*). To identify general QTLs with trait-specific effects, for individual markers, the null hypothesis $\beta_1 = \beta_2 = \ldots \beta_p = 0$ was tested by a Wald test against the alternative hypothesis that at least one of the trait specific effects was nonzero (Zhou and Stephens 2014). To identify consistent QTLs, the null hypothesis $\beta_1 = \beta_2 = \ldots \beta_p = \beta \neq 0$ was tested. To identify adaptive QTLs, contrasts defined on the trait specific QTL effects were tested. For example, suppose the first *p1* of the full set of *p* traits represent responses measured under abiotic stresses, while the second *p2* traits represent responses under biotic stresses. A contrast can now be defined to test the hypothesis whether the QTL effect for abiotic stresses differs from that for biotic stresses: $\beta_1 = \beta_2 = \ldots \beta_{p1} = \alpha_{abiotic}; \beta_{p1+1} = \beta_{p1+2} = \ldots \beta_p = \alpha_{biotic}$ and versus . For the Wald test for the hypothesis $\beta_1 = \ldots = \beta_p$ we first fit the MTMM $Y = XB + G + E$ with *XB* only containing trait specific means μ_1, …, μ_p, and next test hypotheses on the marker effects. The contrast is defined through a partitioning of the traits in two groups (e.g. resistance against biotic or abiotic stress). Using the R-package asreml (Butler, Cullis et al. 2009) we perform Wald tests for the following hypotheses:

1. $H_0 : \beta = 0$, in the constrained model $\beta_1 = \ldots = \beta_p = \beta$.
2. $H_0 : \alpha_1 = \alpha_2$, in the constrained model where α_1 is the effect on all traits in the first group, and α_2 for traits in the second group.

3.6 Simulations

We further compared the different Wald tests using simulations, described in more detail in the supplementary material (SM.12). Specifically, we compared the performance of the general MTMM (i.e. testing the hypothesis $\beta_1 = \beta_2 = \ldots \beta_p = 0$) with the MTMM used for the contrasts (i.e. $H_0 : \alpha_{group\,1} = \alpha_{group\,2}$, where, within two predefined groups of traits, all SNP-effects equal $\alpha_{group\,1}$ respectively $\alpha_{group\,2}$). We simulated phenotypic data for given genotypic data, either assuming SNP-effects positive (but not equal) within one group of traits and negative for the other (Scenario A), or the sign of each SNP effect being chosen randomly (Scenario B). The simulation results as presented in Fig. S11 clearly indicate that the Wald test for the contrast has superior power under scenario A, while the general MTMM

performs best under scenario B. In both cases, univariate analysis of the trait with the highest heritability is outperformed by at least one of the MTMM analyses. As a consequence, univariate GWAS and GWAS with the general and contrast MTMM give different rankings of SNPs.

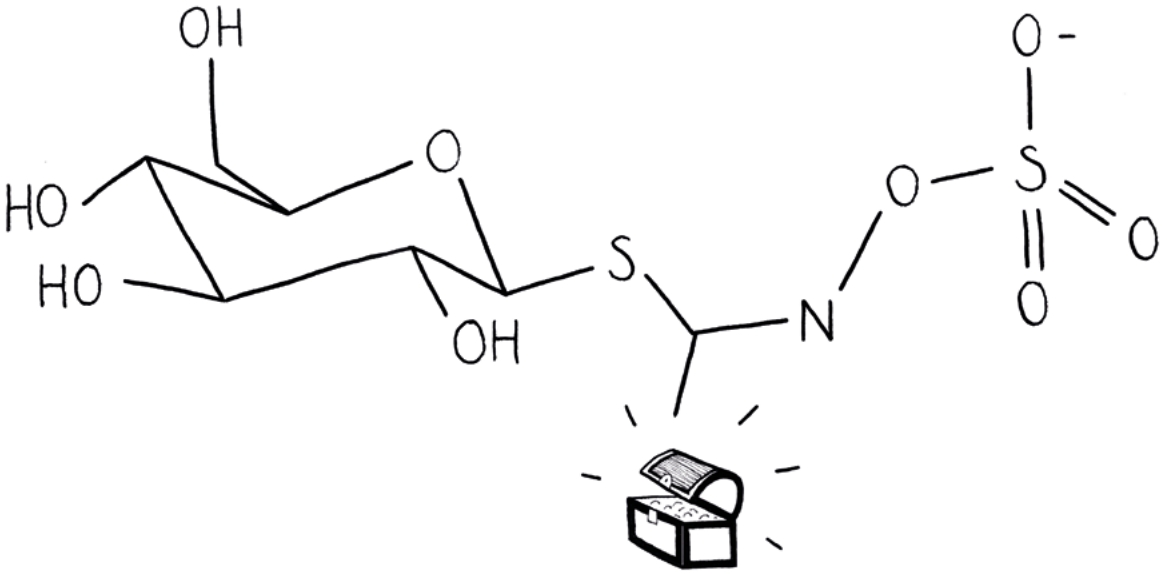
3.7 Selecting candidate genes

A significance threshold of $P < 0.0001$ was chosen after implementation of genomic control (see below). For MTMM this resulted in 43 SNPs meeting this criterion. SNPs within a 20kb region were considered to be part of one LD block. This resulted in 30 genomic regions. For presentation purposes, each LD block was represented in Figures and heatmaps by the SNP with the on average strongest (absolute) effect across all traits. For the GWA contrast analyses, the same procedure was followed to define LD blocks and representative SNPs.

Correcting for genomic inflation

The Wald test is known to suffer from some inflation (Zhou and Stephens 2014), which we correct for using genomic control (GC) (Devlin and Roeder 1999, Devlin, Roeder et al. 2001), which divides the observed test statistics T_1, …, T_p by the genomic inflation factor. For both the unconstrained MTMM and the MTMM for contrasts described above, we observed inflation for small as well as large p-values (i.e. also more p-values close to one than expected). Consequently, the usual genomic control procedures based on the observed versus expected median of test statistics gave too optimistic inflation factors. We therefore applied an alternative genomic control procedure, in which we regress the observed $-\log_{10}(p)$ values on the expected ones, and correct the observed $-\log_{10}(p)$ values for the slope.

Supplementary files can be found online: http://dx.doi.org/10.18174/387714.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Chapter six

Natural variation in glucosinolate profiles and resistance to thrips in Arabidopsis

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Abstract

Glucosinolates (GSLs) are secondary plant metabolites present mostly in the Brassicaceae. The GSL profiles of 349 accessions of the Arabidopsis HapMap population that were exposed to 15 biotic and abiotic stresses correlated most significantly to feeding damage by Western flower thrips (*Frankliniella occidentalis*). Thrips feeding damage also correlated with longitude within Europe, just as reported before for glucosinolates. Western European accessions contained predominantly C4 alkenyl GSLs and were more resistant to thrips than the susceptible Eastern European accessions which predominantly contained C3 hydroxyl GSLs. In a genome-wide association (GWAS) analysis, the C4 alkenyl GSLs that correlated the strongest with thrips resistance mapped to the genomic regions containing genes known to regulate the biosynthesis of these compounds (*MAM, AOP, GS-OH*). However, thrips resistance did not co-localise with any of the GSL genes. Additional screening of a Cvi x L*er* RIL population showed a QTL for thrips resistance on chromosome 2, but no co-localisation with any known GSL genes, nor with thrips resistance loci identified by GWAS. KO mutants and overexpressors of both *MAM-1* and *AOP-2* could also not confirm a causal link between GSLs and resistance to thrips. Despite strong correlations between natural variation in GSL profiles and resistance to thrips, we could not validate a causal link with a genetic basis between the two. It is possible that the relevant doses or combination of specific GSLs was not present in any of the mutants tested in this study. Alternatively, this correlation could be based on independent geographical clines. The limitations of GWAS for the identification of genes determining complex clinal traits are discussed.

Introduction

Glucosinolates (GSL) are secondary metabolites present mostly in the Brassicaceae, for which the ecological importance has been firmly established (Kliebenstein, Kroymann et al. 2005, Hopkins, van Dam et al. 2009). The biosynthetic pathways for induction and production of these compounds have been virtually completely elucidated (Keurentjes, Fu et al. 2006) and for *Arabidopsis thaliana*, a model species belonging to the Brassicaceae familiy, roughly 30 different glucosinolates have been identified (Kliebenstein, Gershenzon et al. 2001, Keurentjes, Fu et al. 2006) (Figure 1,2). Polymorphisms and natural variation in GSL profiles in Arabidopsis make this an attractive system for the study of ecological genetics of plant-herbivore interactions (Moyes, Collin et al. 2000, Kliebenstein, Kroymann et al. 2005). Although variation in aliphatic GSL (the major class of methionine-derived GSLs) contents among plant populations has often been attributed to differences in herbivore pressure, only few studies have actually established a correlation between this variation and resistance to herbivory in natural populations (Gols, Wagenaar et al. 2008, Züst, Heichinger et al. 2012).

GSLs in the Brassicaceae also represent a model system for studying the genetic architecture of plant biosynthetic pathways (Chan, Rowe et al. 2011, Brachi, Meyer et al. 2015). There are four major loci responsible for the diversity of aliphatic GSLs in *A. thaliana* (Figure 1). These loci are: the *MAM* locus, which harbours *METHYLTHIOALKYL MALATE SYNTHASE* (*MAM*) genes that determine the number of methionine side-chain elongations; the *GS-OX* locus, which harbours *FLAVIN-MONOXYGENASE (GS-OX)* genes that oxygenate a methylthio-group to form the methylsulfinyl GSLs; the *AOP* locus, which harbours *ALKENYL HYDROXALKYL PRODUCING (AOP)* genes, adding alkenyl or hydroxy-alkenyl groups to the methylsulfinyl GSLs, and the *GS-OH* locus, which harbours *GLUCOSINOLATE OXYGENATION (GS-OH)* genes carrying out further oxygenation reactions on the alkenyl GSLs. The methionine side-chain elongation steps are governed by the *MAM* genes; most accessions have either two functional *MAM-1* genes arranged in tandem (resulting in the production of C4 GSLs) or a functional *MAM-2* gene and a truncated, non-functional *MAM1* gene (resulting in the production of C3 GSLs). *MAM-3* is responsible for the formation of longer chain length GSLs. The *AOP* locus is also a complex region where *AOP2* and *AOP3* are phylogenetic paralogs physically placed at the same genomic region, thus segregating as alleles of each other. When *AOP3* is functional, hydroxyl (OH) GSLs are produced, when *AOP2* is functional, alkenyl (ALK) GSLs are produced. If neither gene is expressed (*AOP null,* as in Col0), only the precursor methylsulfinyl (MS) GSL is produced (Figure 1). Previous studies have demonstrated a non-random geographic distribution of GSL chemotypes in natural

populations of Arabidopsis (Kerwin, Feusier et al. 2015). Genome-wide association (GWAS) mapping is a powerful tool to study the genetic architecture of complex quantitative traits like the concentration of secondary metabolites, and how these relate to stress resistance (Atwell, Huang et al. 2010, Bac-Molenaar, Fradin et al. 2015, Kloth, Wiegers et al. 2016). Here, we studied the correlation between the GSL content of all 350 lines of the Arabidopsis HapMap population and the resistance/tolerance to 15 different biotic and abiotic stresses that was also assessed on all individual ecotypes of this population. The observed highly significant correlation of specific GSLs with feeding damage by the generalist insect *Frankliniella occidentalis* (Western Flower Thrips) was further investigated with T-DNA insertion and gene overexpression lines, a genome-wide association study, and QTL mapping with a RIL population.

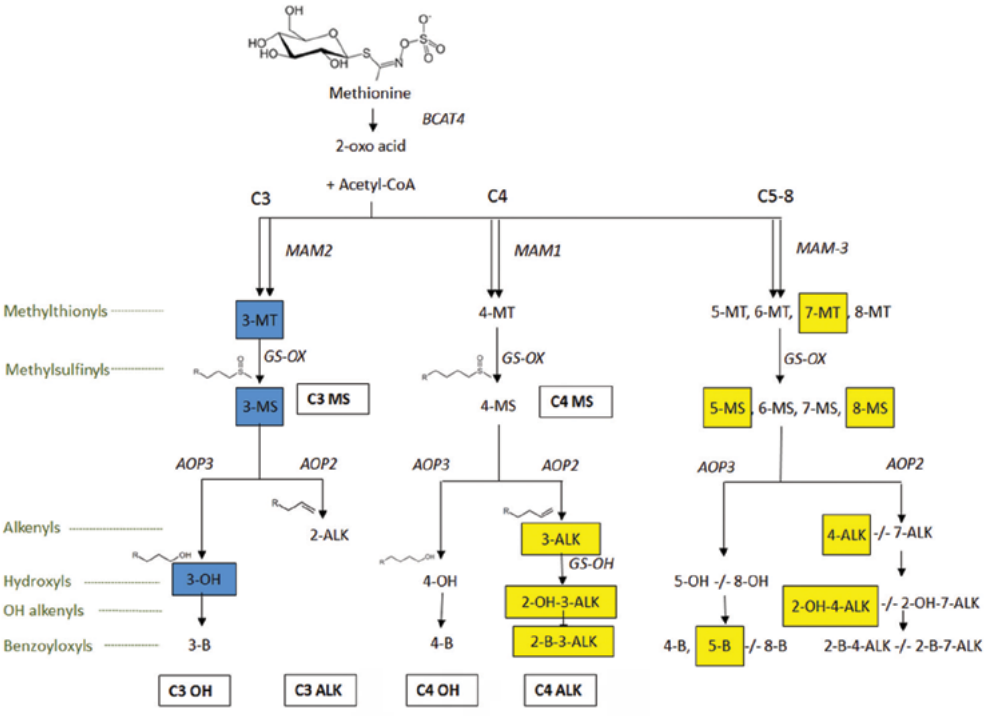

Figure. 1. Schematic overview of the methionine glucosinolate pathway and its relation to thrips resistance. Chemotypes are indicated in boxes. Compounds formed are MT (methylthionyls, e.g. 4-methylthiobutyl glucosinolate), MS (methylsulfinyls, e.g. 4-methylsulfinylbutyl glucosinolate), ALK (alkenyls, e.g. 3-butenyl), OH (hydroxyls, e.g. 4-hydroxybutyl), 2-OH ALK (hydroxyl alkenyls, e.g. 2-hydroxy-3-butenyl glucosinolate), B (benzoyloxyls, e.g. 4-benzoyloxybutyl glucosinolate). Yellow and blue shaded compounds correlated negatively and positively, respectively, with resistance to thrips in the Arabidopsis HapMap population (adjusted $P. < 0.01$, Spearman correlation test). Corresponding proteins of all enzymatic steps are shown next to the arrows.

Results

Natural variation in glucosinolates in the HapMap population

We used the Arabidopsis HapMap population consisting of 360 different Arabidopsis accessions collected across the native range of Arabidopsis (Li, Huang et al. 2010). This population has been genotyped with 214K SNP markers, allowing genome-wide association (GWA) mapping. Using untargeted LC-qTOF-MS analysis, the metabolite profile of all lines in the population was determined in duplicate and 625 unique mass clusters were identified, potentially representing just as many metabolites (Wehrens, Hageman et al. 2016). To correlate the natural variation in aliphatic GSL content in this population to a variety of biotic and abiotic stresses, we putatively identified 23 GSLs based on their retention time and mass spectrum (Table 1). There was considerable quantitative and qualitative variation in the content of these GSLs among the accessions, in line with previous reports on natural variation in GSL content in Arabidopsis (Keurentjes, Fu et al. 2006, Chan, Rowe et al. 2011, Brachi, Meyer et al. 2015).

Correlation between glucosinolates and stress tolerance in the HapMap population

As part of a large concerted effort, the entire HapMap population has been screened for stress resistance to a set of 15 different biotic and abiotic stresses (Thoen *et al.* 2016). These stresses included four abiotic stresses (drought, salt, an osmoticum and heat), seven biotic stresses (the root parasitic plant *Phelipanche ramosa*, the phloem-feeding aphid *Myzus persicae*, the phloem-feeding whitefly *Aleyrodes proletella*, the cell-content feeding thrips *Frankliniella occidentalis*, the leaf-chewing caterpillar *Pieris rapae*, the root-feeding nematodes *Meloidogyne incognita*) and the necrotrophic fungus *Botrytis cinerea* and four stress combinations (fungus and caterpillars, drought and fungus, drought and caterpillars, caterpillars and osmotic stress). The phenotypic information obtained from these assays (Suppl. Table 1) (Thoen et al. 2016) was used in Spearman correlation tests with the GSL levels in all 349 accessions. Both significant positive and negative correlations were observed between some traits and specific GSLs (Suppl. data file 1). For the abiotic stresses, positive correlations were observed between heat stress tolerance and the level of 4-methylsulfenyl glucosinolate (4-MS) and 3-hydroxypropyl glucosinolate (3-OH). A strong negative correlation for heat stress tolerance was observed for levels of 5-benzoloxypentyl glucosinolate (5-B) and 2-propenyl glucosinolate (2-ALK) (Figure 2). Cabbage whitefly resistance correlated weakly negatively with 8-methylthiooctyl glucosinolate (8-MT), 4-hydroxybutyl glucosinolate (4-OH) and 7-methylthioheptyl

glucosinolate (7-MT). None of the detected GSLs correlated with the initial probing behavior of aphids, although aphid reproduction correlated positively with levels of (2S)-2-hydroxy-3-butenylglucosinolate (2-OH-3-ALK). The strongest correlations

Table 1. Glucosinolates in Arabidopsis used in this study

Abbreviation	Full name	Common name	m/z [M-H]⁻	rt
3-MT	3-Methylthiopropyl glucosinolate	Glucoibervirin	406.03073	2.10
3-MS	3-Methylsulfinylpropyl glucosinolate	Glucoiberin	422.0255	1.04
3-OH	3-Hydroxypropyl glucosinolate	Glucoerysimumhieracifolium	376.0385	0.92
3-B	3-Benzoyloxypropyl glucosinolate	Glucomalcomiin	480.06397	5.62
2-ALK	2-Propenyl glucosinolate	Sinigrin	358.0282	1.17
4-MT	4-Methylthiobutyl glucosinolate	Glucoerucin	420.0465	3.15
4-MS	4-Methylsulfinylbutyl glucosinolate	Glucoraphin	436.0411	1.11
4-OH	4-hydroxybutyl glucosinolate	-	390.05341	1.04
3-ALK	3-Butenyl glucosinolate	Gluconapin	372.0428	1.68
2-OH-3-ALK	2-Hydroxy-3-butenylglucosinolate	Progroitin	388.0374	0.93
2-B-3-ALK	2-Benzoyloxy-3-butenyl glucosinolate	-	492.063978	9.6
5-MS	5-Methylsulfinylpentyl glucosinolate	Glucoalyssin	450.0569	1.23
5-B	5-Benzoyloxypentyl glucosinolate	-	508.095276	5.68
4-ALK	4-Pentenyl glucosinolate	-	386.0585	2.78
2-OH-4-ALK	2-Hydroxy-4-pentenyl glucosinolate	Gluconapoleiferin	402.0534	1.39
6-MS	6-Methylsulfinylhexyl glucosinolate	Glucohesperin	464.0712	1.79
7-MT	7-Methylthioheptyl glucosinolate	-	462.0935	10.59
7-MS	7-Methylsulfinylheptyl glucosinolate	Glucoibarin	478.08923	2.08
8-MT	8-Methylthiooctyl glucosinolate	-	476.1092	13.63
8-MS	8-Methylsulfinyloctyl glucosinolate	Glucohiersutin	492.1052	5.14
1-MOX	1-methoxyglucobrassicin	Neoglucobrassicin	477.0643	5.65
4-MOX	4-methoxyglucobrassicin	-	477.0643	7.74
I-3-Y	Indol-3-ylmethyl glucosinolate	Glucobrassicin	447.0537	3.79
4-OHI-3-Y	4-Hydroxyindol-3ylmethylglucosinolate	4-Hydroxyglucobrassicin	463.0487	2.02

were observed with the amount of feeding damage caused by thrips, which varied considerably among the genotypes of the HapMap population. Even though thrips are regarded generalist insects that readily feed on Arabidopsis, we found some accessions that were almost devoid of feeding damage (Thoen, Kloth et al. 2016). 3-Butenyl glucosinolates (3-ALK, 2-OH-3-ALK, 2-B-3-ALK), 4-pentenyl glucosinolate (4-ALK), 2-hydroxy-4-pentenyl glucosinolate (2-OH-4-ALK), 5-methylsulfinylpentyl glucosinolate (5-MS), 5-Benzoyloxypentyl glucosinolate (5-B), 7-Methylthioheptyl glucosinolate (7-MT) and 8-methylsulfinyloctyl glucosinolate (8-MS) all correlated

negatively with thrips feeding damage (Figure 2, Suppl. Figure 1, Suppl. data file). In addition, levels of 3-methylthiopropyl (3-MT), 3-methylsulfinylpropyl glucosinolate (3-MS) and 3-OH correlated positively with the amount of thrips feeding damage observed on these plants (Figure 2, Suppl. data file 1). The strongest correlation was observed for 3-ALK. When accessions that cannot synthesize 3-ALK are deleted from the analyses, we observe that there is also potentially a strong quantitative effect of 3-ALK in regard to thrips resistance (Suppl. Figure 1).

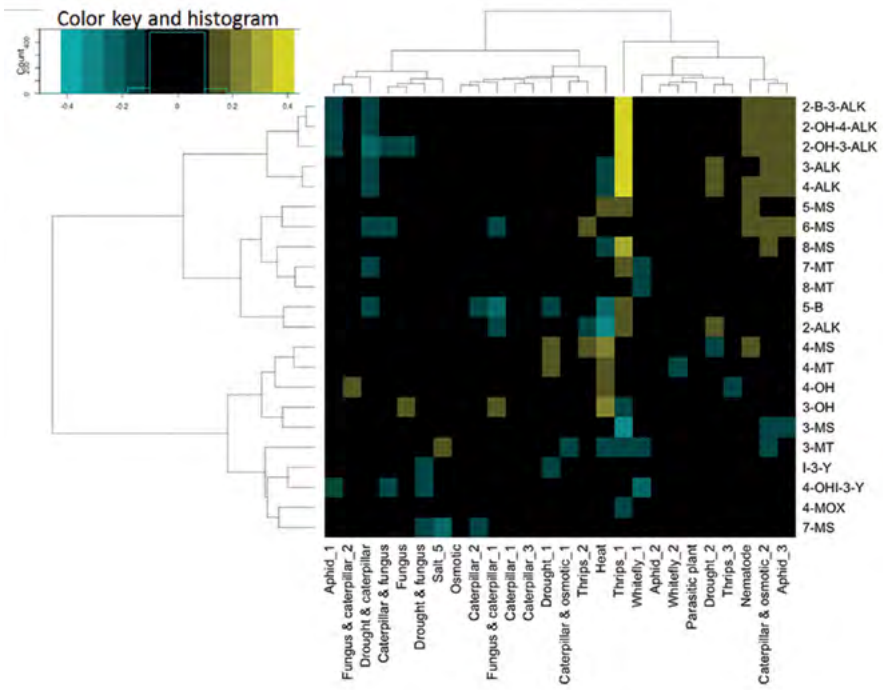

Figure 2. Spearman correlation between stress tolerance and glucosinolates. Yellow indicates resistance/tollerance and blue susceptibility. Traits were clustered hierarchically using Ward's minimum variance method. Traits on the X-axis are described in detail in Suppl. Table 1 and the supplementary methods of Thoen *et al.* (2016). Abiotic stresses include salt (25 mM NaCl), drought, heat (35°C) and osmotic (PEG8000) stress. Biotic stresses include parasitic plants (number of *Phelipanche ramosa* attachments), nematodes (number of *Meloidogyne incognita* egg masses), whitefly (survival (1) and number of eggs (2) of *Aleyrodes proletella*), aphids [probing behaviour (1 and 2) and reproduction (3) of *Myzus persicae*], thrips (feeding damage (1) and preference (2 and 3) of *Frankliniella occidentalis*), caterpillar (*Pieris rapae*), fungus (spreading lesions caused by the pathogenic fungus *Botrytis cinerea*), and combinatorial stresses of caterpillar, drought and fungus. Information on the glucosinolates indicated on the Y-axis can be found in Table 1.

Screening for thrips resistance with reverse genetic tools

As described above there was a positive correlation between feeding damage and C3 GSLs, whereas a number of C4 and longer side chain GSLs correlated negatively with feeding damage, and the strongest correlations are found with lines that contain alkenyls (ALK)(Figure 2). In order to explore whether there is a causal relationship between GSLs and thrips resistance, several mutants and OE lines in different genetic backgrounds were tested. The model accession Col-0 is the background for many mutants. Col-0 is a *MAM1* accession without a functional *AOP* gene (*AOP* null) and with a functional *GS-OH* gene, although ALK-3, the substrate of *GS-OH,* is lacking in Col-0 because of the absence of *AOP-2*. Col-0 can thus be characterized as a C4 MS chemotype. However, we did find small amounts of 3-ALK in Col-0, (Figure 3) in contrast to previous reports where no 3-ALK was detected (Burow, Atwell et al. 2015). The majority of GSLs that correlated negatively with the amount of feeding damage caused by thrips, are not present in Col-0 (Figure 3). GSLs 7-MT and 5-MS are notable exceptions. Previous studies have demonstrated that the *myc2 myc3 myc4* triple mutant (*myc 234*) in Col-0 background is completely devoid of glucosinolates and highly susceptible to larvae of the generalist caterpillar *Spodoptera littoralis* (Schweizer, Fernández-Calvo et al. 2013). In a feeding damage assay with this mutant, we also observed enhanced susceptibility to Western flower thrips (Figure 4a), suggesting that also GSLs of Col-0, that negatively correlated with feeding damage (like 5-MS and 7-MT), could have a negative effect on thrips feeding.

Side-chain elongation of 5-C to 8-C GSLs is controlled by *MAM-3*. Zooming in on specific GSLs, we made use of the fact that the functional *MAM1* and *MAM3* loci present in Col-0 are responsible for the production of the 4-MT and 5-8MT GSL precursors respectively. Thus, we investigated thrips feeding on Col-0 *mam1* and *mam3* mutants to eliminate downstream products. The *mam1* mutant cannot produce 4-MS GSLs, thus 3-MS GSLs will be the pre-dominant GSLs in this line (Textor, Bartram et al. 2004). The *mam3* mutant is completely devoid of long chain GSLs, and, thus, no 5-8MT GSLs and their modified products are produced in this line (Textor, Bartram et al. 2004, Textor, de Kraker et al. 2007). No enhanced susceptibility occurred when either *MAM1* or *MAM3* was knocked out (Figure 4b). This indicates that the potential of 5-MS and 7-MT as resistant factors for thrips are not confirmed in the *mam3* knock-out mutant, thus the *myc 234* mutant might be susceptible for other reasons. It also indicates that the *mam1* knock-out mutant producing 3-MS, will not make plants more susceptible to thrips. This leaves ALK GSLs as the main candidates for further testing.

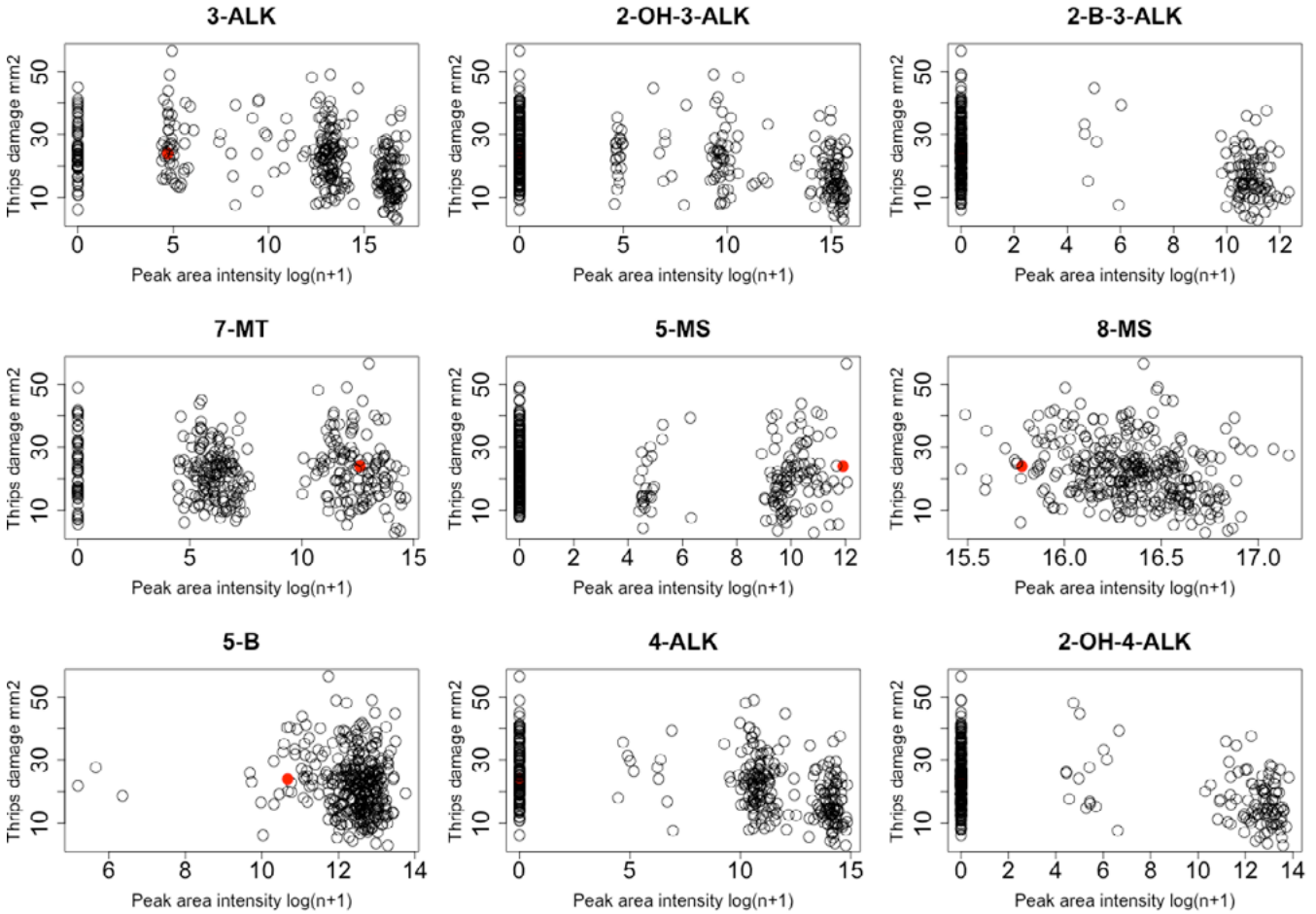

Figure 3. Correlation of GSL content with thrips resistance for the HapMap population. X-axis indicate the log(n + 1) transformed peak area intensities of the representative nine glucosinolates that positively correlated (*P.* < 0.01, Spearman correlation) with thrips resistance (thus negatively with the amount of feeding damage, Y-axis). Red dot indicates Col-0.

To test whether the ALK compounds were crucial, an *AOP2* overexpressor and *GS-OH* knock out in the Col-0 genetic background were tested for thrips resistance. The *AOP2* overexpressor in Col-0 background with a functional *GS-OH* gene, makes predominantly 2-OH-3-ALK, although high levels of 3-ALK (1 - 8 nmoles/mg fresh weight) have also been described for this mutant (Burow, Atwell et al. 2015). The *AOP2/gs-oh* line, an *AOP2* overexpressor in *a GS-OH* knockout line, produces 3-ALK (Burow, Atwell et al. 2015). Both were screened for thrips feeding damage, but also here neither line differed significantly from Col-0 wt in feeding damage levels after six days (Figure 4c).

Correlations of glucosinolate levels with thrips damage were found at the HapMap population level, so it was in principle possible that other transcription factors not present or fully active in Col-0 were critical in creating sufficiently high levels of

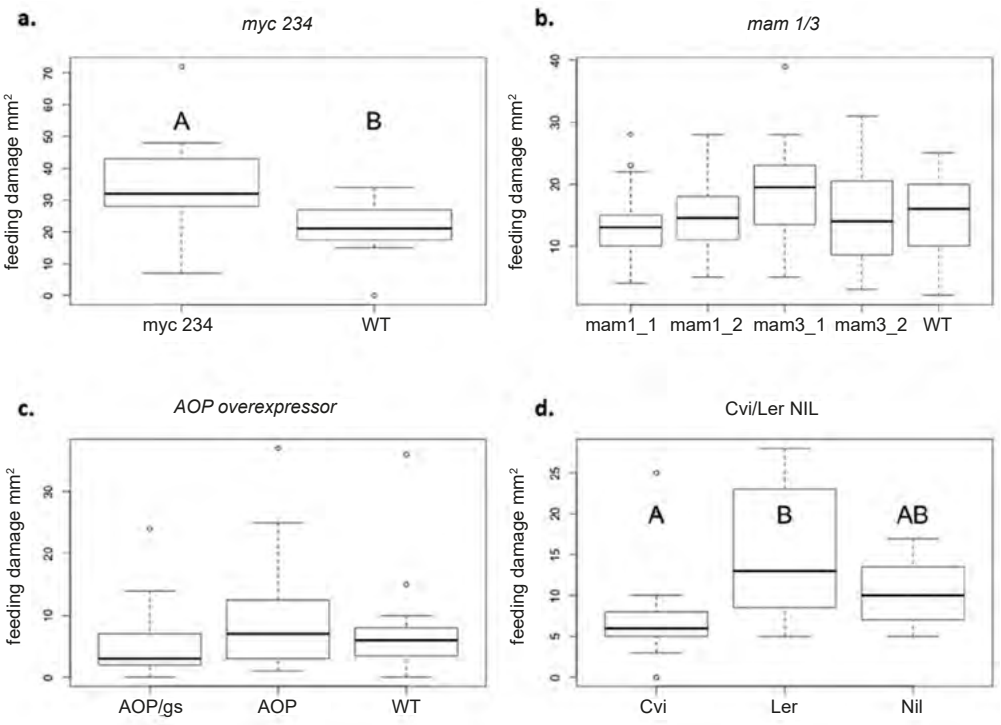

Figure 4. Thrips feeding damage on GSL mutants, overexpression lines and NILs. a. Feeding damage on triple myc knockout (*myc 234*) and WT (Col-0) after six days of thrips feeding (n = 24). **b**. Feeding damage assay on *MAM-1* (N656891 and N682613) and *MAM-3* (N678038 and N653007) KO mutants in Col-0 WT background (n = 20). No significant differences with WT. **c**. Feeding damage assay on *AOP-2* overexpressor (AOP2/gs-oh), an *AOP-2* overexpressor with a *gs-oh* knockout (AOP2/gs-oh) and Col-0 WT background (aop-null/GS-OH). No significant differences with WT. **d**. Near isogenic lines around the *MAM* locus screened for thrips feeding damage. Estimated feeding damage by thrips on parental lines Cvi-0 and L*er*-1 and two NILs. Damage was scored on one leaf in a Petri dish, 5 dpi by 2 WFT nymphs. Different letters indicate significant differences ($P. < 0.05$) based on ANOVA, Tukey HSD.

3-ALK and 2-OH-3-ALK for feeding damage to be affected. We, therefore, analysed NILs introgressed with a Cvi-1 genetic segment around the *MAM* locus in a L*er*-0 background. Cvi-1 and *Ler*-0 accessions were not part of the HapMap panel, but their closely related Cvi-0 and L*er*-1 accessions show similar glucosinolate profiles to Cvi-1 and L*er*-0 respectively, where Cvi-1 can produce ALK GSLs, but L*er*-0 cannot produce these alkenyls. L*er-0* is a thrips susceptible accession with the C3 OH chemotype (*MAM2, AOP3*). Cvi-1 is a resistant accession with the C4 ALK chemotype (*MAM1, AOP2*). The levels of the GSLs that correlated negatively with feeding damage differed between these accessions (Keurentjes, Fu et al. 2006). We used a Near Isogenic Line (NIL) LCN5-2 with a L*er*-0 background that was introgressed around the *MAM* locus with a Cvi genomic region to test differences in thrips susceptibility to C3 OH and C4 OH GLS. This NIL was assayed for thrips resistance, together with its parent lines Cvi-1 and L*er*-0. Based on the location of the introgression (Keurentjes, Bentsink et al. 2007), LCN5-2 was expected to no longer make 3-MS and 3-OH, compounds that negatively correlate with thrips resistance, and was thus expected to become more resistant if it now made C4 glucosinolates (having a functional *MAM1, AOP3* gene, resulting in a presumed C4 OH chemotype). Feeding damage on leaves differed significantly between the two parental lines ($P = 0.0026$, ANOVA). The level of feeding damage on LCN5-2, however, did not differ from either parent (Figure 4d). The NIL could thus be considered to be intermediate resistant to thrips, not as resistant as Cvi-1, but not as susceptible as L*er*-0. We can, however, not conclude whether this is due to the absence of C3 OH GSLs, the presence of C4 OH GSLs, a combination of these, or other factors different in this NIL. A *MAM1* or *AOP2* knock-out mutation in Cvi-1 background could validate explicitly whether C4 glucosinolates or alkenyls, respectively, are responsible for the enhanced resistance to thrips observed in the Cvi parent. However, such mutants are not publicly available and were not tested in the present study.

QTL mapping for thrips resistance with the Cvi x *Ler* RIL population

Limitations in association studies with natural populations, mostly due to population structure and rare alleles with moderate to small effects, can be partially circumvented when combining association studies with classical pedigree-based mapping for the trait of interest (Brachi, Faure et al. 2010, Mitchell-Olds 2010). To this end we used a RIL population for which it was established that parents and RILs differ in glucosinolate content, and that both parents have opposite alleles for the three major genes in the aliphatic GSL pathways (*MAM, AOP* and *GS*-OH), which will result in 8 different chemotypes (Keurentjes, Fu et al. 2006). 94 RILs from a Cvi-1 x L*er*-0 RIL population were analysed for thrips resistance, and QTL mapping was applied to thrips feeding damage (Figure 5a). The strongest signal was found on

chromosome 2 near the Erecta locus at 48.5cM. This locus was also a significant QTL in this RIL population when screening for resistance against the specialist caterpillar *P. xylostella* (Kliebenstein, Pedersen et al. 2002). The linkage disequilibrium (LD) window of 100 KB contained several genes that are involved in plant defence, but none of the aliphatic GSL candidate genes (Suppl. Table 2). Also in proximity of other QTL signals, none of the aliphatic GSL candidate genes were present. In order to compare the results for resistance to thrips with the RIL population with the HapMap population, we can look at the separation of GSL chemotypes based on the *AOP and MAM* loci (omitting *GS-OH* for comparison, because the expression of this gene is hidden in a natural population with *MAM2/AOP2* chemotypes, where the *GS-OH* chemotype can not be determined because *GS-OH* does not act upon 2-ALK, but only 3-ALK). Upon separation of the population into four haplotypes, based on markers located near the *AOP* and *MAM* loci, we found that lines with Cvi-1 parental chemotype (functional *MAM-1/AOP2*) were significantly more resistant to thrips than all other combinations (Figure 5b).

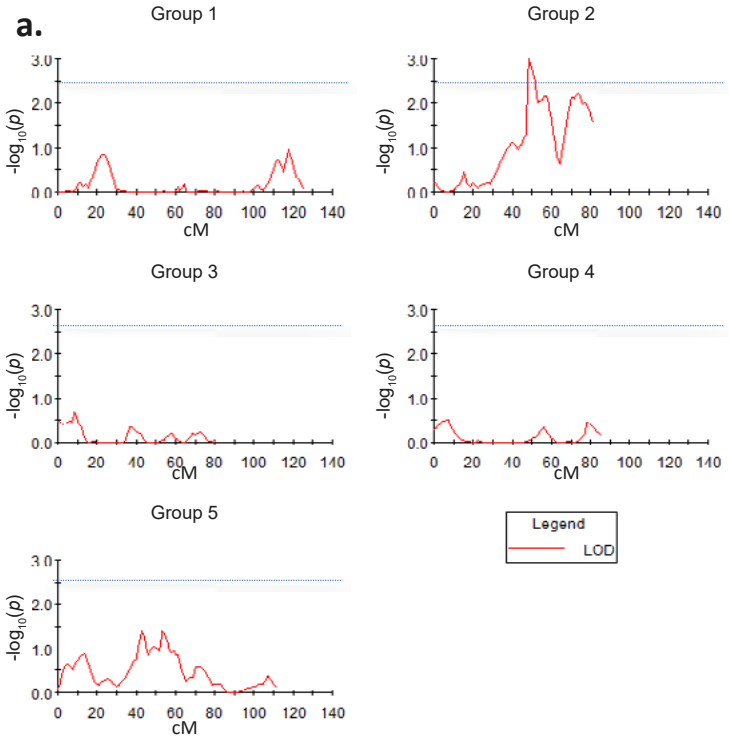

Figure. 5. Cvi x L*er* RIL population and near isogenic inbred lines. a. Red line indicates the LOD score (significant above 2.6). The five groups indicate the five chromosomes of Arabidopsis. **b.** Four different chemotypes in Cvi x L*er* RIL population based on the allelic state of markers AOP (*AOP2 or AOP3*) and Elong (*MAM1* or *MAM2*). Different letters indicate significant differences (*P.* <0.05) based on ANOVA, Tukey HSD.

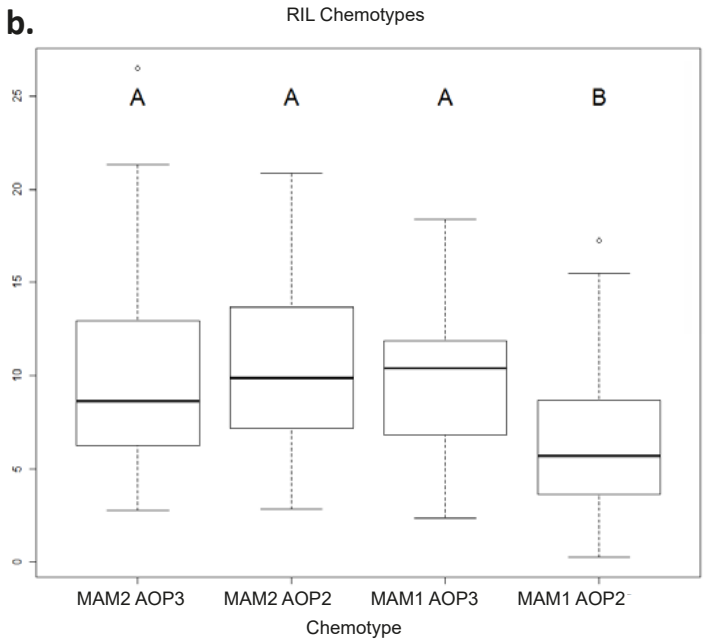

Figure 5. Continued

Genome-wide association mapping of thrips resistance and correlated glucosinolates

The genetic signature of the majority of the GSLs that correlated with thrips resistance was identified using GWA mapping (Suppl. Figure 2). 3-OH, 3-ALK, 2-OH-3-ALK, 4-ALK, 2-OH-4-ALK, and 2-B-3-ALK mapped on the *MAM* locus. A second QTL for 3-ALK was positioned at the *AOP* locus, and for 2-OH-3-ALK and 2-OH-4-ALK a third QTL mapped on the *GS-OH* locus. 3-MS and 8-MS did not map on any known GSL genes. A normalised summary value for all nine GSLs that negatively correlated with thrips resistance (3-ALK, 2-OH-3-ALK, 2-B-3-ALK, 4-ALK, 2-OH-4-ALK, 5-MS, 5-B, 7-MT and 8-MS, Spearman *rho* $= 0.41$) was created to visualize the genetic architecture behind these GSLs. We found significant QTLs for both the *AOP, GS-OH* and the *MAM* locus (Figure 6a). We also performed GWAS with the results of the thrips feeding damage assays. This resulted in a list of candidate genes in LD with SNPs that had a *P* value $< 10^{-5}$ (Suppl. Table 3). These candidate genes can be investigated in more detail in follow-up studies. However, despite the strong correlations with individual glucosinolates, no significant QTLs (above the Bonferroni threshold) were detected for resistance to WFT (Figure 6b). In addition, the peaks with the highest LOD scores did not co-localize with the QTL found on Chromosome 2 in our RIL population. In addition to GWAS with EMMA-X, which corrects for population structure, we performed a GWAS using a Wilcoxon test on all SNPs without correcting for population structure.

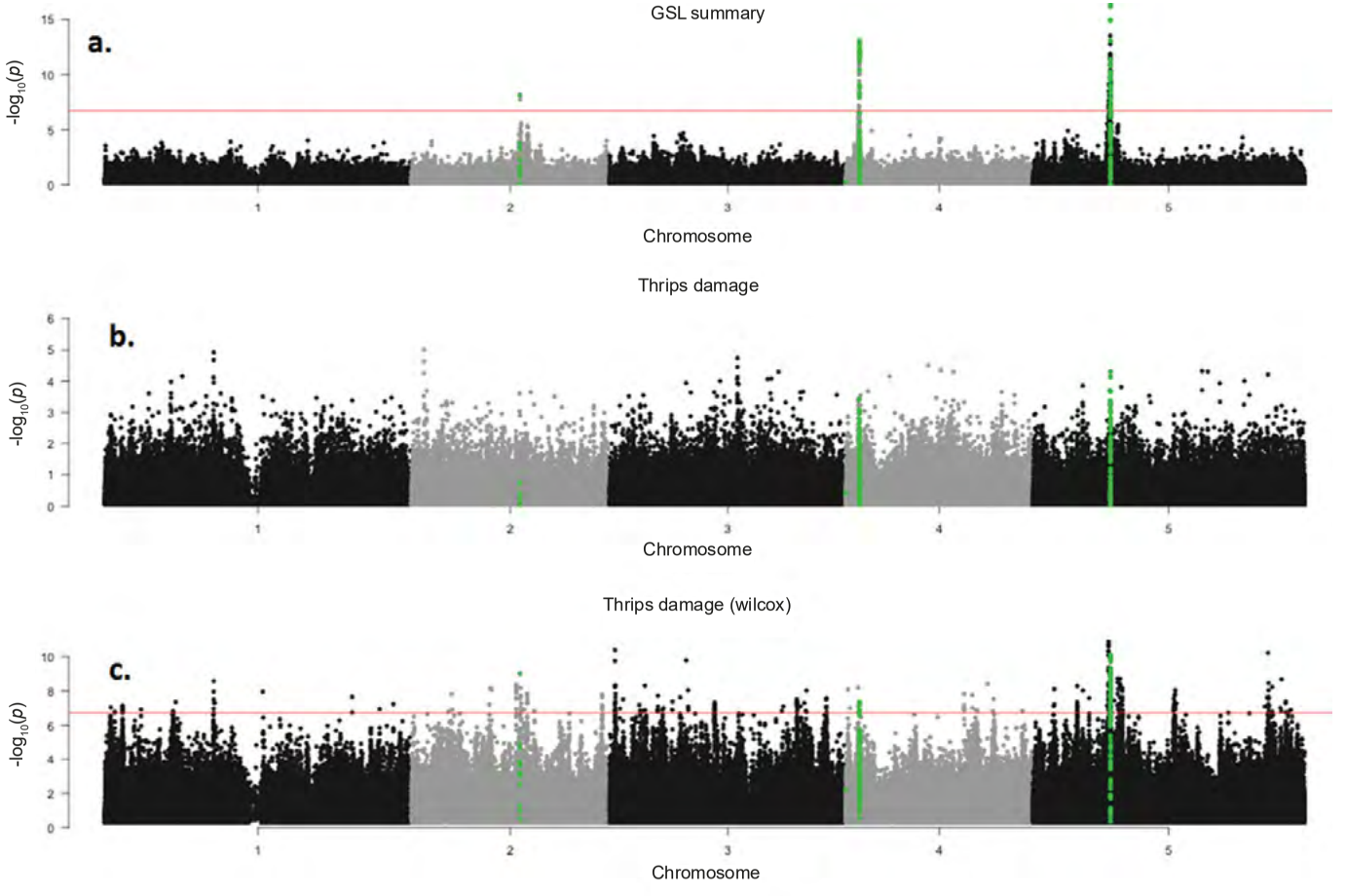

Figure 6. Genome-wide assocation mapping of Glucosinolates and thrips resistance. a, GWAS with a normalised summary value for some GSLs that negatively correlated with thrips feeding damage (3-ALK, 2-OH-3-ALK, 2-B-3-ALK, 4-ALK, 2-OH-4-ALK, 5-MS, 5-B, 7-MT and 8-MS). **b.** GWAS on feeding damage by Western Flower Thrips (EMMA-X). **c.** GWAS on feeding damage by Western Flower Thrips without correcting for kinship (Wilcox test). Green dots are SNPs in a 20KB window of *GS-OH, AOP* and *MAM-1* genes, on chromosomes 2, 4 and 5 respectively. Red line indicates Bonferonni threshold of signficance.

This method brought forward many (false positive) significant QTLs, including known GSL loci. For example, we observed a strong signal ($P < x.10^{-10}$) for the major regulator of the side chain length of aliphatic GSLs, *MAM-1/2* on chromosome 5, as well as a significant QTL for the *GS-OH* and *AOP* loci on chromosomes 2 and 4, respectively (Figure 6c). This finding suggests that the observed correlations between GSL levels and thrips resistance may not have a causal link, but could instead be solely based on population structure. When the predominance of specific short chain glucosinolates (C3 or C4), and the abundance of MS, ALK and OH GSLs are plotted on the map of Europe, geographic patterns of distribution of glucosinolate profiles become evident (Suppl. Figure 3). Indeed, longitude and glucosinolate profiles correlate significantly across Europe, which is in line with previous reports (Züst, Heichinger et al. 2012, Brachi, Meyer et al. 2015). Furthermore, we observe a positive correlation between longitude and the amount of feeding damage by thrips on these accessions (rho = 0.21, p = 0.00005, Figure 7a). When the proportion of C3 across all short chain GSLs on the one hand, and the ratio of MS, ALK and OH on the other hand are considered, six different chemotypes can be distinguished. These chemotypes should reflect the allelic state of the *MAM* locus (*MAM2* = C3, *MAM1* = C4) and the *AOP* locus (*AOP3* = OH, *AOP2* = ALK, *AOP-null* = MS). From these six chemotypes, the accessions producing C4 alkenyls (3-ALK and 2-OH-3-ALK) are significantly more resistant than all other combinations (Figure 7b).

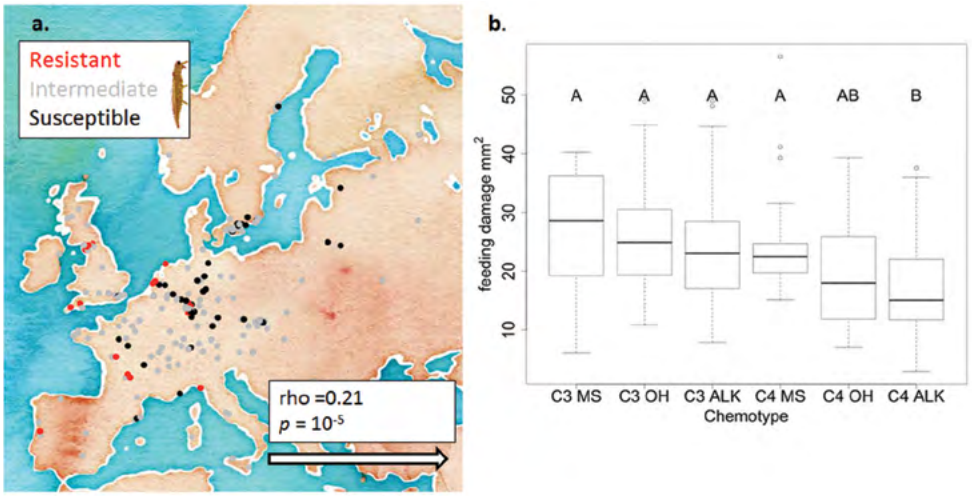

Figure 7. Geographic distribution of thrips feeding damage in the HapMap population and its relation to glucosinolate chemotypes. a. Geographic distribution pattern of thrips feeding damage. N = 311, rho and p values derived from Spearman correlation test of thrips damage and longitude across European accessions. **b**. Chemotype specific differences in the average level of feeding damage. Chemotypes were distinguished in terms of short sidechain elongation (C3 or C4 chemotype) and side-chain modification (methylsulfenyls (MS), alkenyls (ALK) or hydroxyls (OH)) chemotypes. Accessions per chemotype in European selection of the HapMap population are C3 MS (n = 17), C3 OH (n = 82), C3 ALK (n = 97), C4 MS (N = 15), C4 OH (n = 13) and C4 ALK (n = 87). Different letters indicate significant differences (*P.* < 0.05) based on ANOVA, Tukey HSD.

Discussion

Aliphatic GSLs in *A. thaliana* can be used as a model to study the potential underlying selection pressures that generate metabolic diversity. Diversity in aliphatic GSL profiles is mainly controlled by three loci: *MAM* (controlling side chain elongation), *AOP* (modification towards alkenyls or hydroxyls) and *GS-OH* (further modification of alkenyls towards hydroxy alkenyls). In this study we have compared GSL profile compositions with the tolerance/resistance towards 15 different biotic and abiotic stresses in a population of 349 wild Arabidopsis accessions (Chapter 5). We found strong correlations with resistance towards the generalist herbivore *F. occidentalis* (Western flower thrips), for levels of several aliphatic GSLs. This correlation coincides with a geographic correlation of glucosinolate profiles.

Previous attempts to underpin the selective pressure on glucosinolate profiles have ascribed the loss of C3 populations in field trials and its geographic distribution in Europe to the abundance of the specialist aphid *Lipaphis erysimi* (Züst, Heichinger et al. 2012). Both C3 ALK and C4 ALK chemotypes were lost over six generations of field trials in the presence of these aphids. The authors concluded that the loss of alkenyl chemotypes resulted from selection against a costly defence trait that provided insufficient benefits in their experiment with solely phloem feeding aphids. These specialist aphids are not affected by any GSLs, and therefore a tolerance mechanism where Arabidopsis invests all its resources in growth and reproduction is selected for. This could also imply the loss of ALK GSLs, since plants producing these GLSs are much smaller. Our study shows that the C3/C4ALK chemotypes are very common in the HapMap population (Figure 6), thus ALK chemotypes must be maintained by other selective agents. Diversity in GSLs profiles cannot be explained by just one herbivore, but is proposed to be maintained under balancing selection, due to fluctuating fitness benefits that vary with environment and time (Burow, Atwell et al. 2015).

Although GSLs are commonly described to confer resistance to aboveground insect herbivores (Hopkins, van Dam et al. 2009), we know that GSLs can also have an effect on the resistance/tolerance to many other stresses, including bacteria (Bending and Lincoln 2000), root herbivores (van Dam, Tytgat et al. 2009) and heat (Zhao, Zhang et al. 2008). Because metabolomics in the data set used in this study was performed on unchallenged plants, it could thus be that the level of constitutively present GSLs in foliar tissues are not properly representing the actual state of GSLs when plants are tested in detached leaf assays for thrips feeding damage. There are potential mechanisms whereby exposure to abiotic stresses may enhance plant defence against subsequent biotic stressors. In our study, we found many GSLs correlating with one or more biotic or abiotic stresses, and in quite some

cases we observe pleiotropic effects, where the presence of specific GSLs correlates with resistance to one trait, but susceptibility to another trait. For instance, levels of 2-OH-3-ALK correlate positively with resistance against thrips, caterpillars, nematodes and aphids. However, a negative correlation was observed for fungi and the combined stresses drought/caterpillar, fungus/caterpillar. The GLS 2-OH-3-ALK has been reported to negatively influence the performance of the generalist caterpillar *Trichoplusia ni* (Hansen, Kerwin et al. 2008). Another compound that showed both negative and positive correlation based on specific traits is 2-ALK. 2-ALK showed a positive correlation with drought and thrips resistance, but correlated negatively with heat tolerance, initial deterrence of thrips and tolerance towards the combination of fungi and caterpillar stress. It has been shown that levels of 2-ALK enhance resistance to the soil-borne fungal pathogen *Verticillium longisporum* (Witzel, Hanschen et al. 2013). However, the production of alkenyl GSLs is costly and accessions known to produce these compounds are among the slowest growing accessions found in Arabidopsis (Züst et al., 2012). It is thus possible that the larger biomass gained in alkenyl-free chemotypes aids in tolerance against heat and combined stresses, whereas the benefit of making alkenyl GSLs pays off in resistance against herbivores.

The combination of a functional *MAM-1* and *AOP-2* gene enables Arabidopsis accessions to produce the three C4-ALKs; 3-ALK, 2-OH-3-ALK and 2-B-3-ALK (Figure 1). Furthermore, a functional *AOP-2* also enables plants with *MAM-3* to produce 4-ALK and 2-OH-4-ALK. In addition to these alkenyl GSLs, 7-MT, 5-MS and 8-MS do not require a functional *AOP* gene. 5-B requires a functional *MAM-3* and *AOP-3* gene. All these compounds positively correlate with resistance to thrips, measured as the amount of feeding damage of detached leaves (Figure 1, Suppl. data file 1). Also in another dataset obtained from a collection of 595 Arabidopsis accessions (Brachi, Meyer et al. 2015), most of these GSLs positively correlate with our thrips resistance data on the 125 overlapping accessions (Suppl. Table 4), with the exception of the 7-MT and 5-MS GSLs, that did not correlate with feeding damage to thrips in this dataset. In order to validate the relevance of these GSLs for resistance to thrips, we screened several mutant lines (Table 2). However, *mam-1* and *mam-3* mutant lines had levels of resistance comparable to Col-0. This indicates that the production of 3-MS instead of 4-MS (*mam-1*) and the production of no more long side-chain GLSs (*mam-3*) do not by themselves significantly influence resistance to thrips in a Col-0 background. Col-0 contains very little 8-MS and 5-B, and 4-ALK, 2-OH-4-ALK are not present at all, thus this conclusion is limited to levels of 5-MS and 7-MT that are present in Col-0 in relatively high numbers (Figure 3).

The strongest correlation with thrips resistance was observed for 3-ALK and 2-OH-3-ALK (Figure 2, Suppl. data file 1), compounds that are not produced in Col-0 (Figure 3) because Col-0 does not have a functional *AOP* gene. Both compounds require a functional *MAM-1* and *AOP-2*. If the combination of *MAM-1/AOP-2* would render Arabidopsis more resistant to thrips, then both the *AOP2* and *AOP2/gs-oh* lines should have been more resistant to thrips. This, however, is not what we observed (Figure 4c). We did observe enhanced feeding damage in the triple KO mutant *myc 234*, but this mutant is compromised severely in JA-signalling, not just in levels of glucosinolates. The increased susceptibility could thus also be explained by other defence processes no longer activated by JA in that mutant. Mutants in Col-0 cannot fully reflect the metabolome diversity found in the population of 349 accessions because quantitative differences are controlled by several transcription factors. Transcription factors like *MYB28* and *MYB29* influence the quantity of GSLs upon herbivory induction for instance (Burow, Atwell et al. 2015). A different genetic background might have the right combination of GSL biosynthesis genes and transcription factors. Therefore, we screened a NIL in the L*er* background, with a Cvi introgression around the *MAM* locus. The parents L*er* (C3 OH) and Cvi (C4 ALK) showed susceptible and resistant phenotypes to thrips, respectively, while the NIL LCN 5.2 (C4 OH) showed an intermediate level of resistance. Relative to its parent L*er,* the NIL was no longer able to produce 3-OH, a compound that negatively correlated with thrips resistance (Figure 2). Although the NIL did not produce C4 ALK compounds (that positively correlate with thrips resistance), the fact that it can no longer make 3-OH and becomes slightly less susceptible might indicate that 3-OH could function as a feeding stimulant for thrips.

Table 2. Overview of lines used to screen for feeding damage by thrips

Line	Relative resistance[1]	Known chemotype	Reference	Chemotype resistance[2]
Col-0	-	C4 MS	(Kerwin, Feusier et al. 2015)	-
Cvi-1	R	C4 ALK	(Keurentjes, Fu et al. 2006)	R
L*er*-0	S	C3 OH	(Keurentjes, Fu et al. 2006)	S
mam-1	-	C3 MS	(Textor, Bartram et al. 2004)	S
mam-3	-	C4 MS (no LC GSL)	(Textor, Bartram et al. 2004)	?
myc 234	S	No GSL	(Schweizer, Fernández-Calvo et al. 2013)	S
NIL 5.2	-	C4 OH (predicted)	(Keurentjes, Bentsink et al. 2007)	-
AOP2	-	C4 ALK (OH)	(Kerwin, Feusier et al. 2015)	R
AOP2/gs-oh	-	C4 ALK	(Kerwin, Feusier et al. 2015)	R

[1] Observed level in accession/line of difference in thrips feeding damage relative to Col-0. R = Resistant, - = Not significantly different from Col-0, S = Susceptible

[2] Observed association of chemotype to thrips feeding damage in HapMap. R = Resistant, - = Not significantly different from Col-0, S = Susceptible

We also screened a L*er* x Cvi RIL population, for which the GSL content of all individual RILs was already determined (Kliebenstein, Kroymann et al. 2001, Kliebenstein, Pedersen et al. 2002) for thrips resistance. In this population we did not find the same significant correlation between thrips resistance and GSLs as observed in the HapMap population. Furthermore, the QTL found for thrips resistance in this population did not co-localize with any of the known GSL genes, although this QTL was previously reported to be involved in resistance against *P. xylostella* (Kliebenstein, Pedersen et al. 2002). We did observe several potential candidate genes surrounding this QTL, including genes involved in several other biochemical processes. Candidate genes include a *METHYL ESTERASE, SERINE PROTEASE INHIBITOR, UROPORPHYRINOGEN-III SYNTHASE, HEME OXYGENASE* and a *UDP-GLUCOSYL TRANSFERASE*. Also the *ERECTA* gene, which was previously reported to be involved in resistance to bacteria (Godiard, Sauviac et al. 2003) and necrotrophic fungi (Llorente, Alonso-Blanco et al. 2005, Sanchez-Rodriguez, Estevez et al. 2009), is located in the QTL region (Suppl. Table 2).

In addition to QTL mapping with the Cvi x L*er* RIL population, we performed GWAS mapping on the HapMap population for GSL content and resistance to thrips. We did not observe co-localisation of known GSL genes in the GWAS results for thrips resistance (Figure 6, Suppl. Figure 2). The fact that, despite a high narrow-sense heritability estimate of resistance to thrips (0.78), we could not find back genes coding for enzymes involved in GSL biosynthesis in our GWAS, might be explained by population stratification and/or the multigenic and possibly antagonistic pleiotropic effects of a large number of genes controlling plant resistance (i.e. feeding damage) to *F. occidentalis* by different mechanisms. Furthermore, we do observe that some of the methylsulfinyls that correlated with thrips resistance do not map on any of the known GSL genes (3-MS, 5-MS and 8-MS). It is possible that any of the GSLs that did not map on any known GSL gene could explain why thrips resistance did not map on *MAM, AOP* or *GS-OH*. We did not find co-localisation with any GWAS signals from 3-MS, 5-MS and 8-MS with thrips resistance, but maybe the combination of levels of these compounds with other transcription factors that were unaccounted for are responsible for natural variation in thrips resistance. We did not discover significant QTLs (*P.* $<10^{-6.6}$), although some candidate genes underlying SNPs with a *P.* value $< 10^{-5}$ can still yield interesting material for follow up studies (Suppl. Table 3).

So, why do we observe strong correlations between thrips feeding damage and specific glucosinolates, but no enhanced resistance in the mutant, NIL and overexpressor lines tested in this study? One explanation could be dose dependency, wherein despite qualitative differences in GSLs within the mutants and overexpressor lines, the quantitative differences do not reflect the quantitative

differences in the HapMap population. Levels of 3-ALK for instance, show a clear dose dependency effect in relation to thrips resistance (Figure 3, Suppl. Figure 1), so it is not just a black-and-white difference in quality of GSLs that could determine host-plant resistance. Several *MYB* transcription factors can modulate the quantity of GSLs at a constitutive and induced level (Kliebenstein, Kroymann et al. 2001). A second explanation could be the polygenic nature of resistance to insects in plants. Although the genetic architecture behind aliphatic GSL synthesis is well understood, it is just one of the many factors or mechanisms that could influence resistance to thrips. Only modifying GSL content in mutants and overexpressor lines in a Col-0 background, will not take into account all the other factors that could potentially interact with resistance to thrips. A crucial step in the activation of GSLs is the activity of myrosinases that form isothiocyanates. However, alternative products like epithionitriles, nitriles, and thiocyanates with different biological activities can be formed in the presence of specifier proteins (Wittstock and Burow 2007, Mumm, Burow et al. 2008). These, often non-toxic, compounds are formed at the expense of isothiocyanates (Burow, Losansky et al. 2009), and we have not addressed natural variation in these specifier proteins in the Arabidopsis HapMap population. Furthermore, it is likely that generalist insects will have to deal with additional lines of defences that could have additive effects on resistance of the host-plant (Burow, Losansky et al. 2009). These additional lines of defence might involve morphological aspects, or other metabolites. A third explanation may be that the correlation with distribution of glucosinolate profiles and thrips resistance is confounded by a parallel geographic distribution of these traits. The fact that *MAM, AOP* and *GS-OH* are all three found in a GWAS on thrips resistance without correcting for population stratification (Suppl. Figure 3) supports this explanation. The fact that we observe strong dose dependencies of GSLs and feeding damage, and that several GSLs that correlated with feeding damage are synthesised by different genetic pathways support the alternative explanation.

Understanding host-plant resistance to thrips using a metabolomics approach has brought forward several candidate defence compounds. Previous studies in chrysanthemum (*Dendranthema grandiflora*) suggested isobutylamide (Tsao, Marvin et al. 2005) and chlorogenic acid (Leiss, Maltese et al. 2009) as potential deterrent compounds for thrips. In carrot (*Daucus carota* L.), the flavonoid luteolin, the phenylpropanoid sinapic acid and the amino acid β-alanine correlated with thrips resistance (Leiss, Cristofori et al. 2013). In tomato, acyl sugars correlated with lowered thrips feeding damage (Mirnezhad, Romero-Gonzalez et al. 2010) and across pepper (*Capsicum*) cultivars there was a correlation between several tocopherols, alkanes, a sterol and a terpene and thrips resistance (Awang 2013). However, most of these studies did not include follow-up *in planta* bio-assays to

prove the causality of these compounds underlying the phenotypes observed. The present study shows that such a follow-up study is relevant and that conclusions based solely on the correlation of metabolite levels with host-plant resistance are sensitive to false positives. However, the present study also shows that the default approach of following up candidate genes with T-DNA insertion lines in the Col-0 background is useless, if the compounds of interest are not present in Col-0 to begin with. The highly complex and polygenic nature of host-plant resistance to insect herbivores is hard to unravel with single-gene KO mutants. QTL signals might not be as strong and numerous in studies with generalist insects, in comparison to specialist insects (Pfalz, Vogel et al. 2007), due to the broader set of defensive mechanisms that still act on generalist herbivores, but specialist herbivores have overcome.

Conclusion

The strong correlations found in our study indicate a quantitative role for alkenyl glucosinolate as a defensive system against generalist herbivores like thrips, although follow up experiments with knock-out mutants and overexpressor lines could not validate the effect of these GSLs in a Col-0 background. There are two possible explanations for the lack of validation: **1)** The crucial factors that control resistance to thrips may not have been present or in insufficient quantities in the mutants, RILs and NIL screened in this study. The fact that resistance seems to be highly dose dependent makes it possible that the levels of GSLs were not in the right quantity to trigger a resistance response to thrips. Furthermore, the fact that we observed several compounds derived from different genetic pathways indicates that thrips resistance is a consequence of a mixed palette of GSLs not present in any of the mutants tested in this study. **2)** Both GSLs and thrips resistance show a similar geographic distribution, thus the correlation observed could also be a consequence of independent geographical clines. More research should be conducted to confirm the validity of either hypothesis. Disentangling correlation from causality is crucial in explaining the biological significance of natural variation in the secondary metabolome. Untargeted approaches like metabolomics and genome-wide association mapping are a first and crucial step in finding the relevant genes, but the confirmation of their ecological role is challenging when there are so many unforeseen genetic interactions that are overlooked with single-KO or overexpression mutant screens in suboptimal genetic backgrounds.

Methods

HapMap population

The Arabidopsis HapMap population (Baxter, Brazelton et al. 2010) consisted of 360 Arabidopsis accessions . This population was the most genetically diverse subset of a global collection of 5,810 Arabidopsis accession, and thus minimized genetic redundancy and relatedness. These 360 genotyped for 250K bi-allelic SNPs. After quality control and imputation, this set of SNPs were reduced to a set of 214.051 SNPs (199,360 SNPs with a Minor Allele Frequency (MAF) cut-off of 5%). For the chemical profiling with LC/MS all 360 accessions were used. For phenotyping of stress resistance, sets of approximately 350 accessions (depending on the trait) were used. (http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls).

LC/MS dataset

LC/MS on the Arabidopsis HapMap population has been described previously (Wehrens, Hageman et al. 2016). The population has been screened twice, and after batch correction the average peak intensity per representative cluster was used for GWAS and correlation tests, without transformations on the data (log transformation did not results in normal distribution for the majority of the GSL, thus non parametric correlation tests were performed on raw data). For the 'GSL scaled' value, averaged peak intensities were per parent compound were log-transformed. For the log-transformed values per glucosinolate, Z-scores per individual compound were summed to form the summary value of GSL that correlated with enhanced thrips resistance.

Chemotype determination

The mass accuracy of the LTQ-Orbitrap hybrid MS system coupled with retention time of the metabolites measured, allowed us to identify most of the aliphatic glucosinolates described in Arabidopsis. The mean average peak intensity of the parent ion over two replicates was used to calculate correlations and determine chemotypes. For the chemotype determinations, a list of rules were followed to call the allelic state at each locus, based on our knowledge of the aliphatic GSL pathway (see Figure 1) (Chan, Rowe et al. 2011). *MAM1*: non-functional if 3C GSL levels are higher than 4C GSL levels and functional if 4C GSL levels are higher than 3C GSL levels. AOP: *AOP3* if hydroxy-propyl (OH-P) GSL is detected; *AOP2* if 2-ALK or 3-ALK GSL is detected; non-functional if neither OH-P, allyl or 3-ALK are detected

but instead only the precursor methylsulfinyl GSL is detected. GSOH: this locus can only be determined when *AOP2* and *MAM1* are both functional because it converts the 4C ALK to 4C-OH-ALK..*GSOH* is functional when OH-3-ALK is detected and non-functional when 3-ALK is detected, but 2-OH-3-ALK is not detected.

Insects

The Western flower thrips (*Frankliniella occidentalis* (Pergande) were reared in glass bottles on green common bean pods (*Phaseolus vulgaris*). To keep the offspring synchronized, 200 adult females were transferred to bottles with fresh bean pods twice a week. In all experiments L1 or L2 juveniles of approximately 5 days old were used. For the HapMap population, plants were screened as described previously (Thoen et al. 2016). In brief, we screened the population in 5 rounds of 360 accessions. Plants were randomly allocated in blocks (20 accessions per block, 18 blocks per round). Phenotypic information was obtained by estimating the amount of mm^2 feeding damage on one leaf, after exposure to 3 juvenile thrips, for six days. Leaves were cut from plants, and kept turgid in Petri dishes with a diameter of 5 cm, containing a film of 1% technical agar. For the metabolites, we used the mean of two samples after batch correction to apply genome-wide association mapping. Each accession was screened in 5 replicates. For QTL mapping with the Cvi x L*er* RIL population, a subset of 96 (out of the total 161) lines were used, and screened in duplicates. Each duplicate was screened for two individuals, and the average was taken. NIL LCN5-2 had a L*er* background, and was introgressed with a Cvi segment around the MAM locus (Keurentjes, 2005). Twenty replicates per line were used. All plants were grown for 5 weeks at 23°C, 70% RH, 100 μmol m^{-2} s^{-1} light intensity and 8h:16h L:D photoperiod.

Mutant lines

Arabidopsis *mam-1* (SALK_116223C and SALK_086935C) and *mam-3* (SALK_004536C and SALK_007222C) knock-out mutants were obtained from TAIR, and tested for homozygosity with PCR. Seeds from the *myc234* line were kindly provided by Philip Reymond (Lausanne University), seeds from the *AOP-2* and *AOP-2/gs-oh* lines were kindly provided by Daniel Kliebenstein (University of California, Davis).

RIL population

A L*er* x Cvi Recombinant Inbred Line (RIL) population of 161 *Arabidopsis thaliana* lines, derived from a cross between the L*er* and Cvi accessions (Alonso-Blanco et al. 1998), was sown in two time periods. After five days of thrips infestation, the

number of feeding spots was counted by at least two different persons. From this data, the average and standard deviation was calculated for the counting. Leaves with small size or highly curled edges were not included in the analyses. The L*er* x Cvi RIL population was genotyped with 144 SNP markers equally distributed over the genome was used for QTL analyses (IM method) for resistance against thrips with MapQTL (version 6.0) software.

Statistics

For the HapMap population screening on thrips resistance, genotypic means (BLUEs) were calculated using a linear mixed model with fixed effects for round and genotype and a random effect for the block effect, nested within rounds. Correlations between glucosinolate content and stress tolerance were done with Spearman correlation tests using the Hmisc R-package. *P.* values were corrected for multiple testing with the BH method. Comparisons between different chemotypes in resistance to thrips were done with ANOVA for both RIL and NIL assays. Genome-wide association mapping was done using EMMA-X with a minor allele frequency of 5% (199,360 SNPs) and a kinship matrix based on genotypic relatedness among all accessions to correct for population structure. GWAS without correcting for population structure were done with a Wilcoxon signed rank test. All analyses were performed in R.

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Chapter seven

General Discussion

Manus P.M. Thoen

Introduction

Planet earth is a planet of plants and insects. Plants and insects make up the majority of all described species of multicellular organisms (Schoonhoven, van Loon et al. 2005). These two groups of organisms outnumber mammals in evolutionary age, species richness and total biomass. The enormous variation and species richness in the insect world has greatly influenced evolution in the plant kingdom. From mutualistic interactions, like the important role that some insects have in pollination, to more hostile interactions where plants and insects can be considered antagonists. Approximately 50% of insect species are herbivorous. Herbivorous insects are responsible for the consumption of approximately 10% of annual plant biomass in natural habitats and even higher numbers are reported in agricultural systems (Schoonhoven, van Loon et al. 2005). Insect-plant research is a highly dynamic and multi-disciplinary field that integrates ecology, chemistry, behavioral science, genomics and molecular biology to answer questions based in both applied and fundamental research. The focal plant-insect interaction studied in this thesis is that between *Arabidopsis thaliana* ((L.) Heynh) (in short, Arabidopsis) and the minute slender Western Flower Thrips, *Frankliniella occidentalis* (in short, thrips). Arabidopsis is the flagship of plant genomics (Koornneef et al. 2011, Kramer 2015, Weigel 2012) and is also used as a model in evolutionary ecology (Mitchell Olds 2006, Gaut 2012). Its small size, short generation time and limited outcrossing have great practical advantages, although Arabidopsis was initially viewed as a poor choice to study plant-organism interactions precisely for these reasons (Roux and Bergelson 2016). However, it turns out that the diverse habitats in which Arabidopsis occurs and the natural variation it displays for many phenotypic traits, make it a good model to study the eco-evolutionary responses to interactive species such as bacteria (Jakob, Goss et al. 2002), fungi (Adam and Somerville 1996), oomycetes (Holub 2008), viruses (Ouibrahim and Caranta 2013), other plants (Bartelheimer, Schmid et al. 2015) and herbivores (Van Poecke 2007, Whiteman, Groen et al. 2011, Falk, Kastner et al. 2014). Arabidopsis is native in Eurasia, and generally has an early flowering strategy. This implies that plants overwinter in the rosette stage, and flower in early spring (Mitchell-Olds 2001). *Frankliniella occidentalis* is native to North West America (also known as Californian thrips), and as many insect species, is most abundant in the summer (Kirk and Terry 2003). There is thus seemingly both a regional and temporal mismatch in this study system, from which we could presume that Arabidopsis and thrips are not part of each other's evolutionary history. This does, however, not imply that knowledge gained in thrips/Arabidopsis studies is not reflecting eco-evolutionary responses. Thrips are highly polyphagous, having suitable host plants in many different plant families,

including Brassicaceae. The levels of host-plant resistance observed in Arabidopsis might thus be indicative of defensive mechanisms against generalist piercing/sucking herbivores. We need to understand the biology of both plant and insect, to fully grasp the complexity of its interaction. In the Arabidopsis/thrips study model, two distinct questions can be addressed. **1)** What can we learn about Arabidopsis in Arabidopsis/thrips interactions. **2)** What can we learn about thrips in Arabidopsis/thrips interactions? The first question is mainly based on fundamental research, where this specific interaction can help us in understanding how Arabidopsis copes with insect herbivory. How do defenses against generalist insects like aphids and thrips differ from each other in Arabidopsis? Are there trade-offs in resistance/susceptibility with other biotic and abiotic stresses? These questions will be addressed below. Natural variation in host-plant resistance to thrips in Arabidopsis has not been studied before. A panel of 360 Arabidopsis accessions (referred to in this thesis as the Arabidopsis HapMap population, http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls) is a very powerful source of natural variation that can be fully explored at the genetic level with a set of 214K SNP markers. Genome-wide association (GWA) mapping is a relatively new approach that has great potential to study host-plant resistance to insects (**Chapter 2**). The mechanisms discovered with GWA are potentially helpful in gaining a more profound understanding of plant-insect biology. Degrees of resistance to insects are often reported in percentage of leaf consumption or survival rate of the insects. However, these end-point measurements might be a consequence of hundreds of genes interacting with each other, so unraveling novel resistance genes in plants might not point to clear quantitative trait loci (QTL) in population genetics studies like GWA mapping. This leads us to our second question, what can we learn about thrips in Arabidopsis/thrips interactions? The main focus in relation to question 2 is: what is harming thrips? What are effective strategies plants can adopt to rid themselves from thrips? How are thrips affected by these defensive strategies? This question is based in both fundamental and applied science. Thrips are pest insects and there is a high demand to know which plant defenses can counter thrips herbivory. A one-to-one translation of candidate genes discovered for resistance to thrips in Arabidopsis to other crops was not necessarily the aim here. *Brassica's* have unique defensive compounds like glucosinolates, that are not present in crops like pepper and chrysanthemum, for instance, where thrips are problematic (Lewis 1984, Gao, Lei et al. 2012). I therefore opted to dissect host-plant resistance to thrips in component traits based on detailed behavioral changes in the insect. To this end I have developed a novel high-throughput phenotyping platform to analyse thrips behavior automatically to discover changes in behavior that might be indicative of plant resistance mechanisms.

Novel two-choice arena setups for high-throughput screening of insect behavior

A key objective was to investigate how we can better phenotype thrips resistance in large plant panels. The results of this investigation can be found in **Chapter 3 and 4** of this thesis. These chapters describe the first attempts to create a high-throughput video tracking system. The following paragraph will address some additional prototypes that have been developed since then in more detail. Advances in genome sequencing in the last decades made it relatively easy and cheap to generate dense SNP panels for plant populations of interest. It is, however, the phenotyping that is often considered the real bottleneck, and this is especially true for phenotyping host-plant resistance to insects. Phenotyping host-plant resistance based on insect behavior, rather than plant performance has strong potential to unravel specific mechanisms that control host-plant resistance. The genetic factors in plants underlying specific behavioral alterations in insects are potentially less numerous and show stronger effects in QTL and GWAS mapping, than overall end-point resistance traits that entail a high level of complexity. For instance, stronger QTLs were observed for a detailed behavioral parameter in aphid feeding on Arabidopsis (time spent on making short probes) than in the population size of aphids after two weeks of infestation (Kloth 2016). In order to screen the 350 lines of the Arabidopsis HapMap population, we have developed a novel phenotyping platform that could monitor small changes in thrips behavior, in order to link these changes to allelic variants in the Arabidopsis population. Thrips are herbivorous piercing-sucking insects that live on liquids extracted from epidermal or parenchymal cells. Studying the feeding behavior of these tiny insects in more detail will lead to a better understanding of host-plant resistance mechanisms. The use of automated video tracking to phenotype plants for resistance to thrips has been extensively addressed in this thesis (**Chapter 3 and 4)**. A limiting factor with our first setup was the use of 96-well plates where every individual well functioned as a small arena for one two-choice experiment (Thoen, Kloth et al. 2016). This required cutting leaf discs in half, with much mechanical damage as a result, and no clear boundary zone between the two leaf-disc halves (two accessions). To overcome this problem, a number of prototypes for improved assay setups have been developed over the last years. For instance, an improved setup was created to screen thrips behavior in two-choice assays more accurately, allowing simultaneous recording of 60 parallel first choices and follow preference of insects during eight hour recordings (Text box 1, Figure 1a). This new set-up was tested with the two accessions Rmx-A180 and Cur-3, mentioned in **chapters 3 and 4** of this thesis. In this novel 60 two-choice arena platform, we observed similar results where thrips also spent more time on the susceptible Rmx-A180 accession (Nikolaidis 2013). In addition, this system was tested with the *myc 2 myc3 myc 4*

triple (*myc234*) knockout described in **chapter 6**. We found that besides the amount of feeding damage, the behavior of thrips was also different on this mutant that is almost completely devoid of glucosinolates (Schweizer et al. 2013), in comparison with Col-0 WT plants. Thrips preferred the *myc234* mutant, but this preference only became apparent after three hours (Thoen et al. unpublished data). Thus, the possible constitutively present defense differences did not influence thrips preference in this system. The new set-up was also used to screen for host-plant resistance to thrips in 70 chrysanthemum varieties (Nikolaidis 2013). The thick leaves and petals of many plants do not allow illumination from below, which was possible with Arabidopsis. To accommodate for this, we developed a container for the arena setup with strips of led lights on the side, that could illuminate the arenas from above and side to optimize the detection of the insects on thick leaf or flower material (Figure 1a). The thick leaves and petals of many plants do not allow illumination from below, which was possible with Arabidopsis. To accommodate for this, we developed a container for the arena setup with strips of led lights on the side, that could illuminate the arenas from above and side to optimize the detection of the insects on thick leaf or flower material (Figure 1a).

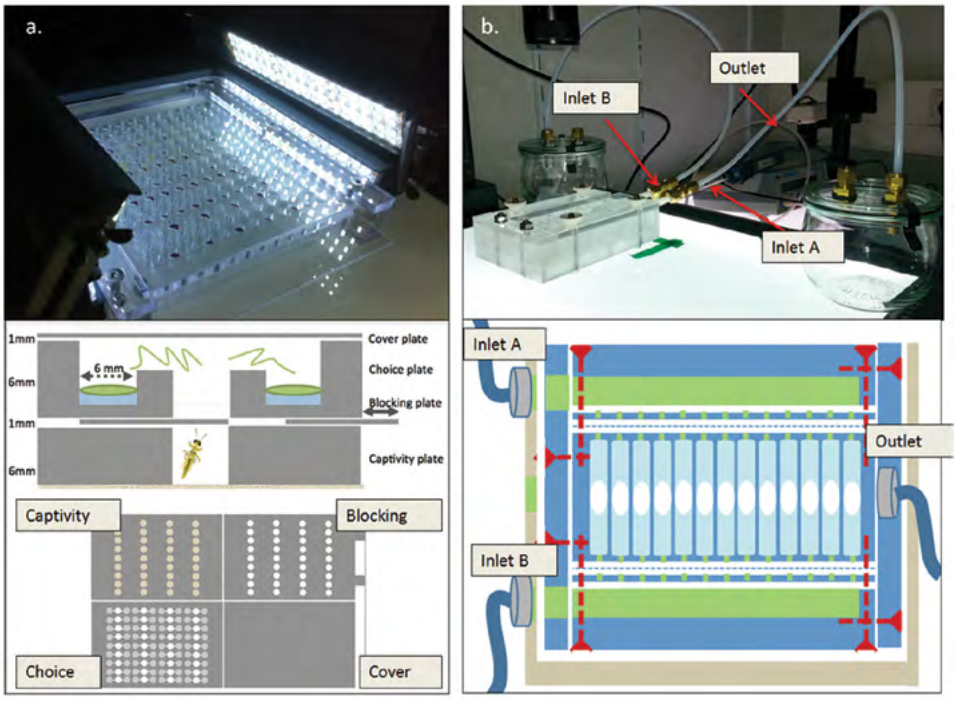

Figure 1. Novel protoypes to study thrips behavior in choice assays. a. High throughput 60 parallel two-choice platform with four separate layers and side lighting to illuminate thick plant material. **b.** Prototype parallel Y-tube assay that can monitor thrips behavior based on olfactory cues with two different odour sources.

Text box 1. Novel high-throughput platform with 60 parallel choice arenas

This setup (Figure 1a) consists of a plate containing 60 parallel mini-choice arenas, using four plates of 2-6 mm thick transparent polycarbonate containing machine milled holes and channels. Every test arena consists of one start zone (captivity plate), a blocking plate, and a test zone (choice plate), that consists of three chambers (2-choice chambers and the entry zone), topped off with a cover plate. The lower layer consists of 60 cells (diameter 6 mm) sealed with a thin mesh. In this captivity plate thrips can be introduced prior to the experiment (one thrips per arena). Thrips are kept in place with the blocking plate. They are able to enter the choice plate when the blocking plate is pushed aside. This choice plate resembles a Y-tube olfactometer experiment, where two different food/odor sources can be used. Thrips can access the two arenas by choosing one of two tunnels that connects the choice arenas to the central cell. Once the blocking plate is removed, all cells open and thrips can move freely between the two zones containing leaf material from the tested accessions, and the neutral zone that separates these arenas. The whole platform can be placed on a flat LED panel with a mounted camera to record the behavior of thrips immediately after the gatekeeper plate is opened.

The use of leaf discs to analyze insect behavior has some major downsides (ten Broeke 2013, Kloth, ten Broeke et al. 2015). The wound response elicited by generating these leaf discs starts a whole array of physiological processes that can result in the emission of volatiles and also otherwise alter the plant's defense and can therefore influence insect behavior. A leaf disc is essentially a dying plant part, and one should exert caution when extrapolating data based on bio-assays with leaf discs to the biology of whole plants that might be much better capable of adequately responding to herbivory. We have made several attempts to screen thrips behavior on intact plants, but the results were never satisfactory due to detection issues. However, we did create a prototype of a high throughput Y-tube olfactometer setup that can assess thrips preference for the headspace volatile blend of intact plants (Figure 1b). In this prototype pumps are used to distribute the headspace volatiles of two plants through twelve independent thrips choice arenas. We performed a pilot study in this parallel two-choice system testing thrips preference for several linalool dilutions in paraffin oil on 1 cm^2 filter paper sections. Linalool is a monoterpene present in many flowers, for which previous experiments in a Y-tube olfactometer demonstrated an attractive effect on thrips (Koschier, de Kogel et al. 2000). We observed a significant preference of thrips for the arm that was loaded with 1% linalool odor, versus the control (100% paraffin oil). Although the twelve arenas simultaneously might not be called 'high-throughput', there is great potential for improvement of this system that would enable screening of insect behavior based on odor cues in a high-throughput manner.

Natural variation in plant resistance to insect herbivory

Understanding of ecological systems is essential for the understanding of the adaptive trajectory of genes associated with variation in biotic interactions (Roux and Bergelson 2016). One of the main questions addressed in this thesis, was whether we can find variation in host-plant resistance/tolerance to insects in Arabidopsis, and what genes are underlying this trait. Before I discuss potential genes underlying host-plant resistance to thrips, it is interesting to look at resistance to thrips in the HapMap population at the accession level. Are there superior accessions that are resistant to many different biotic stresses, or does resistance to thrips come at the cost of susceptibility to other insect herbivores? The 308 accessions in the HapMap population from European origin have been investigated in closer detail for host-plant resistance to four insect species: thrips, the generalist aphid *Myzus persicae* and the *Brassica* specialist caterpillars *Pieris rapae* and *Plutella xylostella* (Davila Olivas, Frago et al. 2016). Ten geographically distinct genetic groups could be identified in this population of 308 European accessions based on a kinship matrix , but only thrips showed a difference in resistance among the genetic clusters. Resistance to aphids and caterpillars did not differ between these genetic clusters. When the accessions were then subdivide into summer and winter annuals significant differences were recorded for all the traits tested. The winter annuals appeared to be more resistant to aphids and thrips, whereas the summer annuals were more resistant to caterpillars. The correlation between different flowering strategies and opposing consequences for resistance against specialist caterpillars on the one hand and generalist piercing sucking insects on the other hand, supports a growing body of evidence where both specialization (Mathur, Ganta et al. 2011, Ali and Agrawal 2012) and insect feeding guild (De Vos, Van Oosten et al. 2005, Broekgaarden, Voorrips et al. 2011) may exert different selective pressure on plants. This does not imply that the insect species used in these studies have exerted these selective pressures, but perhaps some of the herbivores used in this study represent similar herbivores that co-exist with Arabidopsis in nature. Local adaptation within genetic clusters might thus represent a specific defensive strategy that happens to be effective against thrips, but not the other insects tested in this study. Local adaptation might consist of the evolution of tolerance, making plants better equipped to compensate for biomass loss due to herbivore damage. Alternatively, local adaptation can also evolve towards novel resistance mechanisms at the morphological or chemical level. Genome-Wide Association (GWA) mapping is a powerful tool to understand the genetic architecture behind this local adaptation to herbivores. In **Chapter 5**, I have studied the genetic architecture of plant-stress resistance with a multi-trait GWA approach. A genetic network in this study revealed little correlation between the different insect herbivores studied (aphids, whiteflies, thrips and caterpillars). For thrips resistance we observed a weak positive correlation with resistance to drought

stress and Botrytis, and a negative correlation with resistance to parasitic plants. One of the surprising outcomes of this study was the absence of shared major QTLs for host-plant resistance and abiotic stress tolerance mechanisms. Highly significant QTLs, such as those found in GWA studies for flowering-time (Atwell, Huang et al. 2010) and metabolism (**Chapter 6**), should however not be expected for complex multi-genic traits like resistance to insects for instance. There is a bias to focus on QTLs with a strong signal in quantitative genetics, even though theoretical work on adaptive walks through phenotypic optima predicts that the majority of phenotypic trait variation will be found in minor QTLs (explaining less than 20%), rather than major QTLs (Hermisson and Pennings 2005, Louthan and Kay 2011). *RESISTANCE METHYLATED GENE 1* (*RMG1*) is a nucleotide-binding site Leucine-rich repeat (NB-LRR) disease resistance protein recovered from our multi-trait GWA mapping (**Chapter 5**). It was a candidate gene from a minor QTL, but its potential relation to several resistance/tolerance traits were successfully demonstrated with T-DNA insertion lines. Many more candidate genes were discovered in this multi-trait GWA approach, showing that there is no silver bullet explaining 'host-plant resistance' in Arabidopsis. Undoubtedly, many of the candidate genes discovered in **Chapter 5** have a minor but relevant effect on many different stresses, even if they are on QTLs with small effect size.

Natural variation in the aliphatic glucosinolates

Metabolites are crucial in plant biology and there is substantial quantitative and qualitative variation in metabolite composition within plant species (Iason, Dicke et al. 2012). The most abundant and well-studied secondary metabolites in Arabidopsis are glucosinolates. The basic structure of all glucosinolates consists of three building blocks: a β-thioglucose moiety, a sulfonated oxime moiety and a variable side chain that can be derived from the amino acids tryptophan (forming indole glucosinolates), methionine (forming aliphatic glucosinolates) or phyenylalanine or tyrosine (forming aromatic glucosinolates). Previous work has demonstrated a role in host-plant resistance for glucosinolates towards many different biotic interactors with brassicaceous plant species, including mollusks (Falk, Kastner et al. 2014), pathogens (Bending and Lincoln 2000) and nematodes (Potter, Vanstone et al. 1999). However, most numerous are the studies that link glucosinolates to insect herbivory (as reviewed in Hopkins et at. (2009)). Intact glucosinolates may confer resistance to insect herbivory (Kim and Jander 2007), but the defensive properties of glucosinolates are greatly enhanced upon hydrolysis by the enzyme myrosinase. Tissue disruption by cell-destroying insects (like chewing insects, but also cell-content feeders like thrips) create contact between the glucosinolates (that are stored in vacuoles) and the myrosinase. Glucose and sulfate are released as a result of myrosinase activiy,

forming toxic and pungent isothiocyanates, nitriles and oxazolidinethiones in the process. Glucosinolates primarily function as herbivore deterrents, although some fascinating examples exist where specialized insect herbivores evolved to cope with the high levels of glucosinolates and even use them as feeding and oviposition stimulants (Marazzi and Stadler 2004, Wittstock, Agerbirk et al. 2004, Muller 2009). These adaptations include enzymatic detoxification, excretion and sequestration. For instance, larvae of the specialist *P. rapae* redirect the normal course of myrosinase activity with a nitrile specifier protein. When this gut protein comes in contact with intact glucosinolates, nitriles are formed, instead of the more toxic isothiocyanates (Wittstock, Agerbirk et al. 2004). Excretion of intact glucosinolates has been demonstrated in aphids, that do not activate the myrosinase due to their stealthy feeding mode in the phloem, that does not disrupt plants cells (Kim and Jander 2007). The sequestering of glucosinolates in insect herbivores has effects on higher trophic levels. Storing intact sequestered glucosinolates in their hemolymph, protects for instance the harlequin bug, *Murgantia histrionica* from being eaten by birds (Aliabadi, Renwick et al. 2002). Most generalist herbivores have not adapted to glucosinolates and, thus, glucosinolates form an effective line of defense to generalist insects. Previous work on the natural variation in glucosinolates in Arabidopsis demonstrated a non-random pattern of glucosinolate profile distribution in natural Arabidopsis populations (Burow, Halkier et al. 2010). This non-random variation could be caused by natural selection, or non-selective processes such as migration and population structure. An untargeted approach in which glucosinolate profiles are studied in conjunction with many different plant stresses could elucidate whether the observed diversity in glucosinolate profiles is due to fluctuating selection or neutral demographic processes (Züst, Heichinger et al. 2012). Diversity in glucosinolate profiles is proposed to be maintained under balancing selection, due to fluctuating fitness benefits that vary with environment and time (Burow, Atwell et al. 2015). This notion was initially supported in **Chapter 6** where I compared glucosinolate profiles of 349 *Arabidopsis thaliana* accessions with their resistance to a wide range of biotic and abiotic stresses. This study showed that, for instance, levels of progoitrin correlate positively with increased resistance to thrips, caterpillars, nematodes and aphids. However, a negative correlation was observed with resistance to the combinatory stresses drought/caterpillar, fungus/caterpillar and fungi. Progoitrin has also been reported to negatively influence the performance of the generalist caterpillar *Trichoplusia ni* (Hansen, Kerwin et al. 2008). Another compound that showed both negative and positive correlations with specific resistance traits was gluconapin. Gluconapin showed a positive correlation with drought and thrips resistance, but correlated negatively with heat tolerance, initial deterrence of thrips and resistance towards the combination of fungal and caterpillar stress. Glucosinolates also correlated strongly with heat stress tolerance. Recently it

was shown that a myrosinase (*TGG1, THIOGLUCOSIDE GLUCOHYDROLASE1*) catalysing the production of isothiocyanates is highly abundant in guard cells. *Tgg1* mutants are hyposensitive to abscisic acid (ABA) inhibition of guard cell inward K+ channels and stomatal opening, suggesting that the glucosinolate-myrosinase system is required for key ABA-regulated responses of guard cells. These are potential mechanisms whereby exposure to abiotic stresses may enhance plant defence against subsequent biotic stressors (Zhao, Zhang et al. 2008). Thus, in **chapter 6**, we found the levels of many glucosinolates correlating with one or more biotic or abiotic stresses, and in quite some cases we observed potential antagonistic effects, where the levels of specific glucosinolates correlate with resistance to one trait, but susceptibility to another trait. We also observed correlations with the geographic distribution and thrips resistance, and in line with that, correlations with thrips and many different climate variables (Figure 2). Glucosinolate profiles also strongly correlated with latitude, longitude and flowering regime. If geographic genetic clusters represent populations with a unique herbivore community, then this might explain why they correlate so strongly with thrips resistance. But why then do we not find any correlations with the other insect traits? Perhaps the lack of geographic and glucosinolate profile correlations with caterpillar resistance can be explained by the fact that *P. rapae* is a specialist insect that has evolved to cope with large amounts of glucosinolates. For aphids it has been postulated that aliphatic glucosinolates are not that relevant because they remain intact when aphids feed. Indole glucosinolates are more important defense metabolites against aphids, because these will be hydrolysed in the aphid gut (Kim and Jander, 2007). Thrips might thus be the generalist insect that is representative for generalist herbivores that are targeted by aliphatic glucosinolates in Arabidopsis. This might also explain why we have observed such high narrow-sense heritability values for thrips resistance (h^2 = 0.90), in comparison to the other insect traits (Davila Olivas, Frago et al. 2016). There are obvious constraints on correlative approaches as described in **chapter 6** just as in other studies that studied the correlation between glucosinolate profiles and resistance to herbivorous insect species (Züst, Heichinger et al. 2012) and their effects on higher trophic levels (Harvey, van Dam et al. 2011); we have to remain inconclusive about the exact role of specific glucosinolates in host-plant suitability. There are so many factors that are not accounted for due to the heterogeneous background of natural populations. Glucosinolate mutant lines, RNAi and virus-induced gene-silenced plants can offer interesting opportunities to advance our insights in the exact role of glucosinolates (Hopkins, van Dam et al. 2009). However, working with these single gene alterations has other caveats. The diversity in glucosinolates is controlled by many genes, and we do not yet have a good understanding of the extent to which individual genes impact fitness. Comparing a single-gene mutation to its wild-type does not properly reflect the natural variation enclosed in a population. To circumvent this problem, a very interesting approach

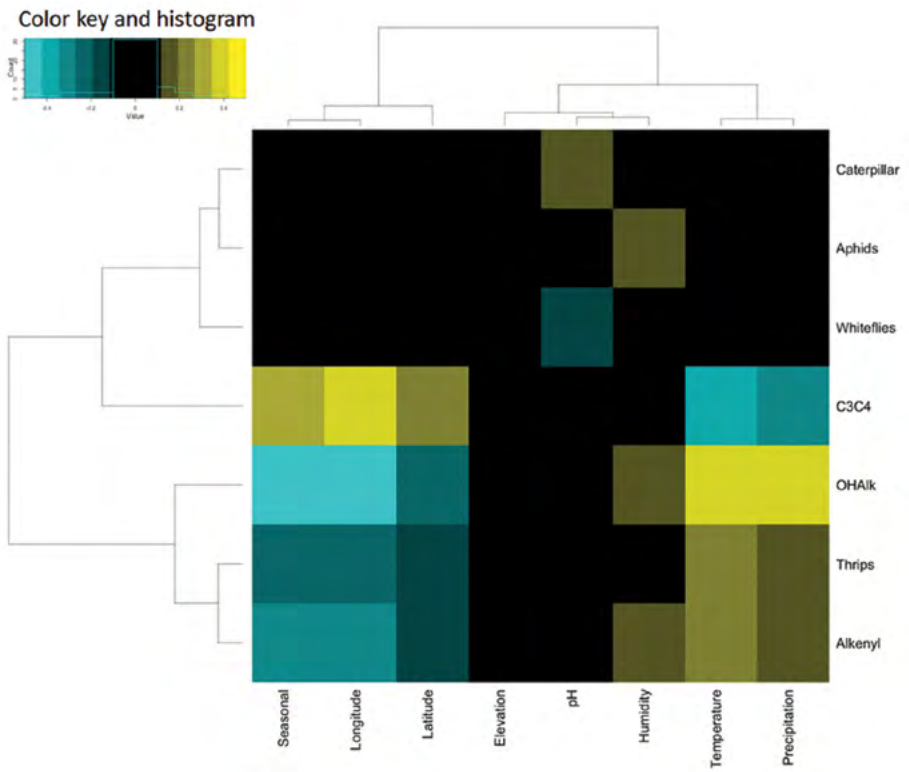

Figure 2. Spearman correlation heatmap of several phenotypes of the Arabidopsis HapMap population with climate data. Y-axis indicate insects studied in chapter 5 and aliphatic glucosinolate profiles based side-chain elongation (C3C4), levels of alkenyl glucosinolate (Alkenyl) and levels of hydroxy-alkenyls (OHAlk). Climate variables on the X-axis indicate fluctuations in seasonal temperature extremes (Seasonal), pH of the soil (pH), annual relative humidity (Humidity), annual mean temperature (Temperature) and annual precipitation (Precipitation).

was undertaken by Kerwin et al. (2015), who used transformation with glucosinolate biosynthesis genes in the Arabidopsis Col-0 background to recreate the natural variation in glucosinolate profiles observed in other Arabidopsis accessions. This synthetic recreation of natural variation allows testing for fitness benefits of specific allele combinations without confounding variation in other regions of the genome. In this study 17 different lines were used that mimicked natural variation in 8 different genes (*MYB28, MYB29, MAM1, GSOX1, GSOX3, AOP2, GSOH, ESP*). Although various combinations in allelic states of these genes could capture most of the naturally occurring chemotypes in Arabidopsis with just these 17 lines, there are in theory 256 allelic combinations possible for these 8 genes. The generation of these 256 lines will fully interrogate the effect of all 8 loci in all possible glucosinolate profile backgrounds. This will undoubtedly be a very powerful tool to study the effect of naturally occurring variation in glucosinolates, and its impact of plant fitness in laboratory and field conditions.

Thrips and terpenoids

Terpenoids represent the largest and most diverse class of metabolites produced by plants. The majority of terpenoids function as chemical protection against biotic and abiotic stresses (Tholl 2006). Although the bulk of the practical work in the past five years of my research consisted of hunting down candidate genes from the GWAS on thrips resistance, not a single candidate gene made it to a chapter of its own. The screening of 30+ knock-out mutants in these candidate genes did not yield conclusive results in most cases. However, there were some genes for which the knock-out mutant did show an interesting phenotype. One of our candidate genes was α-*BARBATENE SYNTHASE* (*At5G44630*). This gene is responsible for all group B sesquiterpenes produced in Arabidopsis flowers, from which α -barbatene, thujopsene and β-chamigrene are the most abundant (Tholl, Chen et al. 2005). Previous work has established natural variation in this gene in Arabidopsis accessions, where some accessions lacking a functional α-BARBATENE SYNTHASE were not able to produce any of the group B sesquiterpenes (Tholl, Chen et al. 2005). In our GWA on thrips resistance, the marker in this gene had a significance value lower than $P = 0.0001$ in all three screenings (end-point feeding assay, preference first hour and preference fourth hour). The knockout mutant of this gene showed an enhanced susceptibility to thrips (Figure 3a), and this results was confirmed in a subsequent screening of this mutant. α-*BARBATENE SYNTHASE* is mainly expressed in flowers, but some of the sesquiterpenes produced by the corresponding enzyme have also been found in the headspace of caterpillar-challenged non-flowering Arabidopsis accessions (Snoeren, Kappers et al. 2010). My hypothesis, therefore, was that there is natural variation in the expression of this gene in the vegetative stage upon herbivory and that this genetic variation must be present in in the promoter region of this gene. This could explain why we did not find any non-synonymous mutations in LD with the significant marker, within the α-BARBATENE SYNTHASE, but we did find three SNPs in LD in the promoter region. A closer look at the expression levels of this gene in 6 accessions clustered in two haploblocks that shared either the Col-0 haploblock, or the opposite haploblock, did however not reveal differences in expression upon thrips herbivory. In both haploblocks α-BARBATENE SYNTHASE was not expressed, also not after 24 hours of thrips herbivory. The creation of different 35S::α-BARBATENE SYNTHASE overexpression lines in a Col-0 background also did not yield further insights. None of the overexpressor lines differed in susceptibility in comparison to the Col-0 WT, although we did not perform chemical profiling on these plants to check whether they were actually producing sesquiterpenes. Using our novel phenotyping platform (Figure 1a) we screened the flowers of the α-barbatene synthase knock-out line (Figure 3b), and here we did observe again an initial preference for the WT over

the knock-out mutant (Figure 3c). It thus seems that the gene could be of relevance in the flowering stage of Arabidopsis for defense, but we could not come up with a satisfactory explanation on why this gene was then picked up in our GWA, that was done exclusively on leaf discs derived from plants in the vegetative stage.

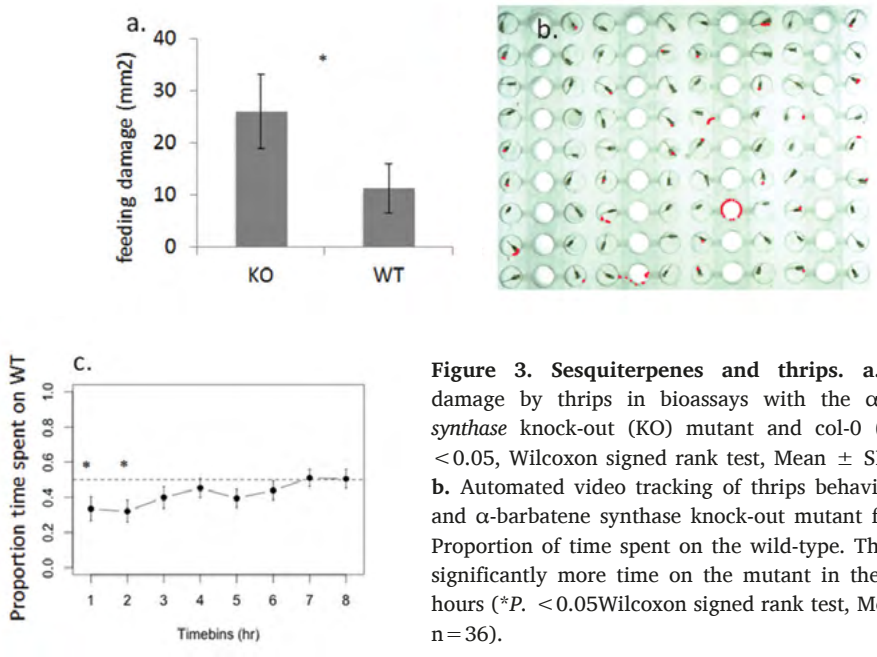

Figure 3. Sesquiterpenes and thrips. a. Feeding damage by thrips in bioassays with the α-*barbatene synthase* knock-out (KO) mutant and col-0 (WT) (**P.* <0.05, Wilcoxon signed rank test, Mean ± SE, n = 20). **b.** Automated video tracking of thrips behavior on WT and α-barbatene synthase knock-out mutant flowers. **c.** Proportion of time spent on the wild-type. Thrips spent significantly more time on the mutant in the first two hours (**P.* <0.05Wilcoxon signed rank test, Mean ± SE, n = 36).

Linalool is another terpenoid that might play a role in thrips resistance. This monoterpene alcohol is also mainly produced in flowers, but also occurs in Arabidopsis plants in the vegetative stage. With GC-MS I found that the resistant Cur-3 accession described in **chapters 3 and 4** had more linalool in its headspace than the susceptible Rmx-A180 (Thoen and Weldegergis, unpublished data). Furthermore, there was an increase in emission rate after 24 hours of thrips infestation and this was stronger in the headspace of Cur-3 plants. There were, however, many other compounds of which the emission also differed between these two lines, most notably 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), α-farnesene, (*Z*)- 3-hexen-1-yl acetate, (*Z*)-ocimene (all more abundant in the resistant Cur-3 accession) and allyl isothiocyanate (more abundant in the susceptible Rmx-A180) accession (Thoen, Weldegergis et al. 2014). In a two-choice assay using automated video tracking and the system described in Figure 1a, we also found a concentration-dependent attraction of thrips to filter paper with linalool (Nikolaidis 2013). The formation of linalool from geranyl diphosphate as a starting substrate is catalyzed

by the enzymes encoded by *TPS14* (producing *3S*-linalool) and *TPS10* (producing *3R*-linalool) in Arabidopsis (Ginglinger, Boachon et al. 2013). These genes were not recovered in GWA for thrips resistance, thus we did not follow up on a possible role of linalool as a herbivore repellent in Arabidopsis. These findings however, in addition to the fact that *TPS10* was a candidate gene in resistance toward the specialist caterpillar *Pieris rapae* (personal communication N.H. Davila Olivas), make this gene and its product, linalool, an interesting subject of future research. Recent work has revealed complex linalool metabolism in Arabidopsis flowers, but the ecological role of these compounds and the observed variation in their emission among plants from natural populations remains to be determined (Tholl, Kish et al. 2004, Ginglinger, Boachon et al. 2013). To further increase the complexness, glycosides of linalool present in transgenic linalool producing plants may be toxic to thrips, while the volatile linalool itself is a potent attractant (Yang, Stoopen et al. 2013). Therefore, there is an interesting potential ecological role for linalool in pollination and potentially a trade-off between plant reproductive strategies and defense mechanisms. Herbivore-induced plant resistance and allocation of resources by plants can potentially conflict with pollinator attraction (Lucas-Barbosa, van Loon et al. 2011, Lucas-Barbosa 2016). Perhaps linalool could have a dual function that circumvents this trade-off. Pollinators will be attracted to floral scents, but plants benefit from short visits, thus compounds that can both attract (initially) and deter (eventually) would theoretically optimize the number of pollination events. Future studies on insects that can function as both friend (pollinator) and enemy (herbivore) could shed more light on this potential role of linalool in ecological systems.

The role of generalist pathogens in maintaining ancient resistance polymorphisms in *Arabidopsis*

A central paradigm in evolutionary ecology of plants is the trade-off between defense and growth (Herms and Mattson 1992). Plants must grow fast to compete, yet maintain the defenses necessary to fend of pathogens and herbivores. But what are plants truly defending themselves against? In trying to explain some of the patterns observed in **chapters 5 and 6**, where the Arabidopsis HapMap population was investigated for the responses to many different biotic and abiotic stresses, it is interesting to try to view these results from an evolutionary perspective. Some accessions may have been resistant to stress 'x', because stress 'x' occurred at the location from which the accessions were isolated, thus eliciting selection pressure on these accessions to 'evolve' resistance mechanisms against stress 'x'. But, most of the

biotic stresses tested in these chapters are not naturally occurring on Arabidopsis. The seasonal mismatch between most insect species, and early flowering plants like Arabidopsis makes the existence of a co-evolutionary arms-race unlikely. So what biotic stresses could be considered crucial in shaping plant defense in Arabidopsis? Most accessions overwinter in the vegetative stage to flower in early spring. Even though herbivores like thrips, aphids and caterpillars are not frequently present in this stage, some herbivores can be found, most notably pulmonates (Harvey, Witjes et al. 2007), which is in line with a small field experiment I did on thrips resistant and susceptible accessions (Thoen and Meldau, unpublished data). I found that two accessions that are highly resistant to thrips in laboratory situations also received less damage in the field. Herbivores found in this field experiment were mostly slugs and flea beetles. Recent work claimed the important role aphids have in co-evolutionary adaptation shaping plant resistance mechanisms (Züst and Agrawal 2016, Züst and Agrawal 2016). The diversity of aliphatic glucosinolates that are discussed in **chapter 6**, have been ascribed to the presence of different aphid species populations (Züst, Heichinger et al. 2012). The side-chain elongation of glucosinolates differed in field experiments with Arabidopsis accessions between the two different aphid treatments. However, after six generations in the field, all alkenyl chemotypes were removed. These alkenyl chemotypes are very common in Arabidopsis, thus the maintenance, rather than the erosion of genetic variation in nature, is not reflected in this field experiment. To study the maintenance of natural variation in defense systems, we must look far beyond proclaimed 'co-evolutionary interactions' between two individual species. To make this point more clear, I'd like to discuss an example concerning *R*-gene evolution in Arabidopsis, and the role of generalist pathogens. *R*-genes are crucial in establishing the recognition of plant pathogen effector proteins and employing the defense arsenal that counters specific pathogens. Because these defenses are costly, individuals within a population lacking these *R*-genes can have a fitness benefit in enemy-free space. In the absence of an attacking pathogen *R*-genes need to be tightly controlled because, once activated, they disturb normal growth and fitness (García, Blanvillain-Baufumé et al. 2010). In nature, stable ancient resistance polymorphisms have been described in many plant species (Roux and Bergelson 2016). The maintenance of such polymorphisms indicates an evolutionary trade-off, where the persistence of polymorphisms renders fitness benefits or penalties depending on the time and environment. Tightly co-evolving host-pathogen interactions could explain the balance in polymorphisms, where the costs of plant defense can be detrimental to the plant in an ecosystem where the specialist pathogen is not present. However, in nature roughly half of all plant pathogens are considered generalists, associated with multiple host plants (Roux and Bergelson 2016). Upon intrusion of plant material, pathogens produce

effector proteins that down-regulate basal defenses. Plants counter pathogens by encoding *R*-gene products that recognize the action of these effectors to establish an adequate defense response. These responses include localized cell death (hypersensitive response) and a systemic production of chemical defenses. This arms race causes pathogens to evolve avoidance of detection, and plants to evolve improved detection (Karasov, Horton et al. 2014), also coined as the zig-zag model (Pritchard and Birch 2014). This model is well described in literature and often found in agricultural systems (mostly in the context of pathogens, although some examples have been described in plant-insect interactions (Stuart 2015)). However, natural ecosystems where longstanding ecological interactions involving hosts and large microbial communities are deemed to be too complex to be described by such a simple model (Karasov, Horton et al. 2014). An alternative way in which *R*-genes might have evolved could be by maintaining ancient polymorphisms through balancing selection, instead of sequential selective sweeps via an arms race. Recent work in the native flax (Thrall, Laine et al. 2012) and Arabidopsis (Karasov, Kniskern et al. 2014) on natural variation in the co-evolution of plant and pathogen populations do indicate that there is a surprising lack of 'classical' arms-race dynamics. Instead, what is observed is the cycling of ancient resistance and virulence polymorphisms, and the maintenance of these polymorphisms is the more correct model to describe the evolution of host-plant resistance. These polymorphisms are maintained through complex and diffuse community-wide interactions. Obligate pathogenic associations between host and pathogen species represent only a fraction of the diversity encountered in nature (Barrett, Kniskern et al. 2009). Host species that often occur in low densities, like Arabidopsis, are rarely attacked by specialist pathogens, yet ancient balanced *R*-gene polymorphisms are common in Arabidopsis (Bakker, Toomajian et al. 2006). Balancing selection maintains co-occurring *R*-gene alleles. Different adaptive processes can generate similar genetic signatures of balancing selection (Karasov, Horton et al. 2014). This polymorphism might be the product of heterozygote fitness advantage, frequency-dependent selection or environmental fluctuations in space and time favoring different resistance and virulence alleles. Community level dynamics must play a key role in explaining the ubiquity of virulence and resistance polymorphisms found in nature. Work on *R*-genes and pathogens in Arabidopsis teaches us that natural variation in defense can not be explained by one generalist pathogen. I believe there is an interesting lesson here to be learned for the field of insect-plant interactions. Whatever variation we find in host-plant resistance to a generalist insect, it is likely not one specific insect that is responsible for this variation. Instead, complex networks of diverse co-occurring plant species and herbivore species should be considered for a robust understanding of the evolutionary underpinning of natural variation in defense systems. Such studies are extremely difficult to accomplish

due to the complexity and labor intensity of insect bioassays. These multi-species interaction studies on generalist pathogens instead of insects seem more attainable in the near future, and would be a very promising endeavor to study plant resistance in a more natural evolutionary context.

Weak crops and super pests

Our continuous efforts over the past ten thousand years of plant breeding in agriculture have led to artificial selection pressures on cultivated crops. Current problems with pest insects in agriculture are two-sided: our crops have been severely weakened and our pests have been severely strengthened. In selecting for higher yield, plants have often unintentionally lost costly defensive mechanisms over the course of many crosses making new cultivars (Chen, Gols et al. 2015). Metabolites like for instance gluconapin have deleterious effects on insects, but will also make plants smaller (Burow, Atwell et al. 2015). The introduction of chemical pesticides in the 20th century made the remaining ancient resistance mechanisms redundant, weakening the genetic strength to fend off herbivores even more. The current state of our 'weak crops' is thus caused by unintentional selective breeding against defensive mechanisms, and this process has been amplified with pesticide use. Pesticide use did not only weaken our crops, it artificially selected 'super pests'. The (ab)use of pesticides in the last 50 years of agriculture led to extreme selection pressures on the herbivores that feed on these crops. This in combination with the common mono-culture in which our crops were cultivated, led to high fitness benefits for those few insects that had mutations that could render them resistant to pesticides. Insecticide resistance has been reported for many pest arthropods, including two-spotted spider mites (Gould, Carroll et al. 1982, Hasibuan, Brown et al. 1990), whiteflies (Wardlow, Budlam et al. 1976) and thrips (Gao, Lei et al. 2012). Adaptation in pest insects due to agricultural practices is not limited to pesticide resistance. An alternative agricultural approach such as rotation has also been countered by evolutionary adaptations in Northern corn rootworms that aligned their life cycle with its host by an extended diapause (French, Coates et al. 2014). So, how to battle these super pests, and how to deal with the 'weakness' of our crops? The answer could very well lie in natural variation of host-plant resistance as a centerpiece in Integrated Pest Management (IPM). Going back to natural populations of the crops we use today, can result in the discovery of defense strategies that have been lost through thousands of years of cultivation. The molecular basis of these strategies can be pinned down by recent developments in quantitative genomics, transcriptomics and metabolomics. Once the causal genes are found, breeding programs can be initiated to restore the ancient strength our crops once had in the

wild. Variation in resistance mechanisms is under balancing selection in nature, where pest insects are very rare. Variation in host-plant resistance in agricultural systems should thus also be achieved for sustainable agriculture. Bringing back ancient resistance mechanisms does not always mean that plants will have a lower consumer value. Some interesting examples exist, where metabolites that act as a line of defense against insect herbivory, have beneficial effects in human consumption. For instance, the glucosinolate glucoraphanin which occurs in high quantities in broccoli, and has anticarcinogenic properties in mammals (Matusheski, Swarup et al. 2006). In the years to come, improved multi-locus and multi-trait GWA models will undoubtedly increase the chances of unraveling the genetic architecture behind host-plant resistance to thrips. In this thesis Arabidopsis was used to study natural variation in plant resistance to insect herbivory. Although not a crop itself, it is closely related to several economically important Brassicaceae species, thus comparative genomics studies can be performed comparing crops like *Brassica rapa* with Arabidopsis (Zang, Kim et al. 2009). The availability of the complete genome of many different accessions, commercially available genome-wide microarrays and knock-out mutant lines, the many high-quality mapping populations and all the other tools and techniques that have been developed for this species, make it a promising plant to study any question that is related to natural variation. The work in this thesis presents a solid basis for further exploration in the genetic architecture underlying host-plant resistance to thrips in Arabidopsis. In addition to the phenotyping tools developed in this thesis (**Chapter 3 and 4**), several candidate genes and compounds (**Chapter 5 and 6**) have been brought forward. Unfortunately, most of the candidate genes that I have further investigated with T-DNA insertion lines did not yield conclusive results. In the cases where T-DNA knock-out mutant showed a significant increase in feeding damage by thrips, I was not able to repeat the outcome in subsequent trials. This could sometimes be explained by variation in the quality of our thrips breeding culture, but it also highlights the importance of robust and accurate phenotyping platforms. The improved hard-ware setup described in this chapter, and the software package described in **Chapter 4** were not yet developed in the initial stage of testing these T-DNA insertion lines. Future efforts in studying the shared genetic signals of defensive traits in Arabidopsis, could include additional organisms that might represent the natural selective pressures that Arabidopsis has endured over the past millions of years even better. To achieve this, it is essential to obtain more information regarding the distribution of biotic interactors of Arabidopsis through its native range. These data are still lacking for the most part (Gloss, Nelson Dittrich et al. 2013). Pulmonates, fungi and microbial pathogens are very interesting candidate organisms to study plant defenses in an ecological context in Arabidopsis, and it would be very interesting to see how the

results found in this thesis are in line with resistance mechanisms active against other organisms that occur naturally in the native range of Arabidopsis. With the ascend of the -omics era in plant breeding, questions on host-plant resistance to thrips might be directly addressed to the variation in crops of interest. Pepper has already been resequenced (Qin, Yu et al. 2014), and it may take only a few more years before also polyploid cut-flowers like Chrysanthemum have their genome sequenced (Zhang, Wang et al. 2013). However, Arabidopsis will remain an essential species in functionally characterizing resistance genes to thrips. Even when entire crop genomes are available in other crops and GWA mapping can be performed to study host-plant resistance to thrips, Arabidopsis will still play a crucial role in the follow-up experiments. Finding candidate genes is easy, confirming their function as resistance genes is the real challenge scientist face. Disentangling correlative patterns from causal allele alterations is essential in genomic studies. These essential validation steps are too often omitted, and they will be a lot harder to perform in plant species other than Arabidopsis due to the lack of genomic tools like the extensive T-DNA library, although the CRISPR-Cas9 technology may provide important novel developments for research on genetic mechanisms in non-model plants (Hsu, Lander et al. 2014). The work presented in this thesis paves the way for these essential confirmation steps. The phenotyping platform to accurately screen large panels of plants is there, several lists of promising candidate genes have been presented and metabolomics aided in the discovery of several promising secondary metabolites that influence host-plant resistance to insects. Validating these genes and compounds can eventually aid marker-assisted breeding to improve host-plant resistance in crops. Using these tools we can and will restore the ancient strengths of wild species into our crops via a profound understanding of insect-plant biology integrated at the molecular, chemical and ecological level.

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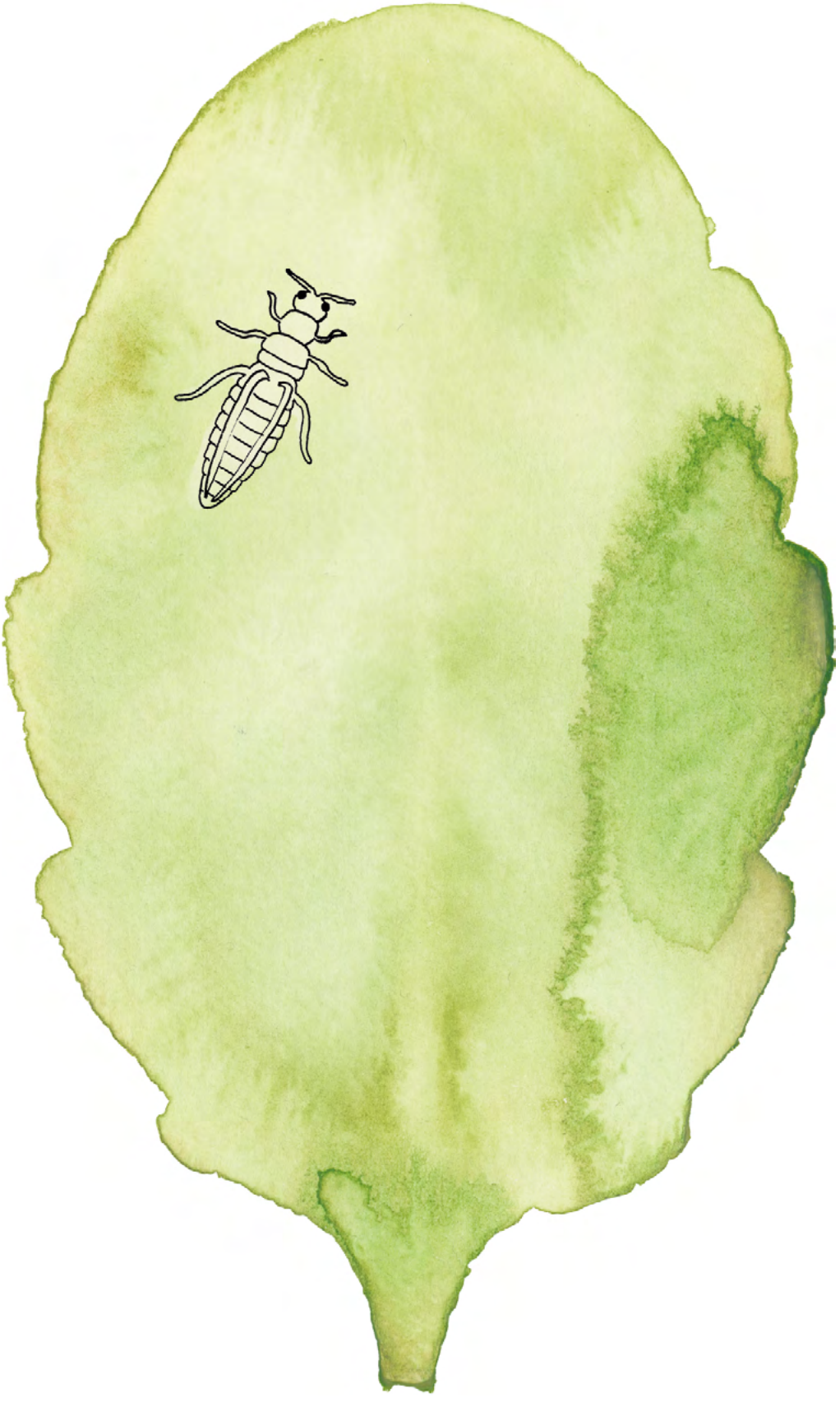

Summary

Summary

Western flower thrips is a pest on a large variety of vegetable, fruit and ornamental crops. The extensive damage these minute slender insects cause in agriculture through feeding, oviposition and the transmission of tospoviruses requires a sustainable solution. Due to their small size, rapid reproduction and resistance to a number of pesticides, this pest insect is difficult to manage. Host-plant resistance is a cornerstone of Integrated Pest Management (IPM). Plants have many natural defense compounds and morphological features that aid in the protection against herbivorous insects. The natural variation in these defensive traits has been a source of inspiration for plant biologists. Understanding the molecular architecture behind host-plant resistance can pave the way for breeding programs of thrips-resistant cultivars. However, the molecular and physiological aspects that control host-plant resistance to thrips are largely unknown.

A novel and powerful tool to study host-plant resistance to insects in natural populations is genome-wide association (GWA) mapping. In the literature review in **Chapter 2,** the unique opportunities of GWA mapping are discussed in the light of discovering genes that control host-plant resistance to herbivorous insects. GWA mapping provides a comprehensive untargeted approach to explore the whole array of plant defense mechanisms. It utilizes natural variation that resides in large plant populations to perform statistical on single nucleotide polymorphisms (SNPs). Increased mapping resolution, reduced research time, and larger allele numbers have been put forward as main advantages over traditional pedigree-based quantitative trait loci (QTL) mapping. Successful GWA mapping requires a high-resolution marker map for a large collection of homozygous plants that encompass relevant natural variation in the plant species of interest. The development of high-throughput phenotyping (HTP) systems is a necessity when large plant panels need to be screened for host-plant resistance to insects. Host-plant resistance to insects is often controlled by many genes that each have a minor effect on the phenotype. It could thus be relevant to dissect host-plant resistance summary traits such as feeding damage, into several component traits extracted from the detailed insect behavior on plants.

An automated video-tracking platform that could screen large plant panels for host-plant resistance to thrips, and dissect host-plant resistance to thrips in component traits related to thrips behavior, was developed (**Chapter 3**). This phenotyping platform allows the screening for host-plant resistance against thrips in a parallel two-choice setup using EthoVision tracking software. The platform was used to establish host-plant preference of thrips with a large plant population of 345 wild Arabidopsis accessions (the Arabidopsis HapMap population) and the method

was optimized with two extreme accessions from this population that differed in resistance to thrips. The behavior of 88 thrips individuals was simultaneously tracked in 88 parallel two-choice arenas during 8 hours. Host-plant preference of thrips was established both by the time thrips spent on either accession and various behavioral parameters related to movement (searching) and non-movement (feeding) events. In comparison to 6-day end-point choice assays with whole plants or detached leaves, the automated video-tracking choice assay developed here delivered similar results, but with higher time- and resource efficiency. This method can, therefore, be a reliable and effective high throughput phenotyping tool to assess host-plant resistance to thrips in large plant populations.

The EthoVision software used in chapter 3 was followed by a manual analysis of the recorded behavior. In **chapter 4** the same video-tracking data obtained with EthoVision software was re-analyzed, now with a novel software package, EthoAnalysis, that allows for automated extraction of more detailed behavioral parameters from the raw tracking data, and automated statistical analysis. Through several parameter iterations optimized settings for 54 variables were generated that described different behavioral characteristics in time, frequency, duration, distance and speed. There were several benefits from using EthoAnalysis to analyze EthoVision data. First of all, the analysis was performed a lot quicker in EthoAnalysis. Secondly, the statistical report that was produced by EthoAnalysis provided a quick overview of relevant differences between two or more tested accessions. Filters automatically removing entire records or events with poor data can be applied in EthoAnalysis, so that more reliable records remain. In comparison, EthoAnalysis can also redefine input thresholds without the need to re-run the entire recording, as with EthoVision. The detailed event statistics that could be extracted from EthoAnalysis allows researchers to distinguish detailed differences in moving and feeding behavior of thrips. The potential of this additional information is discussed in the light of quantitative genetic studies.

Chapter 5 explores stress resistance in the HapMap population on a much broader scale, including a total of 15 different biotic and abiotic stresses ranging from biotic stresses like insects and nematodes, to abiotic stresses like drought and salt. The Arabidopsis HapMap population has been genotyped for 214.000 SNPs. A multi-trait GWA study to unravel the genetic architecture underlying plant responses to the different stresses was performed. A genetic network in this study revealed little correlation between the plant responses to the different insect herbivores studied (aphids, whiteflies, thrips and caterpillars). For thrips resistance a weak positive correlation with resistance to drought stress and Botrytis, and a negative correlation with resistance to parasitic plants was observed. One of the surprising outcomes of this study was the absence of shared major QTLs for host-plant resistance and abiotic

stress tolerance mechanisms. *RESISTANCE METHYLATED GENE 1* (*RMG1*) was one of the candidate genes in this multi-trait GWA study that could be controlling shared resistance mechanisms against many different stresses in Arabidopsis. *RMG1* is a nucleotide-binding site Leucine-rich repeat (NB-LRR) disease resistance protein and its potential relation to several resistance/tolerance traits was successfully demonstrated with T-DNA insertion lines. Many of the candidate genes discovered in **Chapter 5** could have minor but relevant effects on many different stresses, even if they were on QTLs with small effect size.

Glucosinolates are secondary metabolites in Arabidopsis that affect insect-plant interactions. **Chapter 6** uses the same 15 stresses from chapter 5 in a comparison with a metabolomics dataset on this Arabidopsis HapMap population. It was discovered that levels of certain aliphatic glucosinolates correlated positively with the levels of resistance to thrips. This correlation was further investigated with the screening of a RIL (Recombinant Inbred Line) population for resistance to thrips, several knockout mutants and the analysis of co-localization of GWA mapping results between glucosinolates genes and thrips resistance. In a GWA analysis, the C4 alkenyl glucosinolates that correlated the strongest with thrips resistance mapped to the genomic regions containing genes known to regulate the biosynthesis of these compounds (*MAM, AOP, GS-OH*). However, thrips resistance did not co-localize with any of the GSL genes, unless a correction for population stratification was omitted. Additional screening of a Cvi x L*er* RIL population showed a QTL for thrips resistance on chromosome 2, but no co-localisation with any known GSL genes, nor with thrips resistance loci identified by GWA mapping. Knock-out mutants and overexpressors of *MAM* and *AOP* glucosinolate synthesis genes could also not confirm a causal link between glucosinolates and resistance to thrips. It is possible that the crucial factors that control resistance to thrips may not have been present in sufficient quantities or in the right combinations in the mutants, RILs and NIL screened in this study. Alternatively, the correlation between thrips feeding damage and glucosinolate profiles could be based on independent geographical clines. More research should be conducted to assess which of these explanations is correct.

In **Chapter 7**, the general discussion, the results from this thesis are discussed in a broader perspective. Some prototypes of new phenotyping platforms that could further aid screening for resistance to thrips in the future are presented. Natural variation in host-plant resistance to thrips is compared to the variation in host-plant resistance to aphids and caterpillars. The geographic distribution of host-plant resistance to thrips is not evident in the other insects, in line with the distribution of glucosinolate profiles and other climate factors. In addition to glucosinolates, terpenoids are given special attention in this chapter. The chapter concludes with some suggestions for future research in the field of host-plant resistance to thrips.

Acknowledgments

The most valuable outcome of a PhD project is not the book that describes the scientific results, but the personal journey one experienced when completing a PhD. My journey was filled with luminous brilliant minds, lively scientific debates, numerous failed experiments and many pieces of pie during coffee breaks. For most of these things, I am very grateful. Here, I would like to thank those that have contributed in the completion of this thesis, but even more so in the formation of the person I am five years after starting this challenging journey. Please accept my sincere gratitude and apologies to anyone I omitted to mention here.

First of my supervisor Maarten Jongsma. Although we may have disagreed on many things, I have always considered you to be a very inspiring tutor. Thank you for your critical feedback. You demanded clarification on blurry passages of manuscripts that seemed very readable to me. You demanded improvements of experimental set-ups that seemed perfectly fine with me. And yet, in retrospect I (almost) always had to admit that your suggestions were great improvements. My apologies for my occasional stubbornness, and thanks for all your patience with me.

My promotors Harro Bouwmeester and Marcel Dicke. I honestly can not phantom how you are running your lives, spending so much time and devotion to your group and students. In the writing process, you were never the bottleneck. I've learned so much from you both, it was a true honour to be supervised by you.

Gerrie Wiegers, for all your practical help during these years. Rearing thrips, taking care of plants, conducting insect bio-assays and so much more. You were an extremely pleasant person to work with, you deserve a golden statue! It was fantastic to have such a skilled, kind and knowledgeable technician in our team.

Talking of deserving golden statues, thank you Jacqueline Busscher! You did so much molecular work for this project, it's a pity that the majority of it is not included in this thesis. Your positive attitude and your hard work were a blessing, may the Penguin spirit be forever with you!

To complete the EthoGenomics team; Karen Kloth! 'I could not have done it without you' is such an awful cliché, but very true I'm afraid. We started this project five years ago, you focussing on aphids while I focused on thrips. Your discipline, devotion, programming skills and scientific mind-set were essential in my understanding of what a great scientist is all about. I'm sure you will win a Nobel prize one day. Thank you for everything!

A big cheers to Nelson for all the fancy R coding you thought me, the inspiring discussions we had and the great time we had together in our GWAS course in Barcelona and on other occasions.

From the Biometris group I would like to thank Joost van Heerwaarden and Fred van Eeuwijk for their work in the LfN consortium. A special mention for Willem Kruijer, for the many R-scripts you have generated, and the time you invested in the program and me.

Ric de Vos and Henriëtte van Eekelen, your input and knowledge of analytical chemistry was essential for Chapter 6 of this thesis. In collaboration with Joost Keurentjes and Rik Kooke, we have tried to uncover the role of glucosinolate diversity and its potential in plant resistance to thrips. Thank you so much for sharing your time and knowledge.

My dear paranymphs. Dani, I consider you a brilliant friend and tutor. It was a great experience organizing the insect-plant workshop together. Thanks for all your scientific input on a large variety of topics, you are truly a unique, wonderful and inspiring scientist and person, may the force be with you! Jenny, thank you for being so smart, funny and awesome! We have spent much time discussing science and other topics over the past five years. I hope we can work together in the future, although our paths may go very different directions.

All the wonderful people from the Entomology and Plant Physiology department that made this PhD project a very social event. I can't mention you all in person, but thanks so much for all the nice conversations we had over coffee, lunch and Friday afternoon beers. Some of you were the funniest, geekiest, loveliest and most wonderful human beings I have ever encountered in my life. I will never forget you, and I wish you all tremendous amounts of awesomeness in your futures. A special mention for the secretaries Angelique (Entomology) and Rina (Plant Physiology). You are both super kind, and extremely helpful. Your efforts are too often unmentioned in PhD theses acknowledgements.

On a more personal note I would like to thank all my friends and family that supported me over the last five years. My parents, whose support was essential for my academic training. My friends, whose support was essential for my enjoyment of the good life. In some ways it felt like a rather selfish life, using my PhD as an excuse to forget birthdays, cancel nights-out last-minute and just not finding the time to do those things in life that really matter. Please accept my apologies for this, and thank you so much for believing in me. A special cheers to my brother Stef. If it weren't for you, I would never have entered University. You always make me challenge myself in a good way, you are one of the biggest sources of inspiration I have in life.

At last, my dear Suzi. I have learned much from these five years about science and life. You, however, taught me the most essential lesson:

Nothing in life makes sense, except in the light of love.

Manus

Curriculum vitae

Hermanus Paulus Marinus Thoen was born on the 24th of June 1984 in Monster, the Netherlands. His main interests in life are music, sports and nature. As a teenager he was working several years at the outdoor event company of his father. This experience taught him that he loved to entertain large crowds, so he decided to pursue a teaching profession after high school. Upon obtaining his bachelor degree as a biology teacher, he followed his interest in evolutionary biology at Wageningen University. The work he conducted in an MSc thesis on heterokaryon incompatibility in filamentous fungi (Laboratory of Genetics, supervised by Fons Debets) and an MSc thesis on the physiological aspect of insect resistance towards plant defences (Laboratory of Entomology, supervised by Peter de Jong and Manabu Kamimura, Tsubkuba, Japan) were vital in his motivation to pursue a career in science. In 2011, he started as a PhD candidate at Wageningen University, in the Laboratories of Entomology and Plant Physiology. The fruits of this project are presented in this thesis.

List of publications

Karen J. Kloth*, **Manus P.M. Thoen*,** Harro J. Bouwmeester, Maarten A. Jongsma and Marcel Dicke (2012) Association mapping of plant resistance to insects. Trends in Plant Science, 17: 311-319.

Manus Thoen (2014) Rise and fall of the Thunderflies. Proceedings of the Netherlands Entmological Society meeting 25: 23-32.

Ting Yang, **Manus Thoen**, Geert Stoopen, Gerrie Wiegers, Maarten A. Jongsma (2013) Chrysanthemum expressing a linalool synthase gene smells good, but tastes bad to western flower thrips. Plant Biotechnology Journal, 11:7

Karen J. Kloth, Cindy J.M. ten Broeke, **Manus P.M. Thoen**, Marianne Hanhart-van den Brink1, G.L. Wiegers, Olga E. Krips, Lucas P.J.J. Noldus, Marcel Dicke and Maarten A. Jongsma (2015). High-throughput phenotyping of plant resistance to aphids by automated video tracking. Plant Methods, 11:4.

Manus P.M. Thoen, Karen J. Kloth, Gerrie L. Wiegers, Olga E. Krips, Lucas P.J.J. Noldus, Marcel Dicke and Maarten A. Jongsma (2015). Automated video tracking of thrips behavior to assess host-plant resistance in multiple parallel two-choice setups. Plant Methods, 12:1.

Manus P.M. Thoen*, Nelson H. Davila Olivas*, Karen J. Kloth*, Silvia Coolen*, Ping-Ping Huang*, Mark G.M. Aarts, Johanna A. Bac-Molenaar, Jaap Bakker, Harro J. Bouwmeester, Colette Broekgaarden, Johan Bucher, Jacqueline Busscher-Lange, Xi Cheng, Emilie F. Fradin, Maarten A. Jongsma, Magdalena M. Julkowska, Joost J.B. Keurentjes, Wilco Ligterink, Corné M.J. Pieterse, Carolien Ruyter-Spira, Geert Smant, Christa Testerink, Björn Usadel, Joop. J.A. van Loon, Johan A. van Pelt, Casper C. van Schaik, Saskia C.M. van Wees, Richard G.F. Visser, Roeland Voorrips, Ben Vosman, Dick Vreugdenhil, Sonja Warmerdam, Gerrie L. Wiegers, Joost van Heerwaarden, Willem Kruijer, Fred A. van Eeuwijk & Marcel Dicke (2016). Genetic architecture underlying plant stress resistance: a multi-trait genome-wide association mapping approach. (*under review*)

Nelson H. Davila Olivas, Enric Frago, **Manus P.M. Thoen**, Karen J. Kloth, J. van Heerwaarden, Joop J.A. van Loon, Gerrit Gort, Marcel Dicke (2016) Natural variation in stress responses to insects of different guilds and drought differs between life history strategies in *A. thaliana.* (*under review*)

**These authors contributed equally to the manuscript.*

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Manus Thoen
Date: 29 August 2016
Group: Laboratory of Entomology
University: Wageningen University & Research Centre

1) Start-up phase	date
▶ **First presentation of your project**	
EthoGenomics, resistance in *Arabidopsis thaliana* against Myzus persicae and *Frankliniella occidentalis*	Jun 01, 2011
▶ **Writing or rewriting a project proposal**	
Hotel Grant 'QTL mapping in *Arabidopsis thaliana*, to reveal loci involved in host-plant resistance towards thrips'	Sep 2013
▶ **Writing a review or book chapter**	
Review; 'Association mapping of plant resistance to insects', Trends in Plant Science, May 2012, Vol. 17, No.5, p 311. DOI: 10.1016/j.tplants.2012.01.002	2011
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	8.5 credits*

2) Scientific Exposure	date
▶ **EPS PhD student days**	
EPS PhD student day 2011, Wageningen University	May 20, 2011
EPS PhD student day 2012, University of Amsterdam	Nov 30, 2012
EPS PhD student day 2013, Leiden University	Nov 29, 2013
▶ **EPS theme symposia**	
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Wageningen University	Feb 10, 2012
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, University of Amsterdam	Feb 25, 2014
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Utrecht University	Feb 20, 2015
▶ **Lunteren days and other National Platforms**	
NERN 2012	Feb 07-08, 2012
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 22-23, 2013
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 14-15, 2014
▶ **Seminars (series), workshops and symposia**	
Invited seminar: High throughput plant phenotyping (HTPP), a rapidly growing activity, Wageningen	May 10, 2011

EcoGenomics day, Amsterdam	Jun 16, 2011
6th workshop Plant-Insect Interactions (Amsterdam)	Nov 23, 2011
EPS Symposium " Plant Breeding in Genomic Era', Wageningen	Nov 25, 2011
LfN consortium meeting	Oct 27-28, 2012
7th workshop Plant-Insect Interactions (Leiden)	Nov 28, 2012
EPS Symposium 'From Model System to Ecology and Evolution'	Aug 29, 2013
8th workshop Plant-Insect Interactions (Wageningen)	Sep 24, 2013
EcoGenomics day, Leiden	2013
WEES lecture series, consisting of invited seminars and master clasess	2011 - 2015
▶ **Seminar plus**	
WEES Master Class with: Nicole van Dam, Joy Bergelson, Koos Biesmeijer, Frederic Tripet, Jacintha Ellers and more	2011-2015
▶ **International symposia and congresses**	
SIP-14, Wageningen, NL	Aug 13-17, 2011
Cordon Conferance 'Plant-herbivore Interactions', Ventura (USA)	Febr 24-Mar 01, 2013
SIP-15, Neuchatel, Schwitzerland	Aug 17-22, 2014
▶ **Presentations**	
'EthoGenomics: Identifying novel resistance genes in *Arabidopsis thaliana* against Myzus persicae and *Frankliniella occidentalis* by genome-wide association mapping, SIP congress, Wageningen, NL (Poster)	Aug 13-17, 2011
High-throughput phenotyping for genomics, Summer School Natural Variation of Plants, Wageningen, NL (Poster)	Aug 21-24, 2012
High-troughput phenotyping of thrips resistance in *Arabidopsis thaliana*. A genome-wide association study, 7th Plant-Insect Workshop, Leiden, NL (Talk)	Nov 28, 2012
European Ecology and Functional Genomics Symposium (EEFG), Noordwijkerhout, NL (Poster)	Jun 2013
How can we put complex traits on a genetic map? Genome-wide association studies (GWAS) with *Arabidopsis thaliana*, Leiden, NL (Invited talk)	Sep 26, 2013
"Genome Wide Association Mapping for thrips resistance in Arabidopsis", EPS, Lunteren, NL (Talk)	Apr 22-23, 2013
"Rise and Fall of the Thunderflies", NEV Entomologendag, Ede, NL (Talk)	Dec 13, 2013
"The ecological (ir)relevance of Arabidopsis-insect studies", 6th Ecogenomics day, Utrecht, NL (Talk)	Jun 24, 2014
▶ **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
▶ **Excursions**	
PhD labtrip Entomology (Swiss)	Nov 28-Dec 01, 2014
CBSG Matchmaking Event	Oct 18, 2012
Subtotal Scientific Exposure	22.7 credits*

3) In-Depth Studies	date
▶ **EPS courses or other PhD courses**	
6th PhD summerschool 'Environmental signaling', Utrecht, NL	Aug 22-24, 2011
PhD course 'Bioinformatics - a user's approach', 29 August-02 September 2011, Wageningen, NL	Aug 29-Sep 02, 2011
Summerschool 'Natural variation', Wageningen, NL	Aug 21-24, 2012
Course: 'Genomic Data Analysis using HapMap and 1000 Genomes Projects', Barcelona, Spain	Jan 20-24, 2014
▶ **Journal club**	
PhD Entomology discussions	2011-2015
Insect-plant interaction discussions	2011-2015
PPH literature discussions	2013-2014
▶ **Individual research training**	
Noldus course: Observer	Jun 20, 2011
Noldus course: EhtoVision	Jun 22, 2011
Subtotal In-Depth Studies	8.7 credits*

4) Personal development	date
▶ **Skill training courses**	
Competence Assessment	Oct 25, 2011
Statistics Mixed Models	Jun 21 & 22, 2012
Introduction to R for statistical analyses (Wageningen)	Oct 22-23, 2012
PhD workshop carousel	Jun 02, 2014
Scientific writing	Oct 2014
Last stretch of PhD	Dec 19, 2014
▶ **Organisation of PhD students day, course or conference**	
Organisation of 8th Insect-Plant Workshop, Wageningen	Sep 24, 2013
▶ **Membership of Board, Committee or PhD council**	
▶ **Board member of WEES**	Feb 2012-Dec 2014
Subtotal Personal Development	6.5 credits*

TOTAL NUMBER OF CREDIT POINTS* **46.4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

Learning from Nature to protect crops

Plants are under the constant threat of biotic and abiotic stresses. Yet, devastating pests and diseases only rarely occur in nature and plants have managed to sustain for millions of years in this hostile environment. This is due to and has resulted in a tremendous degree of natural variation in mechanisms that plants exploit to defend themselves against pathogens and insects and to deal with abiotic stresses. In agriculture, however, we have exploited only very little of this diversity of defenses and as a consequence environment-malignant pesticides remain a dominant method to control pests and diseases. The current threat of climatic changes and limiting resources for agriculture (water, fertilizer) require improved resistance to abiotic stresses.

Ambition and goal: With this multidisciplinary and innovative STW programme we want to mine the natural reservoir of plant defense mechanisms. This will be done by using state-of-the-art high-throughput technologies to explore the natural potential and exploit mechanisms, genes and markers to develop novel resistance mechanisms against biotic and abiotic stresses for plant breeding.

In nature plants have co-evolved with a large variety of attackers. Therefore, wild species, such as *Arabidopsis thaliana*, harbour a fantastic reservoir of natural adaptive mechanisms to respond to (a)biotic stresses that to date have not been systematically explored. In the past decade, Arabidopsis has been adopted world-wide as the ideal model for plant science and an impressive molecular genetic toolbox has since been developed (e.g. the full genome sequence, the availability of well-characterized Arabidopsis populations, full-genome microarrays and metabolomics protocols). Hence, exploring natural variation in the defense responses of Arabidopsis to a large variety of (a)biotic stresses will yield important new insights into how plants selectively adapt to stresses, and provide novel concepts for sustainable agriculture and resistance breeding.

Objectives

1. To explore natural varation in resistance to abiotic and biotic stresses in Arabidopsis populations through an integrated multidisciplinary approach.
2. To identify mechanisms underlying natural resistance to abiotic and biotic stresses in Arabidopsis
3. To develop methods to analyze complex datasets on different types of resistance
4. To exploit information gained on natural variation in Arabidopsis to identify molecular markers that can assist in breeding for resistance to abiotic and biotic stresses in crop plants.

Focus and results at the end of the programme: To this end Arabidopsis ecotype and RIL populations can be exploited to analyze the degree of resistance to a diversity of microbial pathogens, herbivorous insects and abiotic stresses and their interaction. Using large-scale bioinformatics this information can be integrated with transcriptomics and metabolomics, to select genotypes and lines that can be used for in-depth analysis of the resistance mechanisms. The information gained from this comprehensive approach will lead to the identification of genes and molecular markers for different resistance mechanisms. These mechanisms will be characterized at the molecular, biochemical and physiological level and can subsequently be used to screen large numbers of lines of various crop species for orthologous genes involved in similar resistance mechanisms.

Innovation: Never before has the natural variation in plant defenses against different biotic and abiotic stresses and their interaction been investigated in such a comprehensive, multidisciplinary programme. To date, solutions to individual (a)biotic stresses have been sought. However, this has not resulted in a systems approach that results in durable solutions for a range of stresses.

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