Whitefly resistance in tomato: from accessions to mechanisms

Alejandro Francisco Lucatti

Thesis committee

Promotor
Prof. Dr R.G.F. Visser
Professor of Plant Breeding
Wageningen University

Co-promotors
Dr B.J. Vosman
Scientist, Wageningen UR Plant Breeding
Wageningen University and Research Centre

Dr A.W. van Heusden
Scientist, Wageningen UR Plant Breeding
Wageningen University and Research Centre

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Dr W. J. de Kogel, Plant Research International, Wageningen University and Research Centre

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Thesis

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Alejandro Francisco Lucatti

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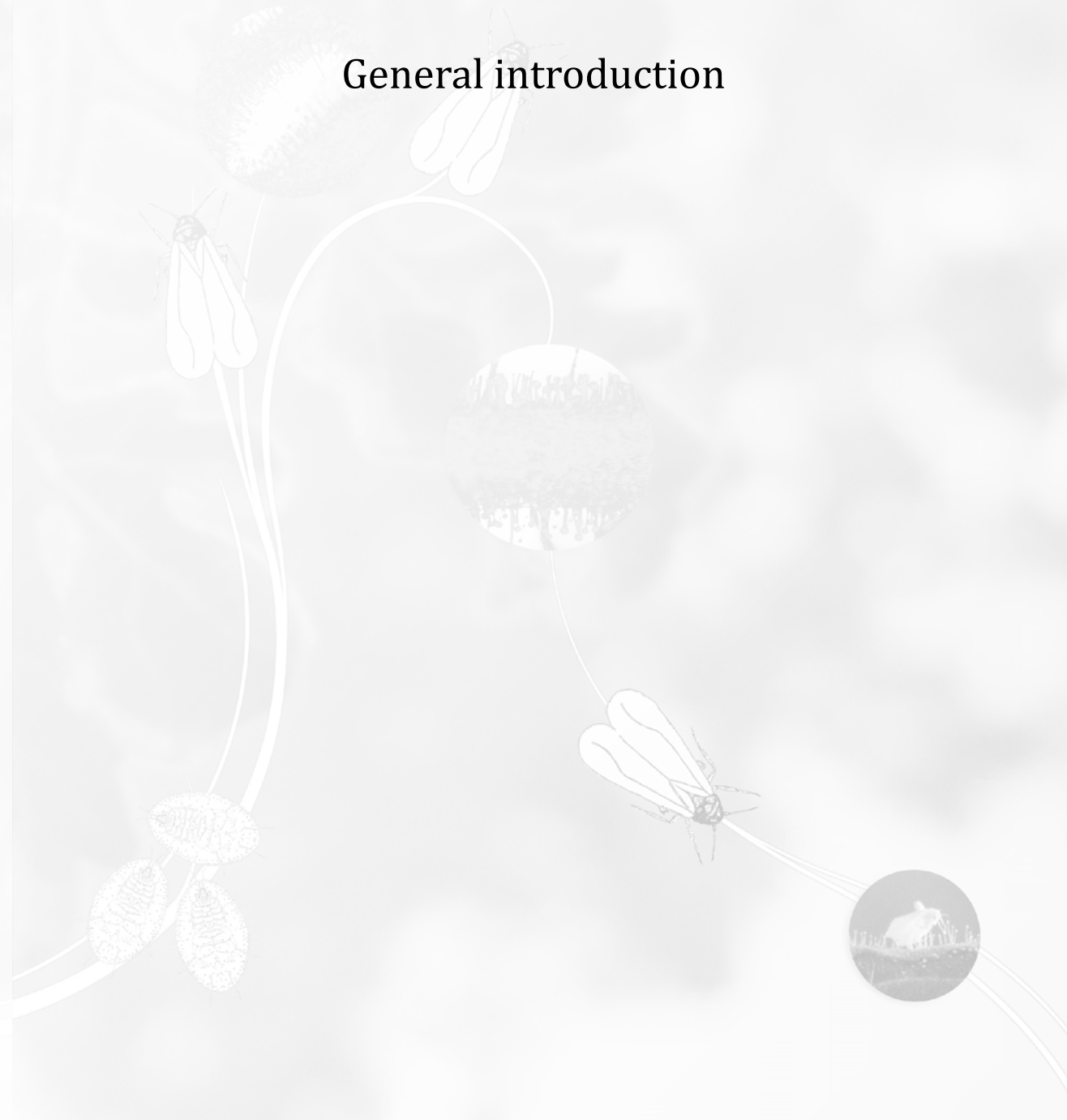
CHAPTER 1

General introduction



Tomato

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops. Molecular data show that tomatoes are members of the *Solanum* genus (Spooner *et al.*, 1993). Tomatoes are native to South America, from the north of Chile to Ecuador including the Galapagos Islands. Tomato species are divided in three sections, section *Lycopersicon*, *Lycopersicoides* and *Junglandifolia*. The *Lycopersicon* section is further divided into four groups (Lycopersicon, Neolycopersicon, Eriopersicon and Arcanum) and comprises thirteen wild tomato species (Peralta *et al.*, 2008).

Whitefly

Whiteflies are classified in the *Aleyrodidae* family with more than 1500 other species. They have an incomplete metamorphosis (Hemimetabolism) consisting of six developmental stages, egg, I instar "*crawler*", II instar, III instar, IV instar "*pupae*" and adult (Figure 1). Whiteflies have a haplo-diploid sexual determination system in which fertilized eggs yield females. Males originate from unfertilized eggs. The sex ratio is generally 2:1 (females: males) and more females develop when males are abundant. The generation time is dependent on temperature and the host plant (Kakimoto *et al.*, 2007, Manzano and van Lenteren, 2009, Tsai and Wang, 1996). In tomato, the mean generation time of *B. tabaci* ranges from 24 to 32 days (Tsai and Wang, 1996, Kakimoto *et al.*, 2007, Islam and Shunxiang, 2007).

Of all whitefly species, *Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I (former *B*-biotype) is one of the most devastating. It has a worldwide distribution, but mainly occupies tropical and subtropical habitats (30ºS in Argentina to 40ºN in the USA), although it also causes damage in more northern zones (Byrne and Bellows Jr, 1991). *Bemisia tabaci* is not one species, but a cryptic species complex in which their members exhibit a considerable amount of genetic variation. However, they are morphologically indistinguishable from each other (De Barro *et al.*, 2011). Recent research based on molecular data of the mitochondrial cytochrome oxidase I (*mtCOI*) gene and mating compatibility experiments suggest that the *B. tabaci* complex consists of at least 36 cryptic species (Dinsdale *et al.*, 2010, De Barro, 2012, Liu *et al.*, 2012, Firdaus *et al.*, 2013b, Tay *et al.*, 2012).

Whitefly and tomato

Bemisia tabaci affects tomato and other crops directly through phloem sap feeding, which results in leaf and fruit spotting, plant weakening, irregular ripening of fruits and honeydew on which sooty mold can grow. *Bemisia tabaci* is responsible for several physiological disorders such as increased stomata resistance, and a reduced transpiration, photosynthesis and chlorophyll content in leaves (Fancelli *et al.*, 2003, Inbar and Gerling, 2008, Muigai *et al.*, 2002). It also affects tomatoes indirectly through

its ability to be the vector of a large number of viruses (Begomovirus, Crinivirus, Closterovirus and Ipomovirus) both in persistent and in semi-persistent circulative manner (Brown and Bird, 1992, Valverde *et al.*, 2004).

Whitefly feeding behaviour

Bemisia tabaci, as other members of the *Aleyrodidae* family, is an obligate phloem-feeder and feeds through its modified mouthparts (stylets). They possess four interlocked stylets that can move independently from each other, sliding back and forward allowing the movement between the mesophyll cells. The interlocked stylets enclose two canals,

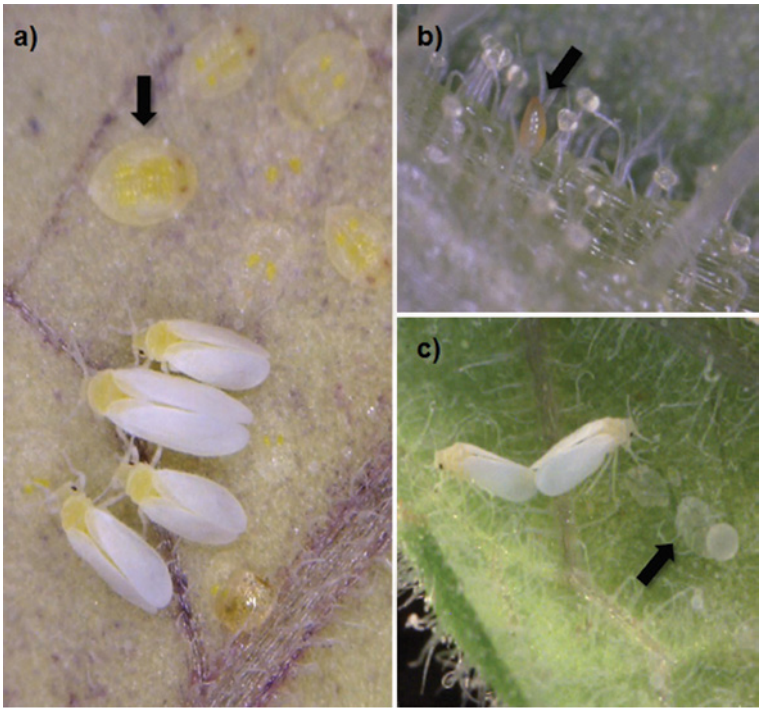


Figure 1: Overview of the different life stages of *Bemisia tabaci*. a) Two couples *B. tabaci* adults and six nymphs, b) close up of a *B. tabaci* egg between tomato trichomes and c) *B. tabaci* adults plus empty pupa cases.

a food and a salivary canal (Miles, 1999, Tjallingii and Esch, 1993). The two canals are independent until almost to the tip of the whitefly labium where they merge in a single canal. In contrast to aphids, whiteflies do a lot less probes (punctures or test of mesophyll cells) on their way to the phloem sieve-element. When they do make a probe; they do not salivate into the cells or ingest cell content (Stansly *et al.*, 2010). During feeding, the whiteflies secrete two types of saliva, sheath and watery saliva (Miles, 1999).

Sheath saliva is secreted during the penetration of the stylets into the plant tissue resulting in a gelled canal from the surface of the leaf to the sieve-tube element. Among

the functions attributed to the sheath saliva is the protection of the moving stylets and minimizing plant wound responses. When the whiteflies reach the phloem, large amounts of watery saliva are injected into the sieve-elements. The watery saliva contains salivary enzymes and metabolites. In the case of aphids, the function is to prevent the sealing response of the sieve-element (Will *et al.*, 2007). It is unknown if the watery saliva of whiteflies has the same function.

Association to primary and secondary endosymbionts

Whiteflies harbour species-specific primary and secondary endosymbionts. Those endosymbionts are obligate bacteria with a smaller genome compared to their closely related free-living relatives. Although all whitefly populations harbour the same primary endosymbiont (*Candidatus* Portiera aleyrodidarum), the presence and ratio of secondary endosymbionts is variable (Skaljac *et al.*, 2010). The primary endosymbionts are present in specific organelles on the whitefly body called bacteriocytes forming the bacteriome. Some secondary endosymbionts are also located in other organs like the abdomen, gut, salivary glands and fat body (Skaljac *et al.*, 2010). Although, primary endosymbionts are almost entirely transovarially (vertically) transferred from mother to offspring via the eggs (Skaljac *et al.*, 2010), secondary endosymbionts have variable degrees of horizontal transmission (Ahmed *et al.*, 2013). One of the main functions of the endosymbionts is to supplement the whitefly diet with amino acids and carotenoids (Santos-Garcia *et al.*, 2012). They might also be involved in the ability of whiteflies to tolerate pesticides and conferring cytoplasmic incompatibility leading to sterile offspring (Ahmed *et al.*, 2013, Skaljac *et al.*, 2010, Kontsedalov *et al.*, 2008, Ghanim and Kontsedalov, 2009).

Whitefly control

Pre-harvest losses of crop production due to pest herbivory are between 10 and 100% when no control is applied (Schoonhoven *et al.*, 2005). Different methods are available for whitefly control. Although several types of biological control are available against whiteflies (i.e. predators, parasitoids, entomopathogenic fungi), chemical control still is an essential component in open field tomato production. The excessive use of insecticides has not only ecological implications but also has resulted in resistance problems (Nauen and Denholm, 2005). Host plant resistance breeding is considered as one of the key methods in insect pest control in crop plants, and especially it is a promising alternative for whitefly control (Bas *et al.*, 1992, Nombela *et al.*, 2000, Firdaus *et al.*, 2013a, Broekgaarden *et al.*, 2011). Host plant resistance is useful as part of an Integrated Pest Management program (IPM) with the intention to combine host plant resistance, biological control and limit chemical control to a bare minimum (i.e. only when strictly needed). Wild relatives of tomato have proven to be useful in breeding programs as source of resistance and quality traits (Yencho *et al.*, 2000, Foolad and

Panthee, 2012, Jimenez-Gomez and Maloof, 2009). In the case of whiteflies, resistance was found in several wild relatives of tomato like *Solanum peruvianum, S. pennellii*, *S. habrochaites, S. lycopersicum var. cerasiforme, S. pimpinellifolium* and *S. galapagense* (Baldin *et al.*, 2005, Fernández-Muñoz *et al.*, 2003, Firdaus *et al.*, 2013a, Firdaus *et al.*, 2012, Freitas *et al.*, 2002, Heinz and Zalom, 1995, Lucatti *et al.*, 2013, Muigai *et al.*, 2003, Muigai *et al.*, 2002, Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012). Despite these efforts, resistant tomato varieties have not been released on the market.

Origin, evolution and maintenance of insect resistance

Not all herbivores become a pest; in a specific crop at one time and location, only a very small number of insects of the total that visit or reach that crop are true pests and require controlling (Schoonhoven *et al.*, 2005). Given the fact that insects are the most diverse group of animals on earth and the existence of more than 400.000 species of insect herbivores it is intriguing that there is not more evidence of high levels of plant damage in nature. This is because resistance is a common phenomenon in nature, being more the rule than the exception. Several hypotheses try to explain the evolution of plant defence (Table 1). These hypotheses are in general not mutually exclusive and most of them are based on the idea that defence mechanisms are costly. A cost can be defined as a trade-off between resistance and fitness under enemy-free conditions (Walters, 2010a). The costs of resistance can be classified as allocation costs, auto-toxicity costs, ecological costs, genetic costs and opportunity costs (Rausher, 2001, Strauss *et al.*, 2002, Walters, 2010a). Allocation costs refer to the costs involved in the mobilization of limited resources that under normal conditions (without herbivory or enemy-free conditions) will be used for growth and reproduction of the plant. Auto-toxicity costs refer to the negative effect of a defence trait on the plant metabolisms. Ecological costs refer to the trade-off between fitness and resistance mediated by interactions with other organisms (Strauss *et al.*, 2002, Walters, 2010a). Genetic costs refer to negative pleiotropic effects between resistance and plant fitness. For example the over-expression of the gene Increased Resistance to *Myzus persicae* 1 (*IRM1*) in *Arabidopsis* produces an increased level of resistance to *Myzus persicae* but at the cost of a reduced size and growth (Chen *et al.*, 2013). Finally, opportunity costs refer to the inability of a plant to compete with others due to a short-term delay in growth due to resistance.

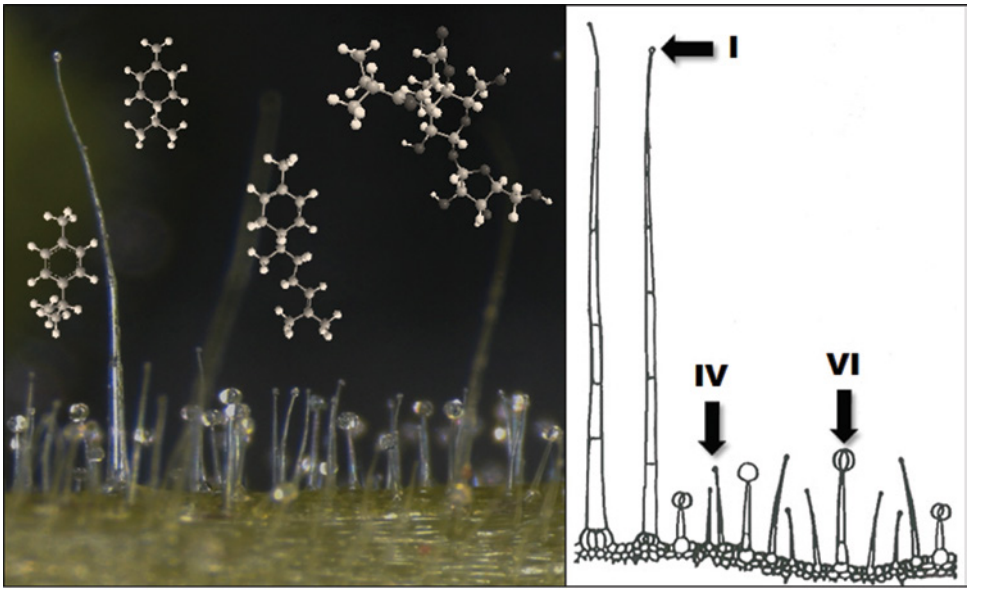


Figure 2: Close up and schematic drawing (adapted from Channarayappa *et al*. (1992)) of glandular trichomes found on *S. galapagense* and chemical structure of the secondary metabolites associated to whitefly resistance of tomato.

Mechanisms of whitefly resistance in tomato

Plants possess a variety of defence mechanisms that can slow down pathogen growth, ward off herbivores and in some cases kill the pathogen/herbivore (Walters, 2010b). Understanding the resistance mechanisms against insects is necessary for successful breeding of resistant varieties. Painter (1951), generalized from a plant breeder's point of view, the mechanisms of plant resistance and grouped them in three categories, non-preference or antixenosis, antibiosis and tolerance. Non-preference mechanisms can be defined as plant characteristics (i.e. colour, shape, odours) that repel insects making a particular plant less attractive for settlement, shelter, feeding and/or oviposition. Antibiotic mechanisms can be defined as plant characteristics that interfere with the insect biology reducing fecundity, size, longevity and increasing mortality. Finally, tolerance can be defined as plant compensation in growth in response to the insect attack. In other words, they can "accept" insect herbivory, while minimizing the reduction in yield. Moreover, plant resistance to herbivory can be further classified as constitutive or inducible and as direct or indirect defences (Schoonhoven *et al.*, 2005). It is important to highlight that the distinction between constitutive and inducible defences, direct and indirect defences is not mutually exclusive. A constitutive defence mechanism may be induced after insect attack (Kariyat *et al.*, 2013) and parasitic insects can use plant defence metabolites as chemical clues in the process of finding their hosts.

Selecting for resistance to insects, involves a set of choice and no-choice experiments. These methods are used to get accurate estimations of the resistance level of a plant genotype and provide an insight in the resistance mechanisms. These experiments measure insect life history parameters to assess resistance. For example, the number of recaptured whiteflies was used to identify non-preferred accessions of tomato (Bleeker *et al.*, 2009). In addition, parameters like insect survival, oviposition rate, nymph survival and developmental period are a few examples of the parameters considered in no-choice tests for whitefly resistance (Bas *et al.*, 1992). Both types of experiments have their advantages and disadvantages, and in breeding for resistance to whiteflies, both type of experiments should be used to give an accurate estimation of the overall resistance level and resistance mechanisms.

In tomato, trichomes and their exudates play a crucial role in insect resistance. Trichomes are hair-like epidermal protuberances showing large variation among tomato species. Based on the absence or presence of a glandular head, tomato trichomes can be divided in non-glandular and glandular. Non-glandular and glandular trichomes are further classified based on the number of basal cells, the length and number of cell in the stalk, on the presence/absence of glandular head and the number of cells on the glandular head (Figure 2) (Channarayappa *et al.*, 1992, Peralta *et al.*, 2008, Luckwill, 1943). Depending on their morphology, non-glandular trichomes function in several processes and have been related to prevention of water loss, regulating plant temperature, affecting photosynthesis, guide pollinators and serve as mechanical barriers to herbivores by reducing insect movement (Wagner, 1991, Wagner *et al.*, 2004).

In tomato, high densities of the non-glandular trichomes type V were correlated with increasing survival and oviposition rates of *B. tabaci* (Heinz and Zalom, 1995, Firdaus *et al.*, 2012, Firdaus *et al.*, 2013a). Glandular trichomes, on the other hand, function as place of synthesis, storage and secretion of secondary metabolites whose principal function can be related to mediate the herbivore-plant chemical interaction (Glas *et al.*, 2012, Schilmiller *et al.*, 2008, Wagner, 1991, Wagner *et al.*, 2004).

Glandular trichomes (type I, IV and VI) of different wild relatives of tomato (*Solanum pennellii*, *S. habrochaites, S. pimpinellifolium, S. galapagense*) were shown to be associated with an increase in resistance against insect herbivores (Antonious *et al.*, 2005, Baldin *et al.*, 2005, Fernández-Muñoz *et al.*, 2003, Firdaus *et al.*, 2012, Freitas *et al.*, 2002, Heinz and Zalom, 1995, Lucatti *et al.*, 2010, Lucatti *et al.*, 2013, Muigai *et al.*, 2003, Muigai *et al.*, 2002, Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012). Glandular trichomes are the place of synthesis of a wide array of secondary metabolites. For example, glandular trichomes type VI of *S. habrochaites* accessions (PI134417, PI134418) are the place of synthesis of methylketones (Antonious, 2001, Fridman, 2005, Ben-Israel *et al.*, 2009, Yu *et al.*, 2010), whereas glandular trichomes type IV of

S. habrochaites accessions (LA1777) are the place of synthesis of sesquiterpenes (i.e. 7-zingiberene, R-curcumene) (Bleeker *et al.*, 2012) and in *S. pennellii* and *S. galapagense* to the synthesis and storage of acyl sugars (Schilmiller *et al.*, 2012, Firdaus *et al.*, 2013a, Lucatti *et al.*, 2013).

Table 1: Hypothesis for the origin and maintenance of plant defences.

Hypothesis	Definition/premise
Growth-differentiation balance hypothesis (GDBH)	There is a trade-off between growth (cell division) and differentiation processes (secondary metabolism) in plants.
Optimal defence hypothesis (ODH)	Herbivory pressure and the fitness consequences of it are important evolutionary forces that vary among plants and parts of the plant. In other words, selection will favour defence traits, when the benefit of that exceeds its cost.
Plant apparency hypothesis (PAH)	States that less apparent plants are more difficult to spot by herbivores and less damaged than apparent plants. In consequence, apparent plants will invest more in defence traits.
The carbon-nutrient balance theory (CNBT)	The variation in types of plant defences are responses to variations in the levels of the carbon-nitrogen ratio.
The growth rate hypothesis (GRH) or resource availability hypothesis	Plants that have evolved in low-resource or stressful environments will exhibit slower growth rates and a particular group of resistance traits (higher level of constitutive resistances). The abiotic environment is the driving force behind the evolution of plant defences.
The univariate trade-off hypothesis	If a defence mechanism is sufficient to confer high levels of defence, selection should not occur for other redundant defence mechanism.
The resistance-regrowth trade-off hypothesis	Given a limited resources, there should be a trade-off between resistance and tolerance traits
Plant defence syndromes	Plants inhabiting a particular abiotic or biotic environment might evolve a set of particular defence traits. A set of multiple co-varying traits associated to ecological interactions is known as syndrome.

Plant responses to whiteflies

Whiteflies use their stylets to penetrate leaf tissue and feed from the phloem of their hosts. During feeding, whiteflies have an intimate and long-lasting interaction with their hosts. Once the feeding site has being established, the whitefly can feed for days (Walling, 2000). Because of their specific form of feeding, phloem feeders produce little injury and limit the local induction of defence responses to a minimal number of cells and tissue types. Among the best known responses to phloem feeders are the induction of hormone dependent pathways (Salicylic acid SA, Jasmonic acid JA, Ethylene ET), the up and down regulation of genes involved in reactive oxygen species responses, the induction of genes related to cell wall modifications, the alteration of photosynthesis rates in the host plant, modification of the source-sink relationships, induction of genes related to nitrogen assimilation and induction of the synthesis of secondary metabolites (Thompson and Goggin, 2006). It has been show that whitefly feeding (adults and nymphs) activates the SA and JA/ET dependent pathways in tomato and *Arabidopsis*

(Kempema *et al.*, 2006, Puthoff *et al.*, 2010, Zarate *et al.*, 2006). It was suggested that whiteflies can modify or alter plant responses by activating or repressing pathways for their own benefit in a so called "decoy" hypothesis (Zhu-Salzman *et al.*, 2008, Zarate *et al.*, 2006, Walling, 2008). Whitefly feeding can induce the genes involved in the biosynthesis of JA (*LOX*, *OPR3*), but do not induce JA responsive genes (*VSP1*) contributing to improve whitefly performance (Zhang *et al.*, 2009).

Genetics of whitefly resistance and related traits

Insect resistance is a complex trait and in most cases quantitatively inherited. For some insects, major genes associated with insect resistance were described. Here we focus on the genes affecting *Hemipterans* in *Solanaceae*, for other insect species Yencho *et al.* (2000) and Broekgaarden *et al.* (2011) give a good overview. One of the few cloned genes involved in insect resistance is the *Mi* gene. In the 1940s, this gene, conferring resistance to a root-knot nematode was transferred from *S. peruvianum* (PI128657) into the cultivated tomato. The *Mi* gene is located on the short arm of Chromosome 6 (Klein-Lankhorst *et al.*, 1991, Kaloshian, 2004) where two clusters (2p and 1p) 300 kb apart were identified, containing seven *Mi* homologues genes in total. The cluster 2p contains 4 *Mi* homologue genes (*Mi-1.4*, *Mi-1.5*, *Mi-1.6* and *Mi-1.7*) and the cluster 1p with 3 *Mi* homologue genes (*Mi-1.1*, *Mi-1.2* and *Mi-1.3*) (Seah *et al.*, 2004). Of the seven homologs, only *Mi-1.2* confers effective resistance against several species of root-knot nematodes (*Meloidogyne spp.*) (Milligan *et al.*, 1998), some isolates of potato aphid (*Macrosiphum euphorbiae* Thomas) (Rossi *et al.*, 1998) (Kaloshian *et al.*, 1995) and the sweet potato whitefly (*B. tabaci*) (Nombela *et al.*, 2003). The *Mi-1.2* gene produces a transcript of approximately 4 kb that encodes a protein of 1,257 amino acids, which is member of a disease resistance-associated plant protein family, characterized by the presence of a nucleotide binding site (NBS) and a leucine-rich repeat motif (LRR) (Rossi *et al.*, 1998, Milligan *et al.*, 1998). Although the *Mi1-2* gene was shown to have an effect on whitefly resistance, the potential application is questionable. When several tomato cultivars differing in the presence of this gene were compared no differences were found (Nombela *et al.*, 2000). Furthermore, a transgenic line (143-11-16-36) carrying the *Mi1-2* gene gave only an effect at lower temperatures (23°C) but not at higher temperatures (27°C) where the insect development is at its optimum (Nombela *et al.*, 2003).

Quantitative Trait Loci (QTL) studies were carried out using resistant wild species of tomato (*S. habrochaites*, *S. pennellii*, *S. pimpinellifolium* and *S. galapagense*) and mapping populations were developed (F_2, RILs, ILs, BC) (Table 2). The use of backcross inbred lines (ILs) helped to identify traits related to insect resistance like the production of monoterpenes, sesquiterpenes and acyl sugars (Schilmiller *et al.*, 2010, Schilmiller *et al.*, 2012, Schilmiller *et al.*, 2008, Van der Hoeven *et al.*, 2000, Bleeker *et al.*, 2012), but failed to identify regions associated to whitefly resistance (Momotaz, 2005, Van den Elsen, 2013) suggesting a polygenic inheritance and possibly epistatic interactions. Maliepaard *et al.* (1995) performed one of the first mapping studies focused on whitefly resistance

in tomato (*S. habrochaites*, CGN1.1561). They identified two QTL reducing whitefly oviposition rate (*Tv-1* and *Tv-2*), two QTLs related to trichome type IV density (*TriIV-1* and *TriIV-2*) and one QTL for trichome type VI density (*TriVI-1*). After this first study, other resistance sources were explored (Table 2). For instance, the wild tomato species *S. pennellii* (LA0716, LA3791) was studied extensively as source of insect resistance and as model to study acyl sugars metabolism (Table 2) (Blauth *et al.*, 1998, Blauth *et al.*, 1999, Mutschler *et al.*, 1996, Leckie *et al.*, 2013, Van den Elsen, 2013). Recently, the focus has shifted to tomato species more closely related to the cultivated tomato. It was found that the resistance in *S. pimpinellifolium* and *S. galapagense* was controlled by major QTLs suggesting a simpler genetic basis (Firdaus *et al.*, 2013a, Salinas *et al.*, 2013). In Table 2 it is clearly shown that there is a co-localization of QTLs affecting whitefly biology with QTLs affecting glandular trichomes (type IV), suggesting an association between glandular trichomes and whitefly resistance.

Priming as an control alternative

Plants are dynamic organisms that are under constant attack. To defend themselves, they developed constitutive resistance mechanisms (i.e. trichomes, cuticle, and wax layers) as a first line of defence. In addition to this, plants can defend themselves by perceiving stress signals (exogenous and endogenous) and responding to them in a specific way through inducing their immune system (Jones and Dangl, 2006). With an inducible immune system, plants obtain flexibility in managing their resources, e.g. to choose between defence and growth by eliciting anti-herbivore defence only when necessary (Erb *et al.*, 2012, Pieterse *et al.*, 2012, Kim and Felton, 2013). These inducible mechanisms can also be triggered in response to exogenous application of endogenous defence hormones (SA, JA and their methyl derivate), as well as in response to some xenobiotic chemicals (benzothiadiazole BTH, β-aminobutyric acid BABA) and sugars (fructose, sucrose, galactinol) (Cohen *et al.*, 1994, Hodge *et al.*, 2005, Nombela *et al.*, 2005, Ton *et al.*, 2005, Bolouri Moghaddam and Van den Ende, 2012). This so-called priming of defence enables plant cells to respond to low levels of a stimulus in a more rapid and robust manner displaying faster and/or stronger, activation of defence responses when subsequently challenged by microbes, insects, or abiotic stress.

Priming is associated with the induction of systemic plant immunity like SAR (systemic acquired resistance) and ISR (induced systemic resistance). The SA-dependant defences can be induced by some analogues of SA like INA (2,6-dicholorodiazole acid), BTH, and by the non-protein amino acid BABA. Priming can also induce JA-dependent defences by treatment with JA and some volatile organic compounds (VOCs). Priming not only allows the plant to cope with attacks in a cost effective way but also seems to help their offspring. *Arabidopsis* plants exposed to an a-virulent strain of *Pseudomonas syringae* or primed by BABA produce descendants that were more resistant to *Hyaloperonospora arabidopsidis* and *P. syringae* pv. *tomato* DC3000 (*Pst*DC3000) (Slaughter *et al.*, 2011).

Table 2: Overview of the QTLs associated to insect resistance, trichome type, density, and acyl sugar metabolism in tomato.

Chr	QTL	Trait	% Explained	Resistance donor	References
1	*TriVI-1*	Density of trichome type VI	n.d.	*S. habrochaites* (CGN1.1561)	(Maliepaard *et al.*, 1995)
	Tv-1	Oviposition rate (*T. vaporariorum*)	6.4		
	Wf-I	Adult survival (*B. tabaci*)	12.1	*S. pennellii* (LA3791)	(Van den Elsen *et al.*, 2013)
		Acyl sucrose accumulation (S3-20-II)	10.6		
2	*Rtu2.1*	Resistance to *T. urticae*	8	*S. pimpinellifolium* (TO-937)	(Salinas *et al.*, 2013)
	Rtu2.2	Resistance to *T. urticae*	31.6		
	TA2A	Density of trichome type IV	2.6	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
		Total acyl sugar accumulation	7.8-11.3		(Mutschler *et al.*, 1996)
	TA2B	Total acyl sugar accumulation	10.7-14.7	*S. pennellii* (LA0716)	(Mutschler *et al.*, 1996)
	Wf-1	Adult survival (*B. tabaci*)	54.1	*S. galapagense* (PRI95004)	(Firdaus *et al.*, 2013a)
		Density of trichome type IV	66.3		
		Oviposition rate (*B. tabaci*)	41.7		
		Pre-adult survival (*B. tabaci*)	13.3		
		Fatty acid chain	n.d.	*S. pennellii* (LA0716)	(Blauth *et al.*, 1999)
3	*3A*	Density of trichome type IV	5.1	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
	3B	% of acylglucoses	50		
	AG3.2	% of acylglucoses	44.6		(Leckie *et al.*, 2013)
		Total acyl sugar accumulation	3.3		
	TA3	Total acyl sugar accumulation	7.9-12.3		(Mutschler *et al.*, 1996)
	Wf-III	Adult survival (*B. tabaci*)	15.6	*S. pennellii* (LA3791)	(Van den Elsen *et al.*, 2013)
		Acyl sugar accumulation	12.6-29		
4	*AG4*	% of acylglucoses	4.6	*S. pennellii* (LA0716)	(Leckie *et al.*, 2013)

Table 2: Overview of the QTLs associated to insect resistance, trichome type, density, and acyl sugar metabolism in tomato.

Chr	QTL	Trait	% Explained	Resistance donor	References
4	*TA4*	Density of trichome type IV	5.2	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
		Total acyl sugar accumulation	7.0-10.3		(Mutschler *et al.*, 1996)
		Total acyl sugar accumulation	13.9		(Leckie *et al.*, 2012)
		Total acyl sugar accumulation	17		(Leckie *et al.*, 2013)
5	*Wf-IV*	Adult survival (*B. tabaci*)	12.3-30.7	*S. pennellii* (LA3791)	(Van den Elsen *et al.*, 2013)
		Oviposition rate (*B. tabaci)*	10.3-29.6		
		Acyl sucrose accumulation	12.5-49.9		
	TA5	Total acyl sugar accumulation	17.2	*S. pennellii* (LA0716)	(Leckie *et al.*, 2012)
	TriIV-1	Density of trichome type IV	n.d.	*S. habrochaites* (CGN1.1561)	(Maliepaard *et al.*, 1995)
	5	Fatty acid chain	n.d.	*S. pennellii* (LA0716)	(Blauth *et al.*, 1999)
6	*6A*	Density of trichome type IV	4.7	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
	iso-even	% *iso*-branched fatty acids	n.d.		(Blauth *et al.*, 1999)
	TA6	Total acyl sugar accumulation	13.4		(Leckie *et al.*, 2012)
	Wf-VI	Adult survival (*B. tabaci*)	10.1	*S. pennellii* (LA3791)	(Van den Elsen, 2013)
		Oviposition rate (*B. tabaci)*	13.9		
7	*7A*	% of acylglucoses	1.3	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
	7B	Density of trichome type IV	2.8		
	7	Fatty acid chain	n.d.		(Blauth *et al.*, 1999)
8	*iso-even*	% *iso*-branched fatty acids	n.d.	*S. pennellii* (LA0716)	(Blauth *et al.*, 1999)
	8	Fatty acid chain	n.d.		
	Wf-VIII	Acyl-sucrose accumulation	12.8-15.7	*S. pennellii* (LA3791)	(Van den Elsen, 2013)
9	*9A*	% of acylglucoses	5.1	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
	R2/9	Density of trichome type IV	69.7	*S. habrochaites* (LA1777)	(Momotaz *et al.*, 2010)

Table 2: Overview of the QTLs associated to insect resistance, trichome type, density, and acyl sugar metabolism in tomato.

Chr	QTL	Trait	% Explained	Resistance donor	References
9	*R2/9*	Oviposition rate (*B. tabaci*)	55.2	*S. habrochaites* (LA1777)	(Momotaz *et al.*, 2010)
	TriIV-2	Density of trichome type VI	n.d.	*S. habrochaites* (CGN1.1561)	(Maliepaard *et al.*, 1995)
	Wf-2	Adult survival (*B. tabaci*)	14.8	*S. galapagense* (PRI95004)	(Firdaus *et al.*, 2013a)
		Density of trichome type IV	8.7		
		Oviposition rate (*B. tabaci*)	11.1		
10	*10A*	% of acylglucoses	4.7	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
		Density of trichome type IV	4.6		
		Total acyl sugar accumulation	13		
	R1/10	Density of trichome type IV	22.5	*S. habrochaites* (LA1777)	(Momotaz *et al.*, 2010)
		Oviposition rate (*B. tabaci*)	15		
	TA10.1	Total acyl sugar accumulation	10.7	*S. pennellii* (LA0716)	(Leckie *et al.*, 2012)
	Wf-X	Adult survival (*B. tabaci*)	16.4	*S. pennellii* (LA3791)	(Van den Elsen, 2013)
		Oviposition rate (*B. tabaci)*	10		
11	*11A*	Density of trichome type IV	8.1	*S. pennellii* (LA0716)	(Blauth *et al.*, 1998)
	AG11	% of acylglucoses	8.3		(Leckie *et al.*, 2013)
	R3/11a	Density of trichome type IV	69	*S. habrochaites* (LA1777)	(Momotaz *et al.*, 2010)
		Oviposition rate (*B. tabaci*)	52.9		
	R4/11b	Density of trichome type IV	n.d.	*S. habrochaites* (LA1777)	(Momotaz *et al.*, 2010)
	R4/11b	Oviposition rate (*B. tabaci*)	43.3	*S. habrochaites* (LA1777)	(Momotaz *et al.*, 2010)
	TA11	% of acylglucoses	17.7-22.2	*S. pennellii* (LA0716)	(Mutschler *et al.*, 1996)
	TA11	Total acyl sugar accumulation	17.7-22.2	*S. pennellii* (LA0716)	(Mutschler *et al.*, 1996)
		Total acyl sugar accumulation	6		(Leckie *et al.*, 2012)
		Total acyl sugar accumulation	3.6		(Leckie *et al.*, 2013)

Table 2: Overview of the QTLs associated to insect resistance, trichome type, density, and acyl sugar metabolism in tomato.

Chr	QTL	Trait	% Explained	Resistance donor	References
12	*Tv-2*	Oviposition rate (*T. vaporariorum*)	8	*S. habrochaites* (CGN1.1561)	(Maliepaard *et al.*, 1995)
	12	Fatty acid chain	n.d.	*S. pennellii* (LA0716)	(Blauth *et al.*, 1999)
Linkage group	*C D I*	2-tridecanone	36	*S. habrochaites* (PI134417)	(Nienhuis *et al.*, 1987)
	D	Density of trichome type IV	n.d.		

Moreover, Rasmann *et al.*, (2012) reported that tomato and *Arabidopsis* plants were more resistant to caterpillar attack, when their parents had been attacked by the same insect species. This trans-generational priming was associated to changes in chromatin structure and in a hypomethylation at CpHpG sites that promote transcription efficiency of defence genes (Luna *et al.*, 2012, Rasmann *et al.*, 2012, Luna and Ton, 2012, Kim and Felton, 2013). So far, the exact relation between priming and the molecular and epigenetic mechanisms is unknown.

Scope and thesis outline

In spite of previous efforts, whiteflies are still a problem in tomato cultivation. The aim of this thesis is to identify and understand resistance mechanisms targeting specific stages of the whitefly life cycle in order to provide breeders with tools for developing whitefly resistant varieties.

In **Chapter 2**, a phenotypic, metabolomic and genetic screening of several accessions of two wild tomato species, native to the Galapagos Islands, is described. The resistance (low adult survival) was associated to high densities of trichomes type IV and acyl sugars. The results were analysed and discussed in an evolutionary context and several accessions that were highly resistant to whiteflies were identified.

Chapter 3 addresses the resistance mechanisms present in a tomato line derived from a *S. habrochaites* accession that affects the preference of *B. tabaci*. The potential metabolites as well as the introgression regions are reported.

Chapter 4 focuses on the fine mapping and description of an introgression fragment on Chr. 5 of *S. habrochaites* reducing exclusively the oviposition rate of *B. tabaci*.

In **Chapter 5**, the possibility to enhance partial resistance by priming is addressed. A set of tomato genotypes differing in their resistance level is treated by drench soil application of priming compounds and analysed for whitefly resistance.

Finally, in **Chapter 6** the results from the experimental chapters are integrated and related to the current knowledge on whitefly resistance in tomato. Furthermore, the potential use of the mechanisms described in this thesis for breeding whitefly resistant varieties is discussed.

Chapter 2

Differences in insect resistance between tomato species endemic to the Galapagos Islands

Alejandro F. Lucatti[1,2], Sjaak van Heusden[1], Ric CH de Vos[3,4,5], Richard GF Visser[1], Ben Vosman[1]

1 Wageningen UR Plant Breeding. Wageningen University and Research Centre. PO Box 386, 6700 AJ Wageningen, The Netherlands.

2 Graduate School Experimental Plant Sciences. Wageningen Campus. Droevendaalsesteeg 1, Wageningen, The Netherlands.

3 Plant Research International, Business Unit Bioscience, Wageningen University and Research Centre, P.O. Box 619, 6700AP, Wageningen, The Netherlands.

4 Centre for BioSystems Genomics, P.O. Box 98, 6700 AB, Wageningen, The Netherlands.

5 Netherlands Metabolomics Centre, Einsteinweg 55, 2333 CC, Leiden, The Netherlands.

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Abstract

Background- The Galapagos Islands constitute a highly diverse ecosystem and a unique source of variation in the form of endemic species. There are two endemic tomato species, *Solanum galapagense* and *S. cheesmaniae* and two introduced tomato species, *S. pimpinellifolium* and *S. lycopersicum*. Morphologically the two endemic tomato species of the Galapagos Islands are clearly distinct, but molecular marker analysis showed no clear separation. Tomatoes on the Galapagos are affected by both native and exotic herbivores. *Bemisia tabaci* is an important introduced insect species that feeds on a wide range of plants. In this article, we address the question whether the differentiation between *S. galapagense* and *S. cheesmaniae* may be related to differences in susceptibility towards phloem-feeders and used *B. tabaci* as a model to evaluate this.

Results- We have characterized 12 accessions of *S. galapagense*, 22 of *S. cheesmaniae*, and one of *S. lycopersicum* as reference for whitefly resistance using no-choice experiments. Whitefly resistance was found in *S. galapagense* only and was associated with the presence of relatively high levels of acyl sugars and the presence of glandular trichomes of type I and IV. Genetic fingerprinting using 3316 SNP markers did not show a clear differentiation between the two endemic species. Acyl sugar accumulation as well as the climatic and geographical conditions at the collection sites of the accessions did not follow the morphological species boundaries.

Conclusion- Our results suggest that *S. galapagense* and *S. cheesmaniae* might be morphotypes rather than two species and that their co-existence is likely the result of selective pressure.

Keywords: *Bemisia tabaci*, *Solanum galapagense*, *Solanum cheesmaniae*, Whitefly, Trichomes, Acyl sugars, Selection pressure

INTRODUCTION

Tomatoes are native to South America and can be found from the north of Chile/ Argentina to Ecuador, including the Galapagos Islands. The Galapagos is a volcanic archipelago of 13 islands located about 1000 km from the coast of Ecuador. On this archipelago, vegetation varies among islands, altitude and cardinal direction (Darwin *et al.*, 2003, Peralta *et al.*, 2008). The Galapagos Islands constitute a highly diverse ecosystem and a unique source of variation in the form of endemic species. There are two endemic tomato species, *Solanum galapagense* and *S. cheesmaniae* and two introduced species *Solanum pimpinellifolium* and *S. lycopersicum*. It is believed that the latter two have been introduced on the islands during the twentieth century, though it is possible that *S. pimpinellifolium* is present on the islands a lot longer (Darwin *et al.*, 2003). The endemic Galapagos Island tomato species have evolved in isolation from the mainland species, resulting in clearly differing morphological features compared to the species that were introduced later. However, natural hybrids have been found (Darwin *et al.*, 2003, Darwin, 2009). The taxonomic state of the Galapagos' endemic tomatoes is under debate and a historic overview is given by Darwin *et al.* (2003). Darwin and co-workers adopted the 'morphological cluster' species concept (Mallet, 1995) to divide the two endemic tomato forms in two species *S. galapagense* and *S. cheesmaniae*. Despite the clear separation obtained on the basis of morphology, it was not possible to separate the two species with molecular markers (Peralta *et al.*, 2008, Nuez *et al.*, 2004, Peralta *et al.*, 2005, Spooner *et al.*, 2005b).

From the total number of endemic species on the Galapagos Islands, 47% are insects (Walsh and Mena, 2013). However, the number of exotic insect species on the Galapagos is increasing due to human activity and now at least 463 exotic insect species can be found on the archipelago, of which 193 species are herbivores and in majority phloem feeders (Causton *et al.*, 2006). Of the exotic insect species 73% are naturalized or are known to feed on endemic plant species. The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is one of the most important invasive insects on the Galapagos Islands, receiving the highest score of invasiveness due to their wide distribution, wide host range and their importance as vector of many plant viruses (Causton *et al.*, 2006, Valverde *et al.*, 2004, Muigai *et al.*, 2002). Recently it became clear that *B. tabaci* is not a single species but a whole complex of at least 36 cryptic species (De Barro, 2012, Dinsdale *et al.*, 2010, Firdaus *et al.*, 2013b, Liu *et al.*, 2012). The first report of *B. tabaci* on the Galapagos dates back to 1998 (Causton *et al.*, 2006). Interestingly, *S. galapagense* was shown to be resistant to this devastating pest insect (Firdaus *et al.*, 2013a, Firdaus *et al.*, 2012, Simmons and Gurr, 2005). This suggests that a general mechanism conferring resistance towards insects is present in *S. galapagense* as the time after introduction of *B. tabaci* is too short for co-evolution. The resistance is associated with the presence of type IV glandular trichomes (Firdaus *et al.*, 2013a, Firdaus *et al.*, 2012).

Selection pressure, co-evolutionary processes, population genetics, bio-geographical variables, and gene dynamics (gene flow, drift, mating systems, etc.) can affect the evolution of resistance genes and the accumulation or the presence of certain metabolites related to resistance (Rausher, 2001, Weber and Agrawal, 2012). For example, the evolution of genes involved in the terpenoid pathway and the relative composition of acyl sugar were suggested to be related to geographic and climatic variables under which populations of *Solanum habrochaites* are found (Gonzales-Vigil *et al.*, 2012, Kim *et al.*, 2012). The Galapagos Islands have proven to be a place of natural experimental conditions to answer questions related to processes like founder effect, genetic drift, divergent selection, ecological opportunity and interaction between endemic and exotic species in the speciation processes (Walsh and Mena, 2013). As *B. tabaci* has an ecological importance on the Galapagos Islands (Causton *et al.*, 2006) and its global importance as a phloem feeding tomato herbivore, we decided to use *B. tabaci* as a model insect to analyse factors underlying insect resistance in relation to species delimitation between *S. galapagense* and *S. cheesmaniae*.

So far, only a limited number of accessions/populations of *S. galapagense* and *S. cheesmaniae* have been evaluated for insect resistance and therefore it is unknown if the insect resistance coincides with the species boundaries (based on the morphological differences). Neither is there any knowledge about the relation between geographical and climatic conditions today on the Galapagos and the occurrence of the two species. Recently it was shown that the whitefly resistance in an accession of *S. galapagense* is most likely based on the production of acyl sugars in the glandular trichomes (Firdaus *et al.*, 2013a). It is unknown if the relative acyl sugar concentration among the different accessions of *S. galapagense* and *S. cheesmaniae* coincides with species boundaries and insect resistance. In the present study, we address the questions raised and discuss the implication in an evolutionary context. We characterized the genetic and acyl sugar variation in 34 endemic tomato accessions and investigated if resistance and chemical variation among accessions are correlated. We demonstrate that *S. galapagense* is different form *S. cheesmaniae* in the resistance towards whiteflies and in the trichome composition. Geographic and climatic variables do not explain the distribution pattern found for the Galapagos' endemic tomatoes. Genetic variation between the two species is almost absent and acyl sugar composition does not completely follow the morphological species boundaries. All together our results suggest that *S. galapagense* and *S. cheesmaniae* might be considered as morphotypes rather than two species and that their co-existence is likely the result of selective pressure.

RESULTS

Resistance to whitefly

The level of whitefly resistance in accessions of *S. galapagense*, *S. cheesmaniae* and cv. Moneymaker was assessed using three parameters, namely adult survival (AS), oviposition rate (OR), and pre-adult survival (PS) (Table 1). The three parameters were highly correlated (Table 2). For AS, significant differences were found among accessions (ANOVA, P< 0.001), with survival rates ranging from 0 to 1. The lowest values for AS were found within the accessions of *S. galapagense*, with five accessions on which all whiteflies were dead after 5 days (AS = 0). Adult survival on all accessions of *S. galapagense* was statistically different from the AS on cv. Moneymaker. None of the *S. cheesmaniae* accessions were statistically different from cv. Moneymaker for the resistance variables.

Table 1: Adult survival, oviposition rate and pre-adult survival of the different accessions of *Solanum galapagense* and *S. cheesmaniae.*

Taxa and accession no.	Adult Survival			Oviposition Rate			Pre-adult Survival		
	n	mean		n	mean		n	mean	
Solanum cheesmaniae		0.89			7.04			0.59	
LA0421	(5)	0.99	*kl*	(5)	6.02	*de*	(3)	0.46	*def*
LA0422	(9)	0.89	*hij*	(9)	6.25	*de*	(5)	0.83	*h*
LA0428	(9)	0.69	*gh*	(9)	6.06	*de*	(5)	0.41	*de*
LA0521	(2)	0.98	*ijkl*	(2)	8.74	*e*	(2)	0.63	*efgh*
LA0522	(8)	0.89	*ijkk*	(8)	5.78	*de*	(5)	0.52	*efg*
LA0528B	(9)	0.85	*hij*	(9)	8.40	*e*	(5)	0.71	*gh*
LA0529	(2)	0.50	*hij*	(2)	5.50	*cde*	(1)	0.48	ND
LA0746	(9)	0.86	*hi*	(9)	6.01	*de*	(5)	0.66	*gh*
LA0932	(6)	0.94	*ijkl*	(6)	7.43	*de*	(2)	0.70	*fgh*
LA1035	(4)	0.97	*ijkl*	(4)	7.33	*de*	(0)	ND	ND
LA1039	(3)	1.00	*l*	(2)	7.11	*de*	(1)	0.35	ND
LA1040	(9)	0.93	*ijkl*	(9)	6.36	*de*	(4)	0.77	*gh*
LA1041	(4)	0.86	*hij*	(4)	7.19	*de*	(0)	ND	ND
LA1042	(9)	0.94	*ijkl*	(9)	8.21	*e*	(5)	0.65	*fgh*
LA1043	(8)	0.95	*ijkl*	(8)	8.77	*e*	(3)	0.74	*gh*
LA1137#	(9)	0.94	*ijkl*	(9)	7.72	*e*	(5)	0.75	*gh*
LA1139	(4)	1.00	*l*	(4)	6.62	*de*	(3)	0.51	*efg*

n: Number of plants per accession.
#: Classified as *S. galapagense* in TGRC, but phenotypically it is a *S. cheesmaniae*.
ND: No determined.
Different letters indicate statistical differences according to LSD test (P<0.05). *S. lycopersicum* cv. Moneymaker is included as a reference.

Table 1: Adult survival, oviposition rate and pre-adult survival of the different accessions of *Solanum galapagense* and *S. cheesmaniae.*

Taxa and accession no.	Adult Survival			Oviposition Rate			Pre-adult Survival		
	n	mean		n	mean		n	mean	
LA1404	(9)	0.90	*ijkl*	(9)	7.59	*de*	(5)	0.66	*fgh*
LA1409	(2)	0.75	*ghi*	(1)	5.45	ND	(1)	0.01	ND
LA1411	(10)	0.95	*ijkl*	(10)	8.65	*e*	(0)	ND	ND
LA1450	(9)	0.91	*ijkl*	(9)	8.86	*e*	(4)	0.64	*fgh*
LA3124	(6)	0.97	*jkl*	(6)	4.76	*cd*	(6)	0.76	*gh*
Solanum galapagense		0.09			0.99			0.12	
LA0438	(3)	0.00	*ab*	(2)	0.00	*a*	(0)	ND	ND
LA0480A	(7)	0.29	*def*	(7)	2.89	*bc*	(3)	0.07	*abc*
LA0483	(3)	0.00	*ab*	(3)	0.00	*a*	(0)	ND	ND
LA0528	(9)	0.22	*cde*	(9)	2.86	*bc*	(5)	0.10	*bc*
LA0530	(5)	0.10	*abc*	(5)	0.20	*a*	(1)	0.50	ND
LA0532	(3)	0.00	*ab*	(3)	0.17	*a*	(0)	ND	ND
LA0748	(8)	0.30	*ef*	(8)	2.48	*bc*	(3)	0.23	*cd*
LA1401	(8)	0.03	*ab*	(7)	0.57	*a*	(1)	0.00	ND
LA1408	(6)	0.00	*a*	(6)	0.00	*a*	(0)	ND	ND
LA1452	(5)	0.00	*ab*	(5)	0.14	*a*	(2)	0.00	*a*
LA1508	(8)	0.11	*bcd*	(8)	1.90	*b*	(4)	0.02	*abc*
LA1627	(9)	0.04	*ab*	(9)	0.70	*a*	(2)	0.02	*ab*
Solanum lycopersicum									
Cv. Moneymaker	(33)	0.93	*ijkl*	(33)	5.55	*de*	(15)	0.51	*efg*

n: Number of plants per accession.
#: Classified as *S. galapagense* in TGRC, but phenotypically it is a *S. cheesmaniae*.
ND: No determined.
Different letters indicate statistical differences according to LSD test (P<0.05). *S. lycopersicum* cv. Moneymaker is included as a reference.

The oviposition rate (OR) ranged from 0 to 8.86 eggs/day/female (Table 1). All *S. galapagense* accessions showed a reduction in OR and were significantly different from cv. Moneymaker. In fact there were three accessions on which no eggs were deposited. All *S. cheesmaniae* accessions were at least as susceptible as the cv. Moneymaker. At the species level, *S. galapagense* showed the lowest OR (OR = 1.3 ± 1.72, P < 0.001).

For several accessions it was impossible to determine PS, as there were no eggs deposited. For two accessions of *S. galapagense* (LA1452 and LA1401) we observed that, although whiteflies were able to lay a few eggs, none of those eggs hatched. The *S. cheesmaniae* accessions were not significantly different from the cv. Moneymaker in the PS values (Table 1).

Table 2: *Pearson correlation among phenotypic resistance characteristics and Acyl sugar accumulation.*

	Resistance variables			Trichome Type					Acyl sugar
	AS	**OR**	**PS**	**I**	**III**	**IV**	**V**	**VI**	
AS		**	**	**	0.13	**	**	0.02	**
OR	1.00		**	**	0.12	**	**	0.02	**
PS	0.81	0.82		**	0.06	**	**	0.16	*
Trichome type I	-0.92	-0.92	-0.84		0.06	**	**	0.07	**
Trichome type III	0.33	0.34	0.39	-0.39		0.07	*	0.19	0.07
Trichome type IV	-0.92	-0.92	-0.84	1.00	-0.39		**	0.06	**
Trichome type V	0.88	0.88	0.73	-0.91	0.55	-0.90		0.37	**
Trichome type VI	-0.50	-0.48	-0.30	0.39	0.28	0.40	-0.20		0.13
Total Acyl sugar	-0.61	-0.60	-0.51	0.64	-0.38	0.64	-0.57	0.33	

Below main diagonal is the correlation coefficient and upper the main diagonal is the P-value associated. Asterisk (*) indicate P ≤ 0.01, double asterisks (**) indicate P ≤ 0.001.

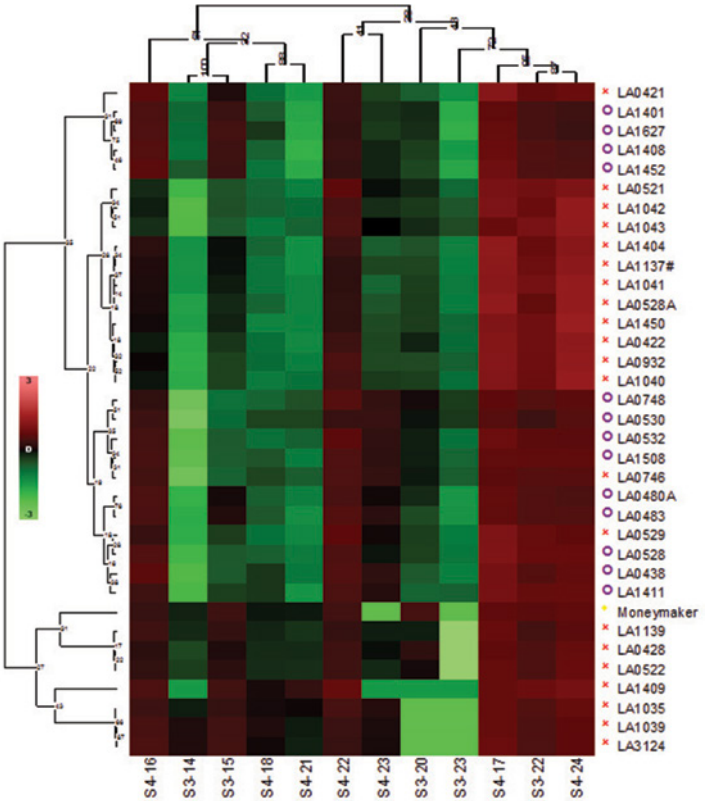

Figure 1: Hierarchical clustering (Pearson correlation, UPGMA), of the different acyl sucroses (columns) and the different accessions (rows). *Solanum galapagense* S.C. Darwin & Peralta (violet circle symbol), *S. cheesmaniae* L. Riley (red cross sign symbol), *S. lycopersicum* L cv. Moneymaker (yellow circle symbol). Colour key is displayed in the figure.

Trichome type and acyl sugar composition

A clear difference in the trichome composition was observed among the different tomato species. All accessions of *S. galapagense* had trichomes of both type I and IV. None of the accessions of *S. cheesmaniae* had trichomes type I or IV, while all of them had trichomes type V and VI in higher or lower densities (Additional file 1: Table S1). Trichome type III was absent on *S. galapagense* and present on 7 of the 22 accessions of *S. cheesmaniae* (Additional file 1: Table S1). A negative correlation between the measured resistance variables and the presence of glandular trichome types I, IV and VI was observed (Table 2).

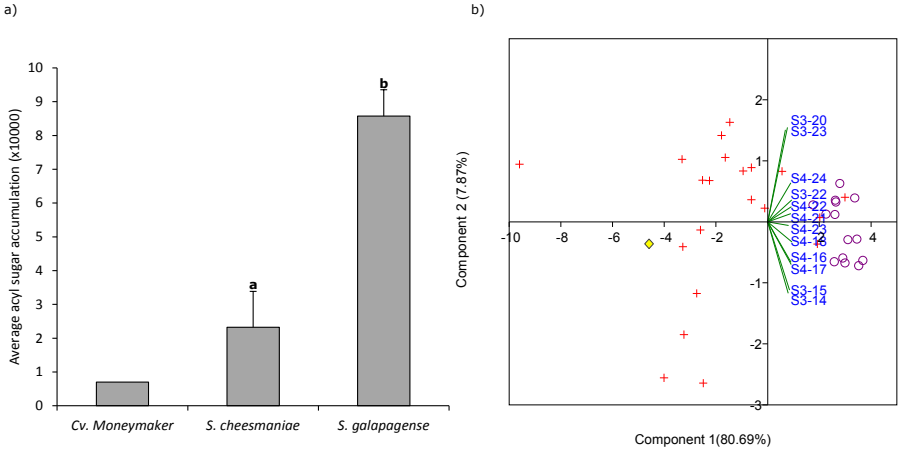

Figure 2: a) Average acyl sugar accumulation per species; values for *S. galapagense* and *S. cheesmaniae* represent means and SD of 14 and 21 plants, respectively. Cv. Moneymaker is included as reference. Different letters indicate statistical differences according to LSD test ($P < 0.05$); b) PCA-biplot score of the accessions of *Solanum galapagense* (violet circle symbol) and *S. cheesmaniae* (red plus sign symbol) based on acyl sugar accumulation. Lines indicate the loadings of the different acyl sucroses.

The LC-MS analysis allowed us to determine the relative abundance of 12 acyl sugars. All of them were acyl sucroses: five with 3 lateral branches (S3-acyl sucroses) and seven with 4 lateral branches (S4-acyl sucroses). A heatmap (hierarchical clustering) visualizing the results is shown in Figure 1 and the relative amounts measured can be found in Additional file 1: Table S1. In Figure 1, we see two clusters: one including 7 accessions of *S. cheesmaniae* and the cv. Moneymaker and the other as an intermingled cluster with all the accessions of *S. galapagense* and the remaining accessions of *S. cheesmaniae*. Total acyl sugar abundance (expressed as the total of acyl sugar peak areas per accession) was higher in plants with a higher resistance level, measured as lower values for AS, OR and PS ($R^2 = 0.37$, $P < 0.001$). In general, *S. galapagense* accessions accumulate higher levels of acyl sugars than *S. cheesmaniae* accessions. Nevertheless, there were four accessions of *S. cheesmaniae* (LA0421, LA0746, LA0521 and LA0529) with levels of acyl sucroses that were as high as those found in *S. galapagense* accessions

(Figure 2, Additional file 1: Table S1). Regression analysis did not indicate a specific relation between acyl sugar accumulation, neither qualitatively nor quantitatively, and the type of trichomes present on a plant. However, we observed a slight positive correlation between total acyl sugars and the presence of glandular trichomes type I and IV (Table 2).

Genetic relationships and correlations among accessions

We used a SNP array (Viquez-Zamora *et al.*, 2013) to determine the genetic relationships between the different accessions. The Neighbour joining tree (Figure 3), based on 3316 markers that showed polymorphisms in or among *S. lycopersicum*, *S. pimpinellifolium*, *S. cheesmaniae* and *S. galapagense*, indicated a tight cluster with all the accessions of *S. galapagense* and *S. cheesmaniae* intermingled. Two accessions of *S. cheesmaniae* (LA3124 and G1.1615) were clearly separated from the rest and are most likely hybrids with either *S. lycopersicum* or *S. pimpinellifolium*. After exclusion of these two deviating *S. cheesmaniae* accessions only 53 polymorphic markers (Additional file 2: Figure S1) were detected within *S. galapagense* and *S. cheesmaniae*. From those 53 SNPs, 44 were polymorphic among *S. cheesmaniae* accessions and 9 among *S. galapagense* accessions. Most of the polymorphic markers were randomly distributed over the two species, 7 of them were found only in one accession and 2 were unique to *S. galapagense*. None of the polymorphic markers were fixed in either species.

Correlation of species presence to geographic/climatic variables

We analysed the available geographic/climatic data for the Galapagos Islands in relation to the location where the *S. galapagense* and *S. cheesmaniae* accessions were collected, but no correlation was detected (Figure 4).

DISCUSSION

Whitefly resistance in S. galapagense, a combination of trichomes and metabolites

A difference in whitefly resistance was observed between *S. galapagense* (resistant) and *S. cheesmaniae* (susceptible). While some *Solanum* species are considered to be resistant, it is important to note that the level of resistance may vary between and within accessions of the same species. Next to resistance to whitefly (Firdaus *et al.*, 2012), such inter- and intra-variation was also described, among others, in a screenings for white mold and late blight resistance in potato (Jacobs *et al.*, 2010, Lokossou *et al.*, 2010, Jansky *et al.*, 2006). We have found resistance to whitefly in all *S. galapagense* accessions, which was accompanied by high densities of trichomes type IV and high acyl sugar accumulation. Based on available data and literature it is not known whether the original founder had type IV trichomes or not. Even though the accessions of *S.*

galapagense differ in the relative amounts of the different acyl sugars present, they were all resistant.

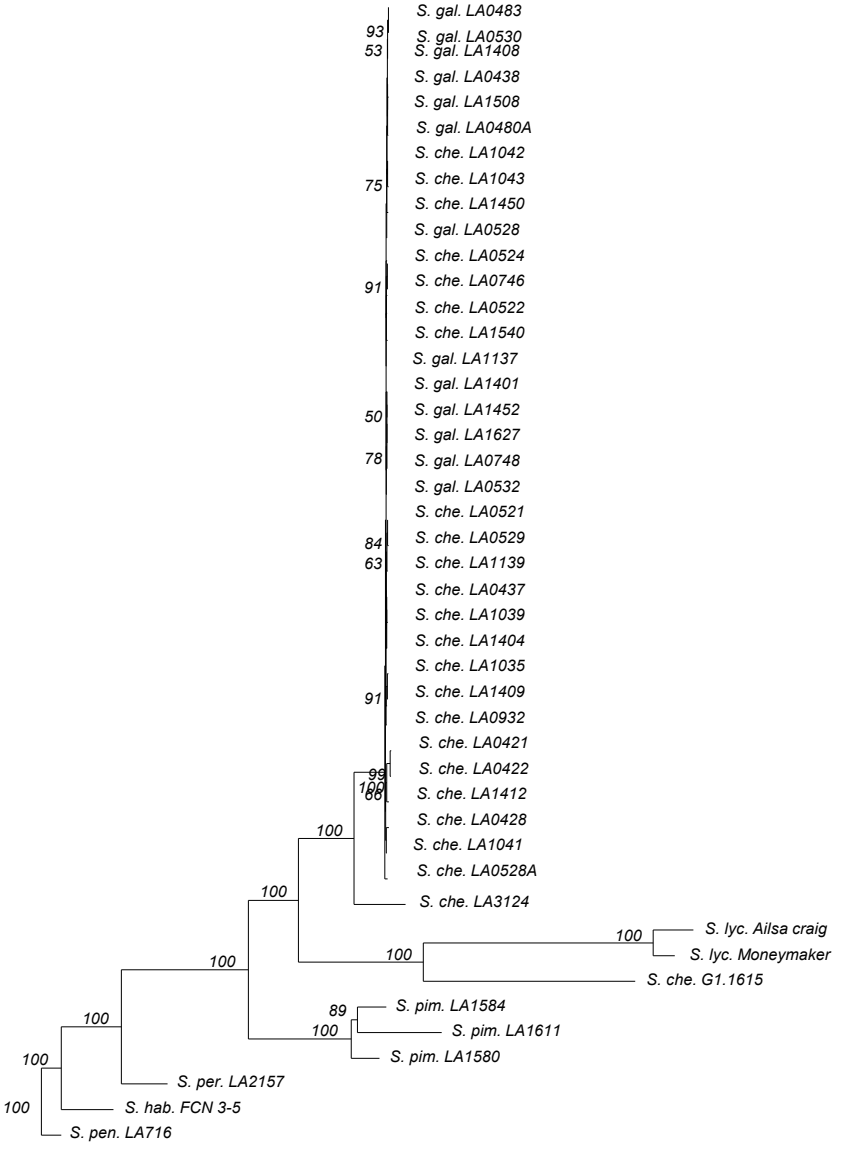

Figure 3: Neighbour-joining tree of the accessions based on Manhattan distances using 3324 polymorphic markers. Per sample the species name is followed by the accession code (*S. che*. for *Solanum cheesmaniae*; *S. gal*. for *S. galapagense*; *S. pim*. for *S. pimpinellifolium*; *S. lyc*. for *S. lycopersicum*; *S. per*. for *S. peruvianum*; *S. hab*. for *S. habrochaites* and *S. pen*. for *S. pennellii*). Bootstrap values (higher than 50) are shown on the branches.

This was not the case for *S. cheesmaniae*. Although some accessions of *S. cheesmaniae* accumulated acyl sugars to levels comparable to those found in *S. galapagense* they were all susceptible, probably because they lack trichome type IV. Contrarily, we also observed that in some accessions of *S. pimpinellifolium*, although they had trichome type IV, the levels of acyl sugars and the resistance variables were not different from those found on cv. Moneymaker (data not shown). The role of glandular trichomes and acyl sugars in insect resistance has been discussed frequently (Firdaus *et al.*, 2012, Leckie *et al.*, 2012, Muigai *et al.*, 2002, Muigai *et al.*, 2003, Simmons and Gurr, 2005). In tomato, the synthesis and accumulation of acyl sugars takes place within the glandular head of the trichome (Schilmiller *et al.*, 2012). Acyl sugars are non-specific resistance components providing resistance to a broad spectrum of insects of different feeding guilds (whiteflies, aphids, leaf miners, caterpillars, etc.) (Glas *et al.*, 2012). Based on our data it is likely that a minimum level of acyl sugars and the presence of glandular trichomes type IV are needed to achieve an effective level of resistance and a fully resistant phenotype. It also suggests that not a specific acyl sugar, but rather the total amount of acyl sugars is important for resistance. Having said so, it is possible that others metabolites, not detected by the LC-MS analysis, may be important as well.

Galapagos' endemic tomato species cannot be differentiated by genetic analysis

The clear morphological differences between *S. galapagense* and *S. cheesmaniae* with regard to their leaf morphology, trichome composition, internode length, among others, were the reason to consider them as two distinct species (Darwin *et al.*, 2003). However, our SNP array analysis based on 5528 markers (Viquez-Zamora *et al.*, 2013), of which 3316 were polymorphic among the Lycopersicon group of *Solanum* sect. *Lycopersicon*, showed only 53 polymorphisms within the two species, with only two alleles specific to *S. galapagense* and no fixed alleles. As to be expected also in the NJ tree the two species could not be separated. Similar observations have been made using AFLP markers (Nuez *et al.*, 2004). The observed low genetic variation among accessions of the two species under study is consistent with the hypothesis that these endemic species are the result of a unique founder event on the Galapagos Islands, followed by morphological divergence. The very obvious morphological differences between *S. galapagense* and *S. cheesmaniae* might be due to a limited number of genetic changes that have not been picked up by our SNP array. For instance, a single nucleotide deletion in the promoter of the *PTS*/*TKD1* gene results in a marked change of leaf complexity as seen in *S. galapagense* (Kimura *et al.*, 2008). *Solanum galapagense* and *S. cheesmaniae* are part of a monophyletic clade within *Solanum* sect. *Lycopersicon*, together with *S. pimpinellifolium* and *S. lycopersicum* (Spooner *et al.*, 2005a). Thus, based on the molecular marker analysis it might be more appropriate to consider them as morphotypes, rather than as different species. In line with this, hybrids between *S. galapagense* and *S. cheesmaniae* have been found (Darwin *et al.*, 2009). In addition, we cannot ignore the clear morphological differences (trichomes,

whitefly resistance, leaf morphology, etc.) found by us and by others (Darwin *et al.*, 2003). All this suggest that the species status of these two tomatoes should be reconsidered carefully taking into consideration the different concepts used to define the species.

Biogeographic variables, phylogenetic studies and experimental approaches can be used to answer questions like adaptation to local environments, divergence and prediction of a phenotype based on the environment where a species was found (Gonzales-Vigil *et al.*, 2012, Jansky *et al.*, 2006, Nakazato *et al.*, 2012, Nakazato and Housworth, 2011, Nakazato *et al.*, 2010).

We observed no correlation between the geographic location where the accession was collected and the occurrence of one of the two morphotypes. Some studies have addressed the population structure of wild tomatoes and external factors causing this. Caicedo and Schaal (2004) observed a clear structure in *S. pimpinellifolium* populations collected from northern to southern Peru, which was consistent with a hypothesis based on genetic isolation by distance. It was also observed that genetically closely related accessions can be found far away from each other. In our analysis, we also saw a correlation between genetic distance and geographical distance (Additional file 2: Figure S1), however this has to be considered with caution as the low level of genetic variation may cause artefacts. Zuriaga *et al.* (2009) extended the analysis of Caicedo and Schaal (2004) by including accessions from Ecuador as well, and they suggested that the population structure could be better explained by ecological and climatic variables rather than by geographic distances. Similar results were obtained for populations of *Solanum habrochaites* (Gonzales-Vigil *et al.*, 2012, Kim *et al.*, 2012) were geographical and climatic variables explain a substantial amount of the variation in terpenoids and acyl sugars. However, in our analysis, we did not see such correlation between climatically or geographical conditions at the collection sites of the accessions and the morphological species boundaries.

Can selection pressure explain morphological differences?

Starting from the generally accepted assumption (Rausher, 2001, Strauss *et al.*, 2002) that maintenance of a constitutive resistance mechanism like the one present in *S. galapagense* (i.e. trichomes, acyl sugars) is energetically expensive and thus only viable when there is a high selection pressure. One possible explanation to consider is that the resistance mechanisms found in *S. galapagense* serves other functions as well. In other words, resistance is a secondary function of a trait evolved in response to some other biotic or abiotic pressure (Rausher, 2001, Flanders *et al.*, 1997). For example, a leaf surface characteristic such as high trichome density may be a direct defence against insects or pathogens like bacteria. It may also be an indirect defence mechanism against viruses although no reports mention the occurrence of whitefly transmitted viruses in *S. galapagense* and/or *S. cheesmaniae*.

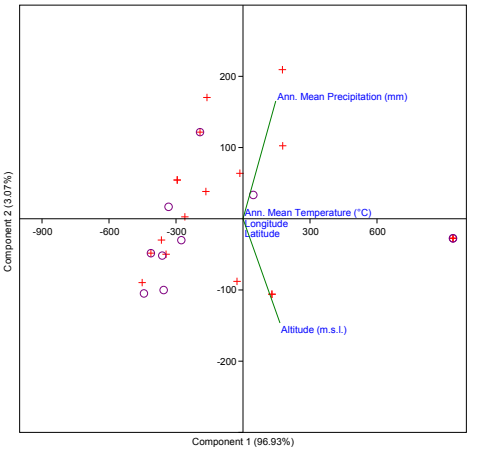

Figure 4: PCA-biplot score of the accessions of *Solanum galapagense* (violet circle symbol) and *S. cheesmaniae* (red plus sign symbol) based on geographic/climatic variables.

Trichomes might be of even greater adaptive value against other environmental stresses such as water loss. Flanders *et al.* (1997), working with potato accessions distributed from USA to Argentina, attributed the observed resistance patterns towards potato herbivores (potato aphid, Colorado potato beetle, potato flea beetle and potato leafhopper) to variation in geographical (altitude) and climatic conditions. Specifically, they found that accessions from hot and dry areas were more resistant to Colorado potato beetle and potato flea beetle. However, considering that *S. galapagense* and *S. cheesmaniae* are found in sympatry in space and time (Darwin, 2009, Nuez *et al.*, 2004) and that within our set of accessions there was no significant difference between *S. galapagense* and *S. cheesmaniae* for any of the climatic/geographical variables, it is unlikely that maintenance of the resistance mechanisms could be explained by this hypothesis.

Another plausible explanation is that resistance traits in tomato (trichomes, secondary metabolites) are under selective pressure. It was observed that *S. galapagense* is more abundant and widespread in undisturbed areas than *S. cheesmaniae* (Nuez *et al.*, 2004). Although it has being proven that trichomes and their exudates also have an effect against other pathogens (Nonomura *et al.*, 2009), we have focused our analysis on whiteflies. Though, *B. tabaci* was only recently found on the Galapagos Islands (1998) (Causton *et al.*, 2006), numerous other herbivores sharing the same feeding guilds (i.e. *Myzus persicae*, *Macrosiphum sp.*) are present (Causton *et al.*, 2006, Wilson *et al.*, 2013). Recently it was shown that even the selective pressure of a single insect herbivore species (either a generalist or a specialist) can be strong enough to shift the allele frequencies of a plant population within a few generations (Agrawal *et al.*, 2012, Züst

et al., 2012, Hare, 2012). Züst *et al.* (2012), working with *Arabidopsis* populations and aphids (a generalist phloem-feeder), reported that the trichome densities of *Arabidopsis* plants in presence of herbivores remains constant over generations and decrease in an herbivore free environment. The authors also proved that aphid populations had an effect on the frequency of the plant genotypes with different aliphatic glucosinolates. It was also proven that there was an indirect effect of herbivory selection pressure in the sense that some accessions were able to compete with others only in the absence of herbivores, showing the ecological benefits of having a resistance trait only in the presence of herbivores. Agrawal *et al.* (2012) provided evidence that herbivory by a specialist insect pest (seed predator moth) can act as a direct selective force in favour of the resistance, but also as an indirect selective force to enhance competitive ability of the plant in the presence of herbivores. All this information provides evidence to support the hypothesis that the current resistance mechanisms present in *S. galapagense* could be maintained by selection pressure, rather than by geographical/climatic variables. This hypothesis can explain how it is possible for the plant to maintain a high level of morphological differentiation (resistance level, trichome composition) with a relatively low genetic variation and in the presence of gene flow.

CONCLUSIONS

Our results show that whitefly resistance was found exclusively in *S. galapagense* accessions and that it was associated with the presence of type IV trichome and high levels of acyl sugars. Our marker and metabolomics data support the hypothesis that *S. galapagense* and *S. cheesmaniae* might be morphotypes rather than two species and that their co-existence is likely the result of a selective pressure.

MATERIALS AND METHODS

Plant materials and growing conditions

In total, we evaluated 35 tomato accessions (Table 1, Additional file 1: Table S1), covering the geographical distribution as much as possible (Figure 5). These included 22 accessions of *Solanum cheesmaniae* L. Riley, 12 accessions of *S. galapagense* S.C. Darwin & Peralta, and 1 accession of *S. lycopersicum* L. as reference. The accessions were grown in a greenhouse at Wageningen UR Plant Breeding, Wageningen, the Netherlands (20 ± 2°C, 70% RH, 16/8 h day/night) in 14 cm pots. The plants were fertilized twice a week and watered once a day. When the plants were six weeks old, they were moved to an insect proof greenhouse. One week before infestation the greenhouse temperature was increased slowly (two degrees per day) from 20 till 27°C to allow plants to adapt to the higher temperature (27 ± 2°C, 70% RH, 16/8 h day/night).

Insect rearing

A non-viruliferous whitefly rearing (*Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I) was maintained on the tomato cultivar Moneymaker for several generations at Wageningen UR Plant Breeding, Wageningen, The Netherlands. The initial inoculum was obtained from a permanent rearing at the Laboratory of Entomology, Wageningen UR, Wageningen, The Netherlands.

No-choice test

Whiteflies were anesthetized using CO_2 and four days old females were selected under a binocular microscope by the morphology of the abdomen. Five females were placed into a clip-on cage (2.5 cm in diameter and 1.0 cm in high). Three clip-on cages per plant and four plants per accession were used. The cages were placed on the first to third fully expanded leaf, counting from the top of the plant, thereby taking care not to break the plant trichomes when assembling the cages. Five days after infestation, the number of death and alive whiteflies, as well as the number of eggs was registered from that day on. The surviving whiteflies were removed from the leaves and the adult survival (AS) and oviposition rate (OR) were calculated according to Bas *et al.* (1992). Due to the fact that the hatching of the eggs was irregular in time, we could not assess the number of newly hatched insects per day in order to calculate the development period (DP). When almost no new whiteflies were seen, the number of empty pupae was recorded and the pre-adult survival (PS) calculated (Bas *et al.*, 1992). A complete randomize assay with four replicas per accession was used. Each replica consisted of the average value of three cages per plant (technical replica). The variables were analysed with a one-way ANOVA followed by a least significant difference (LSD) test (Zar, 2010). An Arcsin (Sqrt) transformation was applied to the variables adult survival (AS) and pre-adult survival (PS), whereas an Sqrt (x + 1) transformation was applied to the variable oviposition rate (OR). All statistical procedures were performed using the statistical software package Infostat Professional (2010) Cordoba, Argentina.

Trichome description

Trichomes present on the abaxial side of the leaf were classified according to type (Channarayappa *et al.*, 1992). For an estimation of trichome density, the abaxial part of three leaflets was observed under the binocular microscope and a visual scale was used to describe it. The scale used was adapted from Simmons and Gurr (2005) and consisted of four categories: 3, Abundant (>5 per mm^2); 2, sparse (5–1 per mm^2); 1, very sparse (<1 per mm2), and 0, absent.

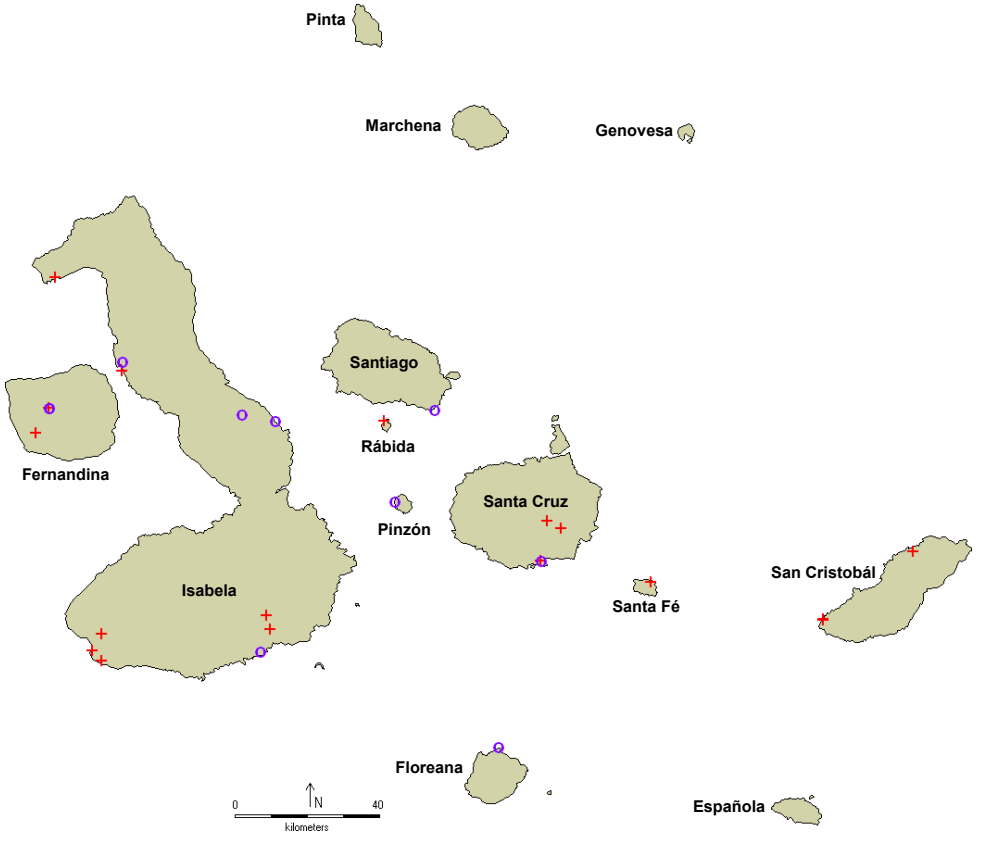

Figure 5: Geographical distribution of the tomato accession on the Galapagos Islands. *Solanum galapagense* S.C. Darwin & Peralta (violet circle symbol); *S. cheesmaniae* L. Riley (red plus sign symbol). Map made with DIVA-GIS version 7.5

Genotyping and phylogenetic analysis

Genomic DNA was extracted from one randomly selected plant per accession as described by Fulton *et al.* (1995). The DNA concentration was adjusted to 50 ng/µl. For marker analysis a custom made single nucleotide polymorphism (SNP) Infinium bead array was used (Viquez-Zamora *et al.*, 2013). On this array 5528 tomato SNPs were present. The marker analysis was performed using the protocol provided by Infinium and carried out by Service XS, Leiden, The Netherlands. Marker data obtained were filtered using the following criteria: 1) monomorphic markers were removed; 2) markers were deleted when the frequency of heterozygotes per marker was equal to or higher than 5%, and 3) markers were deleted when the frequency of no-calls (NC) was equal to or higher than 50%. We choose a threshold of 50% for the NC because

they can be the result of either an amplification problem (true NC) or the presence of a different, informative allele (false NC). The latter situation occurs frequently with more distantly related species (Viquez-Zamora *et al.*, 2013). After filtering a total of 3316 markers were used in the analysis. A phylogenetic tree was reconstructed by Neighbour Joining, using the Manhattan distance (Additional file 1: Table S1). The reliability of the resulting dendrogram was assessed by bootstrap analysis with 1000 replications. The analysis was carried out using the software package PAST (Hammer *et al.*, 2001). Isolation By Distance (IBD, version 3.23) (Jensen *et al.*, 2005) was used to analyse for presence of isolation by distance.

LC-QTOF-MS analysis

Four plants per accession were used for the chemo-profiling. From each plant, one complete leaf (second fully expanded leaf from the top of the plant) was cut, placed into an aluminium envelope and immediately frozen in liquid nitrogen. Each sample was frozen with liquid nitrogen and ground to a fine powder and storage at -80°C until use. Extraction and analysis by Liquid Chromatography-Quadrupole Time of flight-Mass Spectrometry (LC-QTOF-MS) (De Vos *et al.*, 2007, Firdaus *et al.*, 2013a). Four hundred mg of frozen leaf powder was put into a glass tube with 1.2 mL of methanol/formic acid solution (99.9% - 0.1%). The samples were mixed using vortex, sonicated for 15 min and centrifuged at 2500 rpm for 10 min. The supernatant was filtered using a 0.45 μm filter, injected (5 μl) using an Alliance 2795 HT instrument (Waters), separated on a Phenomenex Luna C18 (2) column (2.0 × 150 mm, 3 mm particle size) using a 5–95% gradient of acetonitrile in water (both acidified with 0.1% formic acid) in 45 min and then detected by a Water-Micromass QTOF Ultima MS with electrospray ionization in negative mode (m/z 80–1,500). Annotation of LCMS peaks corresponding to acyl sugars was done on their accurate masses as previously described in Firdaus *et al.* (2013a). The Quanlynx tool of the Masslynx acquisition software was used to calculate the relative abundance (peak area) of the different type of acyl sugars for all samples, based on their specific mass and retention times. The three isoforms of S3-20 (I, II, III) and the four of S3-22 (III – VI), as described before in Firdaus *et al.* (2013a), were considered as single peaks for S3-20 and S3-22, respectively, due to partly overlapping chromatographic peaks of these isomeric compounds in many samples. The peak areas were LOG_2 (x + 1) transformed and auto scaled to the mean. PCA-biplot was done with the software package PAST (Hammer *et al.*, 2001), and all other statistical analysis were performed with the software package GeneMaths XT (version 2.21; Applied Maths, Belgium).

Analysis of geographical distribution and climate data

The collection site information (latitude and longitude) of the accessions was obtained from the Tomato Genetic Resource Centre (TGRC). Locations and climate data were obtained from the WorldClim at 2.5 arc-min resolution and were analysed as proposed

by Gonzales-Vigil *et al.* (2012), and using DIVA-GIS (Hijmans *et al.*, 2012). A regression analysis was carried out between the resistance variables (AS, OR, PS), the metabolites and the climatic/geographical variables (Altitude, Latitude, Longitude, Ann. Mean precipitation, Ann. Mean temperature).

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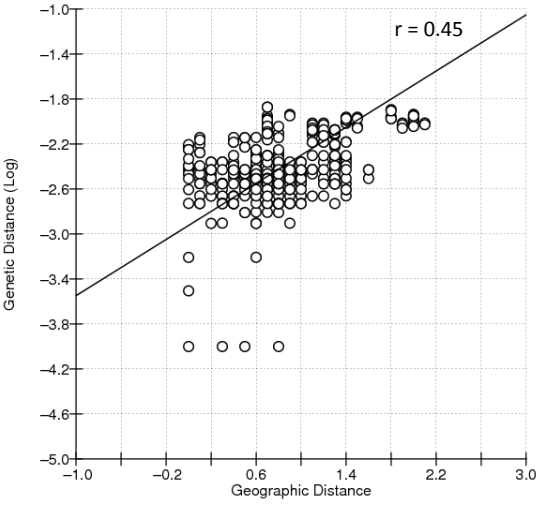
SUPPLEMENTARY DATA

Additional file 1: Table S1: This file includes GeneBank origin, accession numbers, collection sites and all phenotypic information, SNP array data and LC-MS data for the material used in this work. The additional file 1 can be found in this link: http://www.plantbreeding.wur.nl/Publications/Alejandro/Additional file 1_Chapter 2_AFL.xlsx

The Phylogenetic tree was uploaded to TreeBASe.org, and can be found in this link: http://www.plantbreeding.wur.nl/Publications/Alejandro/1_1374317634_Phylogenetic_anaysis_of_S_galapagense_and_S_cheesmaniae.nexrct

Additional file 2: Figure S1: Isolation by distance analysis. This figure describes the relation between the Genetic distances and the Geographic distances.


r = 0.45
Genetic Distance (Log)
−1.0
−1.4
−1.8
−2.2
−2.6
−3.0
−3.4
−3.8
−4.2
−4.6
−5.0
−1.0
−0.2
0.6
1.4
2.2
3.0
Geographic Distance

Chapter 3

Non-preference of *B. tabaci* on tomato genotypes

Alejandro F. Lucatti[1,2], Roland Mumm[3,4], Ric de Vos [3,4,5], Richard GF Visser[1], Sjaak van Heusden[1], Ben Vosman*[1]

1 Wageningen UR Plant Breeding. Wageningen University and Research Centre. PO Box 386, 6700 AJ Wageningen, The Netherlands.

2 Graduate School Experimental Plant Sciences. Wageningen Campus. Droevendaalsesteeg 1, Wageningen, The Netherlands.

3 Plant Research International, Business Unit Bioscience, Wageningen University and Research Centre, P.O. Box 619, 6700AP, Wageningen, The Netherlands.

4 Centre for BioSystems Genomics, P.O. Box 98, 6700 AB, Wageningen, The Netherlands.

5 Netherlands Metabolomics Centre, Einsteinweg 55, 2333 CC, Leiden, The Netherlands.

Abstract

Background- Bemisia tabaci use a set of plant-derived cues in the process of host plant selection. It recognizes mainly monoterpenes (p-cymene, γ-terpinene and β-myrcene, α-phellandrene) and sesquiterpenes (7-zingiberene and r-curcumene). Previously, it was shown that the line FCN93-6-2 was non-preferred by *Trialeurodes vaporariorum* compared to its sibling line FCN13-1-6-1. The FCN lines were derived from a cross between cv. Uco plata INTA and *S. habrochaites* (FCN3-5) followed by a pedigree selection program focused on insect resistance. The aim of this work was to identify chemical cues produced by tomato that affect the preference of the whitefly *B. tabaci* and to identify the chromosomal region(s) of *S. habrochaites* harbouring the genes involved in the preference.

Results- We have characterized two accessions of *S. habrochaites* and four *S. lycopersicum* for whitefly resistance using a series of no-choice and choice experiments. No differences were found among the *S. lycopersicum* genotypes in the no-choice test. The two *S. habrochaites* accessions (CGN1.1561 and in FCN3-5) and the line FCN93-6-2 were non-preferred by *B. tabaci* in two types of choice experiments. The non-preference was independent of trichome type IV and of the presence of methyl-ketones. On average, the non-preferred genotypes had lower concentrations of monoterpenes (β-phellandrene). The non-preference of FCN93-6-2 might be associated with introgressions on Chromosome 5, 6 and 11 originating from *S. habrochaites*.

Conclusion- We identified a tomato line (FCN93-6-2) that is not preferred by the *B. tabaci* both, when the whiteflies were in direct contact with the plant but also when the whiteflies were offered with olfactory cues only. We identify candidate metabolites linked to the no preference. Functional validations of the candidate metabolites and of the different introgressions are still needed.

Keywords: whitefly, *S. habrochaites*, monoterpenes, choice experiment, GC-MS, LC-MS.

INTRODUCTION

Tomatoes are affected by a wide range of biotic stresses, of which whiteflies are one of the most important. Among the whiteflies, *Bemisia tabaci* (Group Mediterranean-Middle East-Asia Minor I) and *Trialeurodes vaporariorum* (Westwood) are the most important. These two whiteflies are highly polyphagous generalists, which can feed on more than 74 different plant families. This number is continuously increasing as these pest insects invade new areas and plant species (McAuslane, 1996). Recent research suggests that *B. tabaci* is not just one species but a complex of at least 36 cryptic species (De Barro, 2012, Firdaus *et al.*, 2013b, Dinsdale *et al.*, 2010, Liu *et al.*, 2012). Whiteflies feed directly from the phloem sap, depleting the plant resources and thereby reducing plant growth. In addition, whiteflies can also cause indirect damage through the transmission of viruses in both a circulative and non-circulative manner (Muigai *et al.*, 2002, Valverde *et al.*, 2004). Phloem consumption on its own can lead to a yield reduction of up to 50% and, when combined with virus transmission, yield losses can go up to 100% (Brown and Bird, 1992, Byrne and Bellows Jr, 1991).

Herbivorous insects use a set of plant derived cues (visual, olfactory, mechano-sensory and gustatory) during the process of host plant selection (Schoonhoven *et al.*, 2005). Attempts have been made to identify the cues in the whitefly-host plant selection. The first studies point out that whiteflies use mainly visual cues in the process of host plant selection, giving a minor role to olfactory cues (Mound, 1962, Vaishampayan *et al.*, 1975). Other studies showed that whiteflies can recognize secondary metabolites and that those chemical cues can affect the host plant preference (Bleeker *et al.*, 2009, Zhang *et al.*, 2004). In *Solanum pennellii* mainly monoterpenes (p-cymene, γ-terpinene and β-myrcene, α-phellandrene) are involved in plant repellence of whiteflies, whereas in *S. habrochaites* (PI127826) sesquiterpenes (zingiberene and curcumene) were the most important (Bleeker *et al.*, 2009). Whiteflies are not only able to use chemical cues in the process of host plant selection, but they are also capable of differentiating between stereoisomers of certain plant metabolites. It was shown that the repellent effect of zingiberene depended on the stereoisomer. Whiteflies were capable of recognizing the stereoisomer 7-epizingiberene and its derivate compound *R*-curcumene, but not zingiberene and *S*-curcumene (Bleeker *et al.*, 2011).

Glandular trichomes of tomato have been shown to be the place of synthesis and storage of several types of secondary metabolites (Glas *et al.*, 2012, Schilmiller *et al.*, 2008). From a metabolic point of view, we can differentiate two types of *S. habrochaites* accessions, those that produce mainly methyl ketones and those that produce mainly sesquiterpenes. In the *S. habrochaites* accessions that accumulate methyl ketones, the synthesis is located in the glandular head of type VI trichomes (Antonious, 2001, Ben-Israel *et al.*, 2009, Fridman *et al.*, 2005); whereas, in the *S. habrochaites* accessions that

accumulate sesquiterpenes, the synthesis is specific for trichomes type IV (Bleeker *et al.*, 2012). In *S. pennellii*, *S. pimpinellifolium* and *S. galapagense* the synthesis of acyl sugars is associated with the presence in high densities of trichomes type IV, but in some accessions of *S. cheesmaniae,* that accumulate high levels of acyl sugars and lack trichomes type IV, it is probable that the synthesis is located in trichomes type VI (Firdaus *et al.*, 2013a, Lucatti *et al.*, 2013, Leckie *et al.*, 2012, Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012a). Previously, it was shown in a free choice experiment that one pre-breeding line (FCN93-6-2) was non-preferred by the greenhouse whitefly (*T. vaporariorum*) in contrast to its sibling line FCN13-1-6-1 and the parental accession cv. Uco plata INTA (Lucatti *et al.*, 2010). The pre-breeding line FCN93-6-2 as well as the sibling line FCN13-1-6-1, were homozygous lines selected from a cross between *S. habrochaites* (FCN3-5, a methyl ketone producer) and cv. Uco Plata INTA. These two sibling lines were selected mainly based on their higher level of resistance to herbivores (*Tuta absoluta* and *Tetranychus urticae*) (Gilardón, 2007). However, there was a clear difference for whitefly preference between these two lines. The mechanisms underlying the difference in preference remain unknown. The aim of this work was to identify chemical cues produced by tomato that affect the preference of the whitefly *B. tabaci* and to identify the chromosomal region(s) of *S. habrochaites* harbouring the genes involved in the preference. We found that the pre-breeding line FCN93-6-2 and two accessions of *S. habrochaites* were differentially preferred by *B. tabaci* compared to the reference cv. Moneymaker. This difference was observed when the whiteflies were in direct contact with the plant but also when the whiteflies could detect olfactory cues only. The three non-preferred genotypes had nine metabolites in lower concentrations (of which seven were monoterpenes) and seven in higher concentrations as cv. Moneymaker. The non-preference of these genotypes might be associated with three introgressions from *S. habrochaites*.

RESULTS

No-choice experiment

The level of whitefly resistance was assessed using the parameters; adult survival (AS), oviposition rate (OR), pre-adult survival (PS) and development period (DP) (Table 1). For all parameters, significant differences were found among the genotypes (ANOVA, $P<0.001$).

Table 1: Results from the No-choice experiment. Mean values (± SD) per genotype and variable.

Genotype	No-choice test							
	Adult survival		Oviposition rate		Pre-adult survival		Development period	
CGN1.1561	0.1	± 0.2[a]	1.5	± 0.71[a]	0.3	± 0.21[a]	25.7	± 0.92[b]
FCN3-5	0.8	± 0.1[b]	5.6	± 1.99[b]	0.4	± 0.11[ab]	25.0	± 0.91[a]
LC138	0.9	± 0.15[bc]	9.1	± 2.43[de]	0.8	± 0.12[d]	25.3	± 0.60[ab]
cv. Uco plata INTA	0.9	± 0.04[bc]	6.7	± 2.68[bc]	0.7	± 0.27[cd]	27.1	± 0.76[c]
FCN93-6-2	0.9	± 0.10[bc]	10.0	± 1.91[de]	0.7	± 0.08[c]	27.1	± 0.70[c]
FCN13-1-6-1	0.9	± 0.05[bc]	10.3	± 2.86[e]	0.6	± 0.06[bc]	27.0	± 0.73[c]
cv. Moneymaker	1.0	± 0.03[c]	7.9	± 1.97[cd]	0.6	± 0.16[c]	26.7	± 0.55[c]

Different letters indicate statistical differences according to LSD (P≤0.05).

For AS, CGN1.1561 had the lowest value, whereas FCN3-5 was not statistically different from two of the susceptible controls (cv. Uco Plata INTA and LC138). With respect to OR, the two accessions of *S. habrochaites* had low values, and the two sibling lines high values. In the case of PS, two main groups were identified; the first group including CGN1.1561 and FCN3-5 (*S. habrochaites*), where less than half of the eggs reached the adult stage. The line FCN13-1-6-1 was shared between the first and the second group including all other genotypes where up to 80% of the eggs reached the adulthood. For DP two groups were identified, LC138 and the two *S. habrochaites* accessions, and a second group containing the rest of the genotypes (Table 1).

Table 2: Results from the trichome type and density measurements. Mean values (± SD) per genotype and variable.

Genotype	Trichome type							
	III		IV		V		VI	
CGN1.1561	0.9	± 0.01[b]	18.1	± 0.33[b]	0.0	± 0.65[a]	3.2	± 0.96[c]
FCN3-5	0.0	± 0.00[a]	1.2	± 0.09[a]	1.1	± 0.10[ab]	0.5	± 0.04[c]
LC 138	0.0	± 0.01[ab]	-	-	2.0	± 0.12[b]	0.1	± 0.02[a]
cv. Uco plata INTA	0.1	± 0.01[c]	-	-	3.4	± 0.27[c]	0.3	± 0.04[b]
FCN93-6-2	0.0	± 0.01[ab]	-	-	3.2	± 0.15[c]	0.3	± 0.04[b]
FCN13-1-6-1	0.1	± 0.01[bc]	-	-	2.8	± 0.16[c]	0.2	± 0.02[ab]
cv. Moneymaker	0.0	± 0.00[a]	-	-	1.8	± 0.18[b]	0.3	± 0.05[b]

Different letters indicate statistical differences according to Kruskal-Wallis test (P≤0.05)

Choice experiment

The leaf disc test showed that the whiteflies had the ability to differentiate among genotypes. The accessions CGN1.1561 (P< 0.01), FCN3-5 (P< 0.01) and the line FCN93-6-2 (P= 0.02) were not preferred by the whiteflies compared to cv. Moneymaker. For the other genotypes, the whiteflies could not make a distinction when compared to cv. Moneymaker (Figure 1).

With the static two-chamber olfactometer test, where no direct contact between the whiteflies and the plant was allowed, the whiteflies were also able to discriminate between genotypes. The accessions CGN1.1561, FCN3-5, and the line FCN93-6-2 were not or less preferred by the whiteflies on at least one time point when compared to cv. Moneymaker, whereas for the other genotypes, the whiteflies were not able to discriminate them from cv. Moneymaker (Figure 2). The leaf disc test was positively correlated with the static two-chamber olfactometer test at 30 minutes after inoculation (r=0.83, P=0.01), 90 minutes after inoculation (r=0.60, P=0.04) and at 120 minutes after inoculation (r=0.77, P=0.02) (Table 3).

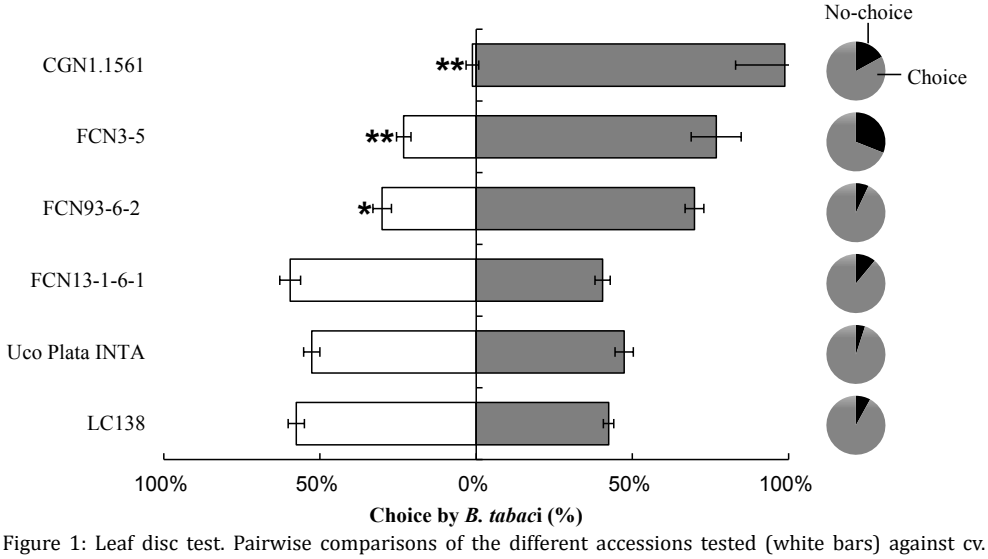


Figure 1: Leaf disc test. Pairwise comparisons of the different accessions tested (white bars) against cv. Moneymaker (grey bars). From top to bottom, CGN1.1561, FCN3-5 (both *S. habrochaites*), FCN93-6-2, FCN13-1-6-1, cv. Uco plata INTA, and LC138 (all *S. lycopersicum*). One hundred whiteflies were used for each comparison. Circles denote proportion of whiteflies that did not make a choice (in black) and those they did make a choice (in grey). Asterisk (*) indicates statistical differences according Mann-Whitney U test (*P≤0.05, **P≤0.01).

Trichome Type and Density

Trichome type IV was exclusively found on the two *S. habrochaites* accessions and

there were differences in the density of trichome type IV between CGN1.1561 and FCN3-5 (Table 2). Trichome type V was the most abundant type of trichome on the *S. lycopersicum* genotypes. Different trichome densities were observed among the *S. lycopersicum* accessions for type V and VI (Table 2). A significant negative correlation was found between the presence of trichome type IV and AS, OR and PS; and also between the density of trichome type VI and OR and PS. A positive correlation was found between the density of trichomes type V and OR and PS (Table 3).

Untargeted metabolomic analysis

In the GC-MS analysis, 149 compounds were detected. The principal component analysis showed two clear clusters, the cluster with the *S. habrochaites* accessions (CGN1.1561 and FCN3-5) and the cluster with the *S. lycopersicum* genotypes (FCN13-1-6-1, FCN93-6-2, cv. Uco plata INTA, LC 138 and cv. Moneymaker) (Figure 3). Several methyl ketones (2-tridecanone, 2-undecanone, 2-pentadecanone, etc.) were found in high abundance exclusively in the two *S. habrochaites* accessions (CGN1.1561 and FCN3-5) without differences between accessions (Additional file 1). Differences were found for eight compounds between preferred and non-preferred genotypes, seven monoterpenes and one alkane (Table 4). All monoterpenes were detected in lower relative concentrations in the non-preferred accessions (Table 4). In the LC-MS analysis, 323 compounds were detected and eight of them were related to whitefly preference. They were identified as acyl sugars (S3:10, S3:18 II), a steroid glycoside (dehydrotomatoside), a flavonoid (myricetin methyl ester) and four unknowns (Table 5). From those eight, seven metabolites were detected in higher abundance and one in lower abundance in the non-preferred accessions. Figure 4 depicts a correlation analysis over the statistically significant metabolites (both GC-MS and LC-MS). It can be seen that the relative abundance of all the GC-MS metabolites is positively correlated among each other. The same is true for the LC-MS metabolites with the exception of the compound 4771. This compound is the only one that is present in higher concentrations in the preferred genotypes. Between the GC-MS and the LC-MS data, an overall negative correlation was observed.

Chromosomal regions associated with non-preference

A SNP array (Víquez-Zamora *et al.*, 2013) was used to determine the size and the position of the introgressions in the genotypes under investigation. FCN93-6-2 had three introgressions not present in its sibling line FCN13-1-6-1. The three introgressions are located on Chromosome (Chr.) 5 (between 41.9Mbp and 60.62Mbp with 480 annotated genes), Chr. 6 (0.53Mbp to 2.92Mbp with 248 annotated genes) and Chr. 11 (4.94Mbp to 5.38Mbp with 57 annotated genes). A search on the tomato reference genome was performed for the annotated genes on the introgressions on Chr. 5, 6 and 11. As filtering criteria we used the key words “terpene” and “flavonoid” to get the genes involved

Table3: Spearman correlation among variables measured in the no-choice test, the leaf disc test, the static two-chamber olfactometer test and the trichome density.

	No choice test				Leaf -disc	Static two-chamber olfactometer test					Trichome type			
	AS	**OR**	**PS**	**DP**		**10 min.**	**30 min.**	**60 min.**	**90 min.**	**120 min.**	**III**	**IV**	**V**	**VI**
AS	-	*	0.09	*	*	0.23	*	0.12	0.07	0.14	0.23	**	*	0.06
OR	**0.90**	-	0.07	0.11	**	0.23	*	0.23	0.14	0.12	0.14	**	**	*
PS	0.46	0.49	-	0.12	*	0.20	*	0.23	0.20	0.06	0.18	**	*	*
DP	0.64	0.37	0.31	-	0.14	*	*	0.11	0.20	0.17	0.09	0.06	0.20	0.14
Leaf-disc test	**0.81**	**0.83**	0.66	0.26	-	0.12	**	0.09	*	*	0.23	**	**	**
Choice_10	0.03	0.03	-0.09	-0.60	0.31	-	0.18	0.17	*	*	0.11	0.23	0.18	0.23
Choice_30	**0.81**	**0.60**	**0.71**	0.60	**0.83**	0.14	-	0.06	*	*	0.18	**	*	*
Choice_60	0.34	0.06	0.03	0.38	0.44	0.20	0.58	-	*	0.07	*	0.18	0.23	0.11
Choice_90	0.49	0.26	0.09	0.09	**0.60**	0.71	0.66	0.75	-	*	0.18	0.14	0.17	0.12
Choice_120	0.29	0.31	0.54	-0.20	**0.77**	0.60	0.60	0.52	0.66	-	0.20	0.09	*	*
Trichome type III	-0.06	-0.26	-0.14	0.43	-0.03	-0.37	0.14	0.75	0.14	0.09	-	0.18	0.14	0.17
Trichome type IV	**-0.86**	**-0.85**	**-0.85**	**-0.54**	**-0.85**	0.07	**-0.85**	-0.14	-0.27	-0.44	0.17	-	**	*
Trichome type V	0.64	**0.83**	0.77	0.09	**0.89**	0.14	0.60	0.03	0.20	0.66	-0.26	-0.85	-	**
Trichome type VI	-0.58	-0.66	-0.77	-0.26	**-0.89**	-0.03	**-0.71**	-0.41	-0.31	-0.77	-0.20	0.78	-0.89	-

Below main diagonal is the correlation coefficient and upper the main diagonal is the P-value associated. Asterisk (*) indicate P≤0.05, double asterisks (**) indicate P≤0.01.

in these pathways (Chibon *et al.*, 2012, Sato *et al.*, 2012). For genes involved in the terpenoid pathway, we found nine candidate genes, two located in the introgression of Chr. 6 and seven located in the introgression on Chr. 5.

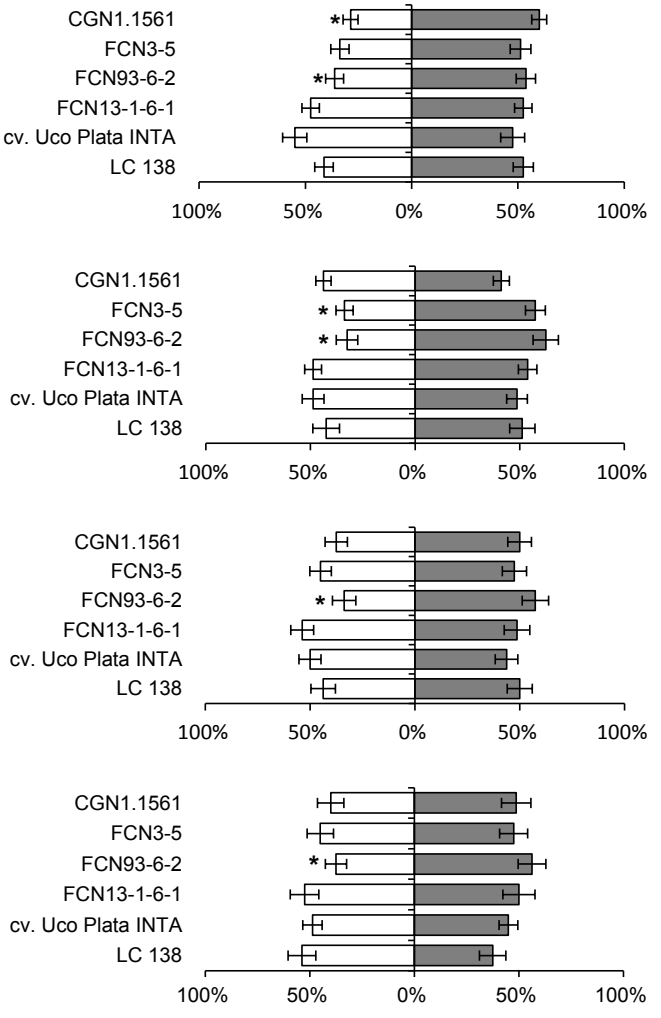


Figure 2: Static two-chamber olfactometer test. Pairwise comparisons of the different accessions tested (in white) against cv. Moneymaker (in grey) at different time points (from top to bottom 30, 60, 90 and 120 min). Asterisk (*) indicates statistical difference according Binomial distribution test (P≤0.05).

Of the seven on Chr. 5, the genes Solyc05g026590.1.1 and Solyc05g026600.1.1 were monoterpenes synthases. The first gene (Solyc05g026590.1.1) codes for a Beta-ocimene synthase that catalyse the synthesis of (E)-beta-ocimene and myrcene from geranyl diphisphate (solgenomics.net). The second gene (Solyc05g026600.1.1) codes for a

Table 5: Relative abundance of metabolites (mean ± SD) that show significant differences among preferred and non-preferred accessions.

	ID	Putative identity	Non-preferred			Preferred			
			CGN1.1561 (n=5)	FCN3-5 (n=5)	FCN93-6-2 (n=5)	cv. Uco plata INTA (n=5)	LC 138 (n=5)	FCN13-1-6-1 (n=5)	cv. Moneymaker (n=4)
Volatiles (GC-MS)	54	α-Pinene	1678 ± 102	1726 ± 79	1958 ± 62	17133 ± 910	11882 ± 1613	10628 ± 1245	3349 ± 205
	235	2-Carene	6699 ± 762	5501 ± 104	6626 ± 348	74884 ± 4851	41176 ± 5765	55776 ± 6437	16839 ± 1208
	281	α-Phellandrene	1917 ± 98	1995 ± 71	1985 ± 19	18940 ± 1591	9718 ± 1710	11386 ± 1897	3019 ± 238
	348	β-Cymene	3220 ± 182	3786 ± 231	3397 ± 119	22386 ± 4021	8357 ± 1055	8726 ± 705	3867 ± 74
	427	β-Phellandrene	15396 ± 2465	29951 ± 4180	19767 ± 1500	217756 ± 15982	134604 ± 19985	139513 ± 17051	50030 ± 2617
	523	g-Terpinene	688 ± 49	958 ± 67	745 ± 48	6098 ± 855	3852 ± 734	3653 ± 614	1154 ± 108
	565	Terpinolene	658 ± 51	706 ± 27	561 ± 25	5357 ± 605	2590 ± 471	2674 ± 582	890 ± 28
	7161	Tetratetracontane	420748 ± 46595	477714 ± 33369	799718 ± 101751	1166633 ± 140895	1571938 ± 156534	1073648 ± 110650	994935 ± 115168
			CGN1.1561 (n=2)	FCN3-5 (n=2)	FCN93-6-2 (n=2)	cv. Uco plata INTA (n=2)	LC 138 (n=2)	FCN13-1-6-1 (n=2)	cv. Moneymaker (n=2)
Non-volatiles (LC-MS)	755	Unknown	11161 ± 44.4	7934 ± 54.5	8556 ± 155.7	4621 ± 88.8	5854 ± 230	6255 ± 124.8	5358 ± 104.7
	1074	2-propanol	2660 ± 25.3	1208 ± 116.2	1353 ± 27.8	344 ± 0.3	458 ± 11	470 ± 26.1	434 ± 10.8
	2080	Unknown	1948 ± 14.1	1701 ± 20.3	1443 ± 121.2	719 ± 10.3	773 ± 14.5	917 ± 59.3	899 ± 51.6
	2531	Unknown	1304 ± 42.7	1365 ± 80.2	1203 ± 13.6	797 ± 4.5	993 ± 22.4	900 ± 37.5	933 ± 28.6
	3968	S3:10	704 ± 0.1	648 ± 14	635 ± 1.5	544 ± 2.9	594 ± 19.6	577 ± 3.7	568 ± 12.9
	4771	Unknown	2859 ± 109.2	3039 ± 108.2	3099 ± 77.4	6020 ± 57.6	4668 ± 367.3	4399 ± 368.4	5190 ± 242.8
	4993	Myricetin methyl ester	486 ± 0.2	464 ± 9.6	528 ± 4.7	402 ± 0.2	412 ± 3.7	429 ± 12.6	424 ± 8.5
	8241	S3:18 II	2611 ± 113.4	2253 ± 480.8	1634 ± 119.9	603 ± 15.8	891 ± 90.6	1136 ± 193.6	1028 ± 130.5

Limonene synthase ((R)-limonene synthase) involved in the synthesis of limonene from geranyl-diphosphate (solgenomics.net). For genes related to the flavonoid pathway, we found one candidate gene (Solyc06g007960.1.1) located in the introgression of Chr. 6, which codes for an O-methyl-transferase.

DISCUSSION

Genotypes with different effects on whitefly behaviour

Based on the no-choice results (OR and AS) and the choice experiments (both leaf disc and static two chamber test), a clear level of whitefly resistance in *S. habrochaites* was found. The level of resistance in the accession CGN1.1561 (AS and OR), can be explained by the presence of trichomes type IV in high densities, but not by the abundance of methyl ketones, as these metabolites were found at comparable levels in CGN1.1561 (AS=0.1 ±0.23) and in FCN3-5 (AS=0.83 ±0.10) (Additional file 1). Although methyl ketones were reported to be involved in the resistance to several types of insects including whiteflies (Antonious, 2001), they are apparently not the only important compounds. Similar observations were made in a free-choice experiment, where the percentage of the recaptured whiteflies was not correlated with the presence of methyl ketones (Bleeker *et al.*, 2009). Some components are associated with an increased susceptibility, like the presence of trichomes type V or a higher oviposition rate. A positive correlation was found between the densities of trichomes type V and OR. Whiteflies might even use this type of trichomes (V) to attach and protect their eggs (Heinz and Zalom, 1995).

Solanum habrochaites was shown to be not preferred by the greenhouse whitefly and to interfere in the oviposition behaviour of *Tuta absoluta* (Lucatti *et al.*, 2010, Proffit *et al.*, 2011). In the choice test, whiteflies were able to differentiate between accessions in both types of choice tests. The results of the two types of choice assays were positively correlated for at least one time point. In summary, the resistance in CGN1.1561 is dependent on high densities of glandular trichomes but not on the accumulation of methyl ketones.

Role of chemical cues in host preference of whiteflies

We were able to identify several volatile and non-volatile compounds associated with preference of the whitefly. Eight non-volatile compounds (LC-MS data) were found in different concentrations in the preferred and non-preferred genotypes. Of those eight, an acyl sugar was present in relative higher concentrations in the non-preferred genotypes (Figure 4). Acyl sugars are known to be important for insect resistance in tomato acting mainly as toxic compounds (Glas *et al.*, 2012, Kang *et al.*, 2010, Leckie *et al.*, 2012, Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012, Firdaus *et al.*, 2013a).

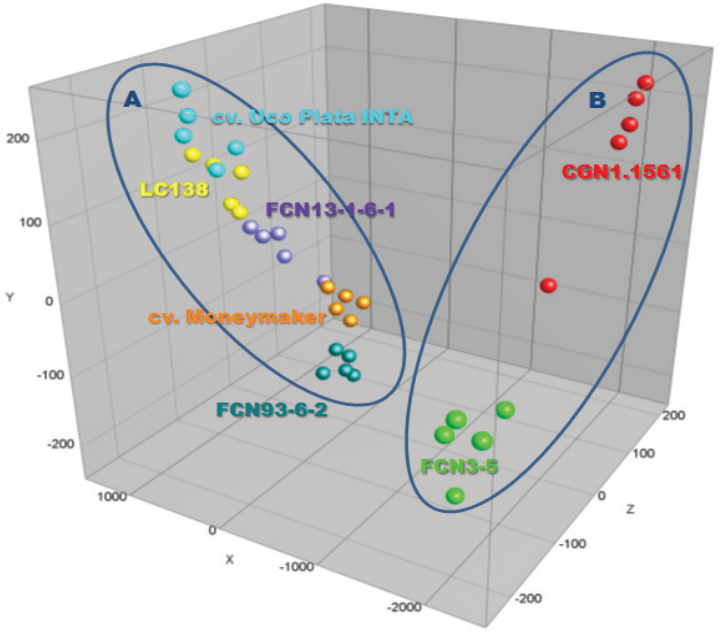


Figure 3: Principal component analysis of the different accessions used based on GC-MS metabolites. The *S. lycopersicum* accessions form the cluster "A", and the two *S. habrochaites* accessions form the cluster "B".

However, the presence of this acyl sugars did not reduce AS in FCN93-6-2. The toxicity of this type of compounds is dependent on the presence of type of glandular trichomes (type IV), the densities of those glandular trichomes and/or the concentration of the acyl sugars in the glands (Firdaus *et al.*, 2013a, Lucatti *et al.*, 2013, Glas *et al.*, 2012, Leckie *et al.*, 2012). Such a dose-toxicity relation was already described for the green peach aphid (*Myzus persicae*), where the settlement of the aphids was reduced after increasing the concentration of acyl sugars (Rodriguez *et al.*, 1993). This phenomenon of dose related toxicity was also observed for the Colorado potato beetle (*Leptinotarsa decemlineata*), where the mortality rate increased with an increase in sesquiterpene concentration and also for whitefly (*B. tabaci*) where the antenna response was proportional to the concentration of 7-epizingiberene (Bleeker *et al.*, 2011, Carter *et al.*, 1989).

In the GC-MS analysis, we identified eight compounds in lower relative concentration in the non-preferred pre-breeding line and in the two *S. habrochaites* accessions (Table 4, Figure 4). Whitefly non-preference in *S. habrochaites* is believed to be associated with the presence of high concentrations of methyl ketones or sesquiterpenes (Antonious, 2001, Ben-Israel *et al.*, 2009, Fridman *et al.*, 2005, Glas *et al.*, 2012, Kennedy, 2003, Bleeker *et al.*, 2009, Bleeker *et al.*, 2011, Bleeker *et al.*, 2012). None of these compounds were

detected in the line FCN93-6-2. In our experiments, we found seven monoterpenes in lower relative concentration in the non-preferred accessions compared to the preferred ones. Terpenes and sesquiterpenes were identified as key signals not only for the attraction of pollinators, but also as chemical clues used by parasitoids and predators of herbivores (Tholl, 2006). A previous study on the metabolic variation in tomato accessions of *S. habrochaites* showed that the relative composition of terpenes varied among accessions and geographic origin, from accessions having one predominant component (i.e. *S. habrochaites* LA2109) to others with a more complex chemical profile (e.g. *S. habrochaites* LA2107) (Gonzales-Vigil *et al.*, 2012). In that study, it was also seen that most of the *S. habrochaites* accessions had relative lower concentrations of monoterpenes, especially of β-phellandrene/limonene, when compared to *S. lycopersicum* (M82).

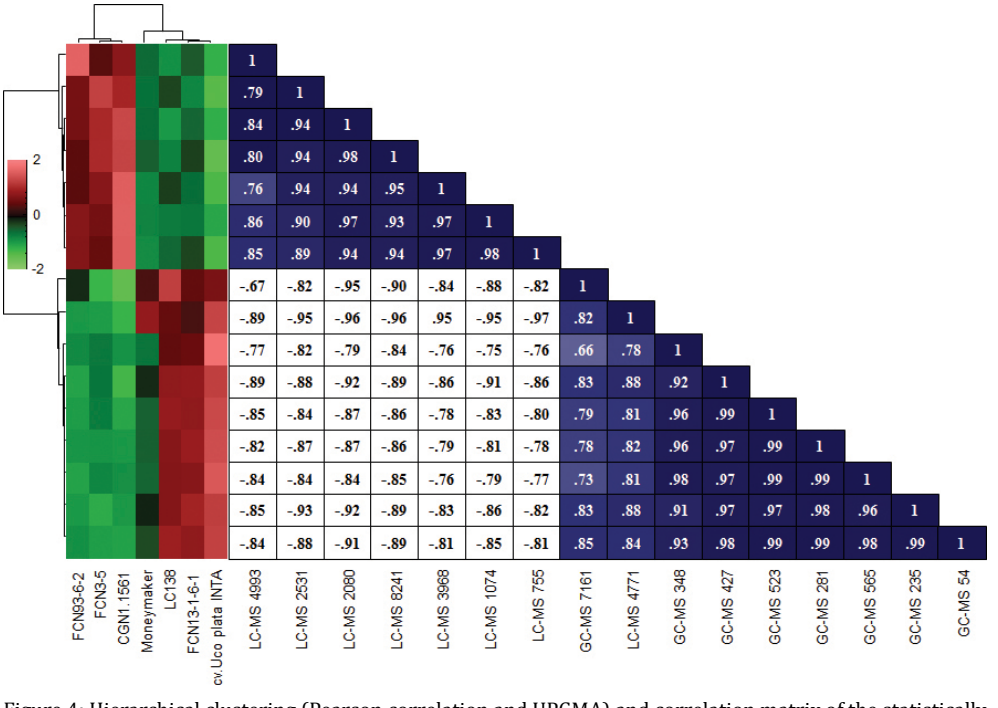


Figure 4: Hierarchical clustering (Pearson correlation and UPGMA) and correlation matrix of the statistically different metabolites (both, GC-MS and LC-MS) between preferred and non-preferred genotypes. Colour key is indicated in the figure.

Also in our material, the non-preferred accessions (two *S. habrochaites* and FCN93-6-2) had considerably lower concentrations of monoterpenes, especially of β-phellandrene, α-Pinene and 2-carene. The synthesis of monoterpenes is catalysed by terpene synthases and in tomato located in the glandular head of trichomes type VI (Glas *et al.*, 2012, Schilmiller *et al.*, 2009). It has been reported that the mutation *Od-2* (odourless

tomato) located on Chr. 11 interferes with trichome type VI formation and with the synthesis of monoterpenes (i.e. α-pinene, 2-carene, α -phellandrene, α -terpinene, limonene, and β-phellandrene), increasing the level of insect susceptibility (Kang *et al.*, 2010). In our material, we have not observed a difference either in the type or morphology of trichome type VI and also at the level of AS and OR of FCN93-6-2, which was comparable to cv. Moneymaker. This suggests that we are looking at a mechanism that might affect the synthesis/release of monoterpenes without affecting trichome morphology or density. Although the close relation between FCN93-6-2 and FCN13-1-6-1, a difference in whitefly preference and metabolite profiles (both GC-MS and LC-MS) was observed indicating that the factor underling the whitefly preference should be located in the unique introgressions of FCN93-6-2. The *Od-2* mutant (located on Chr. 11) had a lower monoterpene emission (Kang *et al.*, 2010), however the physical position of that mutation and the introgression on Chr. 11 of FCN93-6-2 do not co-localise. Schilmiller *et al.* (2009) showed that an introgression on the top of Chr. 8 containing the gene *PHS1* (phellandrene synthase 1) in tomato resulted in a reduction in the emissions of monoterpenes. Chromosome 6 is known for the presence of genes involved in sesquiterpene biosynthesis. Two sesquiterpene synthase genes (*Sstle1-A, Sstle1-B*) involved in the synthesis of β-caryophyllene and α-humulene, were mapped on Chr. 6 (van der Hoeven *et al.*, 2000). After checking on the tomato reference genome for the annotated genes in the introgression regions on Chr. 5, 6 and 11 and filtering by the keyword "terpene", we found two other candidates genes involved in the synthesis of monoterpenes. In summary, we have characterized a tomato line (FCN93-6-2) that is not preferred by the *B. tabaci.* The resistance mechanism(s) is independent of trichome type IV and methyl ketones. We were able to identify metabolites and candidate genes related to whitefly preference in this breeding line. A functional validation of the candidate metabolites by testing the pure synthetic compounds, by developing isogenic lines containing each individual introgression and finally by cloning the candidate gene(s) will be the next step in this research.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

The plant material assessed in this study is listed and described in Table 5. The two pre-breeding lines, FCN13-1-6-1 and FCN93-6-2 are the result of the cross between cv. Uco plata INTA and *S. habrochaites* (FCN3-5) followed by a pedigree selection program focused on insect resistance (Gilardón, 2007).

The plants were grown in a greenhouse at UNIFARM, Wageningen University and Research Centre, The Netherlands (20 ± 2°C, 70% RH, 16/8h day/night) in pots (14cm

in diameter) filled with soil compost mixture until they were six weeks old. At that moment, the plants were moved to an insect proof greenhouse where they were infested with whiteflies. Before infestation, the temperature was increased slowly (two degrees per day) from 20°C to 27°C in order to allow plants to adapt to the higher temperatures (27 ± 2°C, 70% RH, 16/8h day/night). Throughout the experiment, the plants were watered once a day and fertilized once a week.

Insect Rearing

A non-viruliferous whitefly rearing (*Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I) (Firdaus *et al.*, 2013b) was maintained on the susceptible cv. Moneymaker at Wageningen UR Plant Breeding, Wageningen, The Netherlands. The initial inoculum was obtained from a rearing at the Laboratory of Entomology, Wageningen UR, Wageningen, The Netherlands.

Table 5: Description of the plant material used in this study.

Name	Description and species	Characteristic	Reference
CGN1.1561	Wild relative. *S. habrochaites*	Resistant to *B. tabaci*. Source of QTL related to a reduction in oviposition rate.	Maliepaard *et al.*, 1995
FCN3-5	Wild relative. *S. habrochaites*	Selection of the accession PI134417 based on level and uniformity of *Tuta absoluta* resistance. Whitefly and spider mite resistant.	Gilardón, 2007, Lucatti *et al.*, 2010
cv. Uco plata INTA	Cultivar. *S. lycopersicum*	Tomato cultivar carrying the genes *Mi1-2* and *Tm2²*	Lucatti *et al.*, 2010
FCN93-6-2	Pre-breeding line. *S. lycopersicum*	Line resistant to *T. absoluta* and non-preferred by *Trialeurodes vaporariorum.* Long shelf life.	Gilardón, 2007, Lucatti *et al.*, 2010
FCN13-1-6-1	Pre-breeding line. *S. lycopersicum*	Line resistant to *T. absoluta* and preferred by *Trialeurodes vaporariorum.* Long shelf life.	Gilardón, 2007, Lucatti *et al.*, 2010
cv. Moneymaker	Cultivar. *S. lycopersicum*	Tomato used as susceptible reference.	Maliepaard *et al.*, 1995
LC138	Breeding line. *S. lycopersicum*	Tomato used as susceptible reference.	Lucatti *et al.*, 2010

No-choice experiment

Five whitefly females (four days old) were anesthetized (using CO_2), selected under a binocular microscope, and placed into a clip-on cage (2.5cm diameter and 1.0cm high). Three cages per plant, ten plants per genotype, were put on the abaxial part of the first to third fully expanded leaf counting from the top of the plant. Five days after inoculation, the number of alive and dead whiteflies was recorded and the surviving whiteflies were removed. The number of eggs was counted, and the Oviposition rate (OR) and Adult survival (AS) were calculated according to Bas *et al.* (1992). As soon as the first adults started to hatch from the fourth nymphal stage "pupae", the leaf was cut and put in wet OASIS® floral foam. The number of new empty pupae was recorded daily until nearly no adults emerged anymore (approximately one week) and the development period (DP) calculated. At this moment, the total number of nymphs (empty plus full pupae cases) was counted, and pre-adult survival (PS) calculated (Bas *et al.*, 1992). The variables were analysed with a one-way ANOVA followed by a least significant difference (LSD) test (Zar, 2010). An Arcsin (Sqrt) transformation was applied to calculate adult survival (AS) and pre-adult survival (PS), whereas a square root of (x+1) transformation was used for oviposition rate (OR) and development period (DP). The statistical analyses were performed using the statistical software package Genstat (15th edition).

Choice experiments

Two types of choice tests were used, a leaf disc test and a static two-chamber olfactometer test. In the leaf disc test, both olfactory and taste/surface cues are considered whereas in the two-chamber olfactometer only olfactory cues play a role in the decision making of the whitefly. Both choice tests were performed in a growth chamber at 25 ± 1°C, 70% relative humidity, 16/8h day/night.

Leaf disc test

Leaf samples were harvested and placed in a petri dish with 1.5% water agar with the abaxial surface of the leaf facing the inner part of the cage (Figure 5a). Each accession was compared to cv. Moneymaker. The lid of the cage had four holes from which two were covered with cv. Moneymaker and two with another sample. Twenty non-sexed whiteflies were anesthetized with CO_2 and placed inside each cage. Whiteflies were allowed to choose for a period of 24 hours. After that period, the number of whiteflies on each sample was recorded. The position of the samples with respect to the light source was randomly distributed to avoid an interference of the light on the whitefly behaviour. In total ten cages per comparison were used in a complete random design. The data were analysed using the Mann-Whitney U using the statistical software package Genstat (15th edition).

Static two-chamber olfactometer test

To test the effect of odours only, we used a static two-chamber olfactometer (11.2cm diameter, 18cm high) (Figure 5b). With this setup, the whiteflies do not have direct contact with the plant. A fully expanded leaf (first to fourth from the plant apex) of cv. Moneymaker and of another tomato sample were cut and inserted in wet OASIS® floral foam to keep the leaf turgid. The floral foam was covered with aluminium foil to prevent a possible effect of it on the decision taken by the whiteflies. Ten non-sexed whiteflies were anesthetized with $CO_{2,}$ placed inside each cage, and after five minutes recovery time, the experiment started. The choice of the whiteflies was recorded at 10, 30, 60, 90 and 120 minutes after the start of the experiment. Sixteen replicas per accession were used and the data were analysed using a Binomial test implemented in the statistical software package Genstat (15th edition).

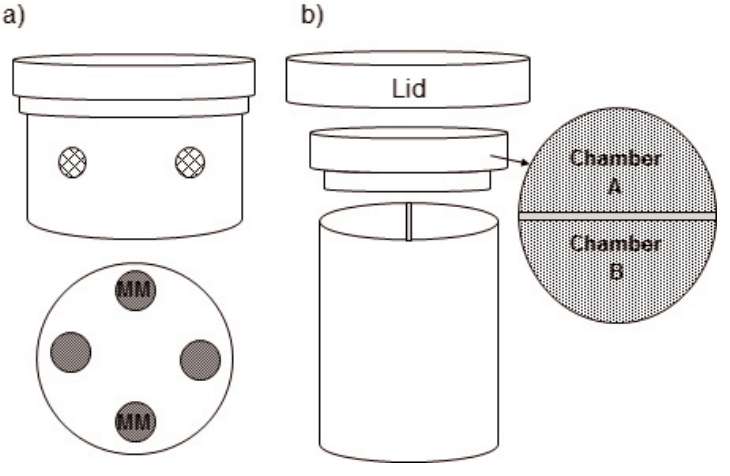


Figure 5: Different olfactometers used in the choice tests. a) Leaf disc test set-up; b) Static two-chamber olfactometer test set-up.

Trichome Type and Density

The number of trichomes per square millimetre was counted next to the main vein on the abaxial side of the first to fourth fully expanded leaf from the apex using a stereomicroscope (40x). For trichome description, we followed the classification proposed by (Channarayappa *et al.*, 1992). The data were analysed using the Kruskal-Wallis test as implemented in the statistical software package Genstat (15th edition). Spearman correlation tests was performed, in order to correlate the variables measured in the no-choice test, in the two types of choice test (leaf disc and static two-chamber olfactometer) with the trichome densities.

Untargeted metabolomic analysis

Sample preparation

Five plants per accession were grown at the same time and under the same conditions as the plants used for the no-choice experiment. From each plant, one complete leaf (second fully expanded leaf from the top of the plant) was cut, placed immediately into an aluminium envelope and frozen in liquid nitrogen. Each sample was ground under liquid nitrogen to a fine powder and stored at -80 °C until processing.

GC-MS Analysis

Per plant, 400 mg of frozen leaf powder was transferred to a reaction tube with 3 ml of anhydrous dichloromethane (>99.8%, Sigma-Aldrich) as solvent and 0.75 µg per ml heptadecanoic acid methyl ester was added as internal standard. Samples were vortexed and centrifuged at 1500 rpm for 10 min. Residual water was removed by passing the supernatant through a sodium sulphate (Na_2SO_4) bed. Extracts were analysed by injecting 1 µl on a 7890A gas chromatograph (GC Agilent Technologies) coupled to a 5975C MSD in spitless mode (Agilent Technologies). Chromatography was performed using a Zb-5MS column (Phenomenex, 30 m, 0.25 mm inner diameter, and 0.25 µm film thickness) with 5 m retention gap. Injection temperature was 250 °C, and temperature of column was programmed at 45 °C for 1 min, increased by 10 °C min^{-1} to 300 °C, and kept at 300 °C for 7 min. Column flow was set at 1 ml min^{-1}, using Helium as carrier. The column effluent was ionised by electron impact at 70eV and mass spectra were obtained with a scanning range of 35-400 m/z.

LC-QTOF-MS Analysis

Extraction and analysis by Liquid Chromatography-Quadrupole Time of flight-Mass Spectrometry (LC-QTOF-MS) were carried out as described by (De Vos *et al.*, 2007, Firdaus *et al.*, 2013a). In brief, two replicas per genotype were used, where each replica consisted of a balanced mix of 5 plants (five independently grounded leaves). Per replica, 400 mg of frozen leaf powder was put into a glass tube with 1.2 ml of methanol/formic acid solution (99.9 % - 0.1 %). The samples were mixed using a vortex, sonicated for 15 min and centrifuged at 2500 rpm for 10 min. The supernatant was filtered using a 0.45 µm filter, injected (5 µl) using an Alliance 2795 HT instrument (Waters), separated on a Phenomenex Luna C18 (2) column (2.0 × 150 mm, 3 mm particle size) using a 5–95% gradient of acetonitrile in water (both acidified with 0.1% formic acid) in 45 min and then detected by a Water-Micromass QTOF Ultima MS with electrospray ionization in negative mode (m/z 80–1,500). Annotation of LC-MS peaks corresponding to acyl sugars was done based on their accurate masses as described previously (Firdaus *et al.*, 2013a). The Quanlynx tool of the Masslynx acquisition software was used to calculate the relative abundance (peak area) of the different centrotypes for all samples, based on

their specific mass and retention times (De Vos *et al.* 2007). The peak areas were LOG_2 (x+1) transformed and auto scaled to the mean.

Data Analysis and Compounds Identification

MetAlign metabolomics software package (www.metalign.nl) was used to perform peak alignment and noise reduction, and MSClust software package (www.biotools.wurnet.nl) was used for data reduction by clustering several peaks into putative metabolites (Lommen, 2009). Putative metabolites were identified corresponding the obtained mass spectra to the NIST library (National Institute of Standards and Technology, Gaithersburgh, MD, USA), the Wiley online library, and the Wageningen Natural compounds spectral library. Putative metabolites were fully identified comparing the spectra and the retention index with the pure compound, when available. Prior to statistical analysis, the metabolites were Log transformed and auto scaled to the mean.

The raw data from GC-MS analysis were processed using an untargeted metabolomics approach. MetAlign package (www.metalign.nl) was used for baseline correction and peak extraction (s/n>3) and alignment (Lommen, 2009). Mass signals that were below s/n of 3 were randomized below the noise level. Mass signals that were present in ≤ 4 samples were discarded. Signal redundancy per metabolite was removed by means of clustering and mass spectra were reconstructed (Tikunov *et al.*, 2012). Metabolites were identified by matching the mass spectra of obtained metabolites to authentic reference standards and the NIST08, Wiley, and Wageningen Natural compounds spectral library and by comparison with retention indices in the literature (Strehmel *et al.*, 2008). Prior to statistical analysis, the metabolites were Log transformed and auto scaled to the mean. To select metabolite compounds putatively related to whitefly preference a t-test, followed by False Discovery Rate correction (Benjamini & Hochberg, 1995), was applied to compare the preferred vs. non-preferred accessions. As FCN93-6-2 has plenty differences when compared to the two *S. habrochaites* accessions; the candidate metabolites were further analysed assuming the following criteria; the metabolites had to be statistically different between FCN93-6-2 and cv. Moneymaker and not be statistically different between FCN93-6-2 and the two *S. habrochaites* accessions. In order to test for relations between volatile (GC-MS) and non-volatile (LC-MS) compounds, a Pearson correlation analysis was done. Prior to the correlation analysis, the data was averaged per genotype, LOG_2 (x+1) transformed and auto-scaled to the mean. Statistical analyses were done using the software package GeneMaths XS 2.0 (www.applied-maths.com).

Genotyping, relationship analysis and target gene identification

Genomic DNA was extracted using the KingFisher Flex magnetic particle processor (Thermo Life Sciences) according to the manufacturer's protocol. The DNA concentration

was adjusted to 50 ng/ul. For marker analysis, a custom made, single nucleotide polymorphism (SNP) Infinium bead array was used. On this array 5528 tomato SNPs are present (Viquez *et al.* 2013). Marker analysis was carried out by Service XS Leiden, the Netherlands, according to the Illumina® Infinium HD Ultra Assay protocol (www.illumina.com). After removing all monomorphic markers, a total of 3002 SNP markers were used in the analysis. The web service *Marker2seq* was used to find and select candidate genes (Chibon *et al.*, 2012).

SUPPLEMENTARY DATA

Additional file 1: This file includes the GC-MS and LC-MS data for the material used in this work and can be found in this link: http://www.plantbreeding.wur.nl/Publications/Alejandro/Additional file 1_Chapter 3_AFL.xlsx.

Chapter 4

QTL mapping in *S. habrochaites* for reduced whitefly fecundity

Alejandro F. Lucatti[1,2], Fien R.G. Meijer-Dekens[1], Richard G.F. Visser[1], Ben Vosman[1], Sjaak van Heusden[1]*

1 Wageningen UR Plant Breeding, Wageningen University and Research Centre. PO Box 386, 6700 AJ Wageningen, The Netherlands.

2 Graduate School Experimental Plant Sciences. Wageningen Campus. Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands.

Submitted

Abstract

Background- Host plant resistance has been proposed as one of the most promising for whitefly management. In 1995, one of the first mapping studies focused on whitefly resistance in tomato was done and two QTL reducing whitefly oviposition rate (*Tv-1* and *Tv-2*) were found. After this first study, several others have followed, but all other QTLs affecting whitefly oviposition were highly correlated with a reduction in whitefly survival. Is that the reason why, the aim of our study was to further characterize *Tv-1* and *Tv-2*, which were independent of adult survival and to determine their role in resistance against *Bemisia tabaci.*

Results- To test this, from selected F_2 plants we did three successive backcrosses without phenotyping. Twenty-three F_2BC_3 plants were phenotyped for whitefly resistance. Differences were found for oviposition rate of *B. tabaci* exclusively. The most resistant F_2BC_3 had in common an introgression on Chromosome 5. To validate this result, $F_2BC_4S_1$ and $F_2BC_4S_2$ families were developed. A 3.06 Mbp introgression on top of Chr. 5 was responsible of the reduction in oviposition. This reduction was independent of the presence of trichomes type IV.

Conclusion- Contrary to expectations, we found an additional region of 3.06 Mbp at the top of Chr. 5 (*OR-5*) affecting only Oviposition rate. This phenotype was independent of the presence of *Tv-1* and *Tv-2* and of the presence of trichomes type IV.

Keywords: *Bemisia tabaci*, Oviposition rate, fine mapping, *Tv-1*, *Tv-2*, trichome type IV.

INTRODUCTION

Tomato is one of the most important vegetables worldwide. It is host for a broad range of pathogens and pests. Among the pests affecting tomato production whiteflies are the most important in terms of costs and distribution. There are more than 1500 species of whiteflies, of which *Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I and *Trialeurodes vaporariorum* (Westwood) are the most important in commercial tomato production. *Bemisia tabaci* affects tomato production directly (i.e. phloem consumption, irregular ripening of the fruits) and indirectly (virus transmission) causing yield losses that can range from 50% to 100% of the total production (Brown and Bird, 1992, Byrne and Bellows Jr, 1991).

Among the possible control methods, host plant resistance has been proposed as one of the most promising for insect pest management (Broekgaarden *et al.*, 2011, Schoonhoven *et al.*, 2005). Resistance to whiteflies was found in several wild relatives of tomato (*S. pennellii*, *S. habrochaites, S. lycopersicum var. cerasiforme, S. pimpinellifolium, S. galapagense*) (Baldin *et al.*, 2005, Fernández-Muñoz *et al.*, 2003, Firdaus *et al.*, 2012, Freitas *et al.*, 2002, Heinz and Zalom, 1995, Lucatti *et al.*, 2013, Muigai *et al.*, 2002, Muigai *et al.*, 2003, Rodriguez-Lopez *et al.*, 2011). In these species, whitefly resistance is associated with the presence of high densities of glandular trichomes (type I, IV and VI) and with the presence of specific secondary metabolites (i.e. 7-epizingiberene, 2-tridecanone, acyl sugars) (Rodríguez-López *et al.*, 2012, Firdaus *et al.*, 2013a, Lucatti *et al.*, 2013, Bleeker *et al.*, 2012).

The use of backcross introgression lines (ILs) was proposed as a method to identify genomic regions important for whitefly resistance. These IL lines helped to identify regions and genes involved in traits previously related to insect resistance, like the production of monoterpenes, sesquiterpenes and acyl sugars (Schilmiller *et al.*, 2010, Schilmiller *et al.*, 2012, Schilmiller *et al.*, 2009, Van der Hoeven *et al.*, 2000), but failed to identify regions associated to whitefly resistance in terms of adult survival or oviposition rate (Momotaz *et al.*, 2010) suggesting a polygenic inheritance and possibly epistatic interactions. Quantitative Trait Loci (QTL) studies were carried out using the same resistant wild species of tomato to develop segregating populations (Table 1). Maliepaard *et al.* (1995) performed one of the first mapping studies focused on whitefly resistance in tomato. They were able to identify two QTL reducing whitefly oviposition rate (*Tv-1* on Chromosome 1 and *Tv-2* on Chr. 12), two QTLs related to trichome type IV density (*TriIV-1* on Chr. 5 and *TriIV-2* on Chr. 9) and one QTL for trichome type VI density (*TriVI-1* on Chr. 1). After this first study, others have explored different resistance sources and more QTLs were described. A summary of the QTLs related to whitefly resistance in tomato is given in Table 1. Except the QTLs described in Maliepaard *et al.* (1995), all other QTLs affecting whitefly oviposition were highly correlated with a

Table 1: Overview of the QTLs found associated to whitefly resistance in tomato.

Trait	QTL	Chr.	Resistance donor	% Explained	References
Adult survival (*B. tabaci*)	*Wf-1*	2	*S. galapagense* (PRI95004)	54.1	Firdaus *et al., 2013*
	Wf-2	9		14.8	
	Wf-I	1	*S. pennellii* (LA3791)	12.1	Van den Elsen *et al.,* 2013
	Wf-III	3		15.6	
	Wf-IV	4		12.3-30.7	
	Wf-VI	6		10.1	
Oviposition rate (*B. tabaci*)	*Wf-1*	2	*S. galapagense* (PRI95004)	41.7	Firdaus *et al., 2013*
	Wf-2	9		11.1	
	R2/9	9	*S. habrochaites* (LA1777)	55.2	Momotaz *et al.,* 2010
	R1/10	10		15	
	R3/11a	11		52.9	
	R4/11b	11		43.3	
	Wf-IV	4	*S. pennellii* (LA3791)	10.3-29.6	Van den Elsen *et al.,* 2013
	Wf-VI	6		13.9	
	Wf-X	10		10	
Oviposition rate (*T. vaporariorum*)	*Tv-1*	1	*S. habrochaites* (CGN1.1561)	6.4	Maliepaard *et al.,* 1995
	Tv-2	12		8	
Pre-adult survival (*B. tabaci*)	*Wf-1*	2	*S. galapagense* (PRI95004)	13.3	Firdaus *et al.,* 2013
Density of trichome type IV	*Wf-1*	2	*S. galapagense* (PRI95004)	66.3	Firdaus *et al.,* 2013
	Wf-2	9		8.7	
	TriIV-1	5	*S. habrochaites* (CGN1.1561)	n.d.	Maliepaard *et al.,* 1995
	TriIV-2	9		n.d.	
	R2/9	9	*S. habrochaites* (LA1777)	69.7	Momotaz *et al.,* 2010
	R1/10	10		22.5	
	R3/11a	11		69	
	R4/11b	11		n.d.	
	TA2A	2	*S. pennellii* (LA0716)	2.6	Blauth *et al.,* 1998
	3A	3		5.1	
	TA4	4		5.2	
	6A	6		4.7	
	7B	7		2.8	
	10A	10		4.6	
	11A	11		8.1	
Density of trichome type VI	*TriVI-1*	1	*S. habrochaites* (CGN1.1561)	n.d.	Maliepaard *et al.,* 1995

reduction in whitefly survival, suggesting that a low oviposition rate is most likely a consequence of a low survival rate. The aim of our study was to further characterize the QTLs found by Maliepaard *et al.* (1995), which were independent of adult survival and to determine their role in resistance against *Bemisia tabaci.* For this, we have gone from selected F_2 plants to $F_2BC_4S_2$ populations and contrary to expectations we found an additional region at the top of Chr. 5 (*OR-5*) affecting only Oviposition Rate.

Table 2: Primers and restriction enzymes for CAPs analysis.

Marker name	Primer sequence	Restriction enzyme
TG59	AACTCTACGCTGCACTGCTG CTGAAGCTCCACCTTGAGGTG	Hpa II
TG17	GGTCTTCCCTTCGTCATTCAT GTTATTCGGTTCTTGTTCTTCACG	HpyCH4 IV
CD2	CAGCTGCAACTCCACTACCA GGGCTTGAAGAACTGCACTC	Mwo I
TG68	TTTGATTACACCTGCCTTTACATA CATGTCAAGGGGATTGAACA	Dde I

RESULTS

Plant material development started from F_2 plants containing *Tv-1*, *Tv-2* or both QTL using the markers shown in Table 2. Three successive marker assisted backcrosses were carried out with selection for the presence of at least one QTL (Figure 1). The criteria used for selection was that the plants should have at least one of the linked markers in heterozygous state. Twenty-three F_2BC_3 plants were selected for phenotyping and genotyping to confirm the presence of *Tv-1* and *Tv-2*. As reference lines, we included *S. habrochaites* (CGN1.1561) and *S. lycopersicum* cv. Moneymaker. CGN1.1561 had the lowest values (AS= 0.1 ± 0.21 females/day and OR= 0.2 ± 0.30 eggs/female/day). Among the twenty-three F_2BC_3, differences were found for adult survival (AS) and oviposition rate (OR). For AS, only three F_2BC_3 plants were considered more resistant (Table 3). Whereas, for OR a gradient was observed, with fourteen F_2BC_3 plants having statistically lower values than cv. Moneymaker (Table 3).

To confirm the CAPs results and to know the size of the introgressions, the twenty-three F_2BC_3 plants were extensively genotyped using the Infinium bead array. The four F_2BC_3 plants with the lowest OR (PV101092-2, PV101088-2, PV101087-3 and PV101088-5) shared an introgression on Chr. 5, but had differences in the presence of the regions *Tv-1* and/or *Tv-2* (Table 3). Plant PV101088-8 also had the same introgression on Chr. 5, but it had an OR not significantly different from cv. Moneymaker (Table 3).

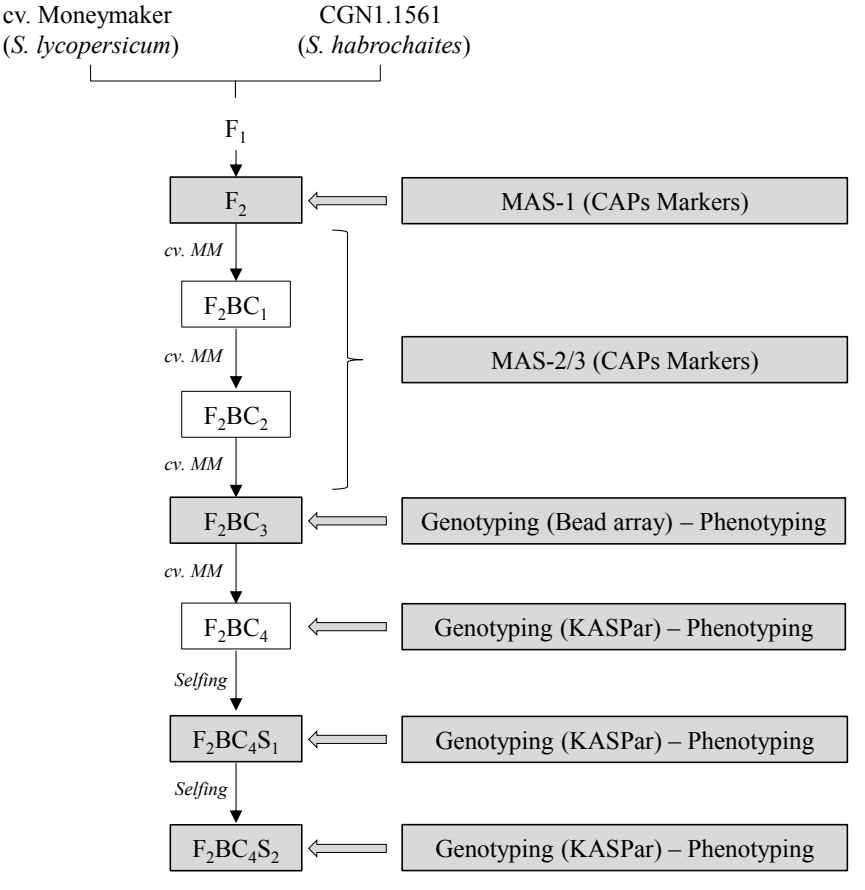


Figure 1: Overview if the pedrigree scheme and the plant material

To further understand the role of the introgression on Chr. 5, five F_2BC_3 plants (PV101092-2, PV101088-2, PV101087-3, PV101093-1, PV101087-2) were selected based on the difference in OR and on the presence/absence of *Tv-1*, *Tv-2* and the introgression on Chr. 5.

Table 3: Results of the no-choice experiment (mean) and Infinium array of selected F_2BC_3 plants

Genotype	n	Adult survival mean		Oviposition rate mean		Chromosome 1 *Tv-1*	Chromosome 5	Chromosome 12 *Tv-2*
CGN1.1561	(4)	0.10	a	0.15	a			
cv. Moneymaker	(3)	0.99	cd	5.53	f			
PV101092-2	(3)	0.14	a	0.58	ab			
PV101088-2	(3)	0.75	ab	1.32	bc			
PV101087-3	(3)	0.92	abc	1.65	bc			
PV101088-5	(3)	0.61	ab	1.81	bc			
PV101089-3	(3)	0.99	cd	2.04	cd			
PV101092-7	(2)	0.73	abcd	2.06	cde			
PV101093-5	(2)	0.74	abcd	2.06	cde			
PV101090-1	(2)	1.00	d	2.12	cde			
PV101092-4	(4)	0.98	bcd	2.26	cde			
PV101087-1	(3)	0.92	abcd	2.36	cde			
PV101087-4	(3)	1.00	d	2.54	cde			
PV101090-2	(3)	0.81	abcd	2.93	cde			
PV101092-6	(4)	0.96	bcd	2.95	cde			
PV101089-5	(4)	1.00	d	2.98	cde			
PV101089-7	(1)	0.98	abcd	3.15	cdef			
PV101093-1	(3)	0.98	bcd	3.40	def			
PV101087-2	(4)	1.00	d	3.45	def			
PV101093-6	(3)	0.99	bcd	3.52	def			
PV101088-8	(3)	0.97	abcd	3.63	def			
PV101089-8	(4)	0.98	bcd	3.93	ef			
PV101089-9	(2)	0.96	abcd	3.99	def			
PV101088-6	(3)	0.96	abcd	4.10	ef			
PV101093-3	(3)	1.00	d	5.57	f			

Different letters indicate statistical differences according LSD ($\alpha<0.05$). Areas filled in black represent homozygous markers (S. habrochaites allele). Areas filled in grey represent heterozygous markers. Non-filled areas represent homozygous markers (cv. Moneymaker allele). n.d.: no data. On Chromosome 1 and 12 are indicated the physical position (tomato genome assemble version ITAG2.3) of Tv-1 (76.7 to 90.0 Mbp) and Tv-2 (4.6 to 63.5 Mbp).

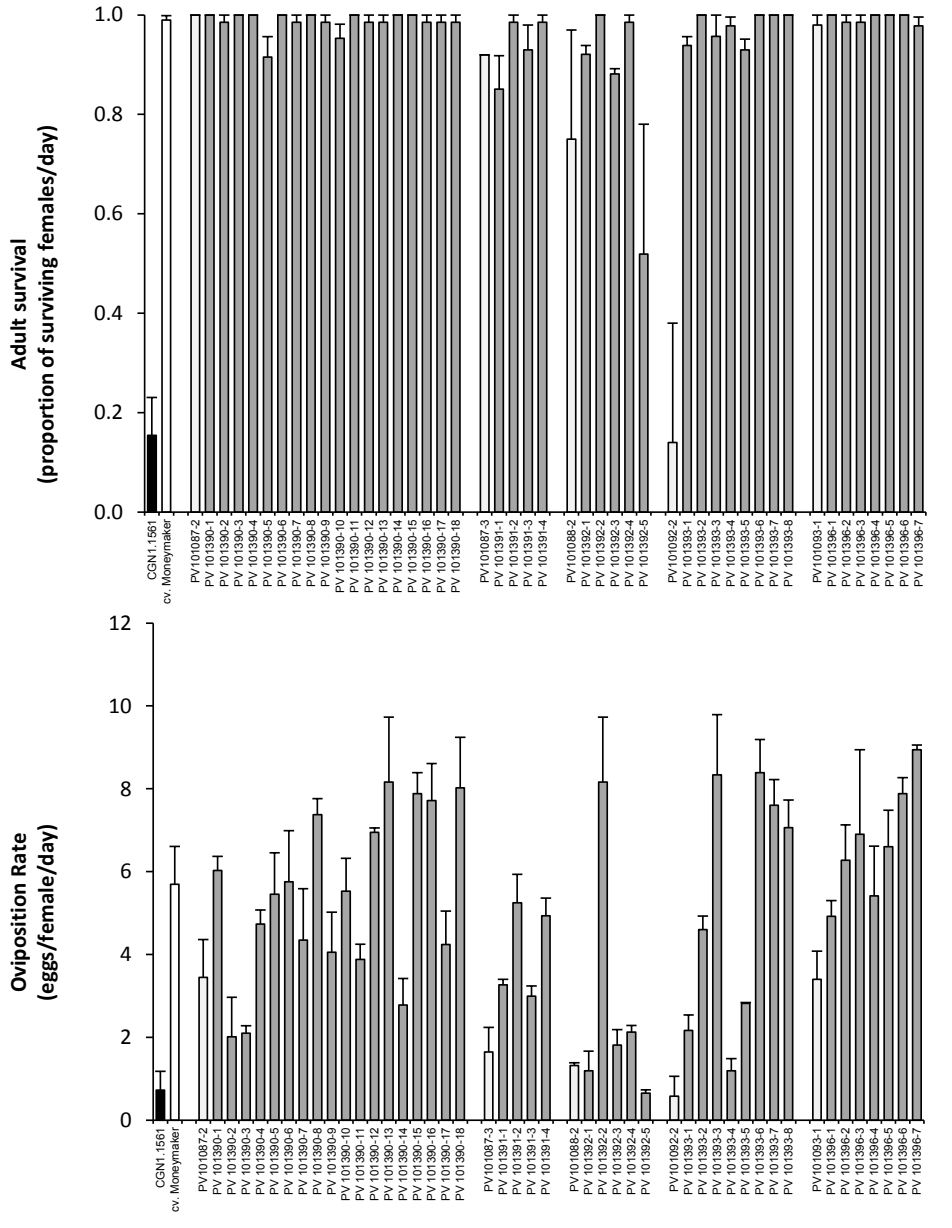


Figure 2: Phenotypic distribution of the F2BC4 plants for AS (a) and OR (b) grouped by family. *Solanum habrochaites* (CGN1.1561) is in black and cv. Moneymaker is in white. The first sample of each family is the parent of that family.

The plants PV101092-2, PV101088-2, PV101087-3, contain the Chr. 5 (61.27 Mbp) introgression, whereas PV101093-1, PV101087-2 lack it. The lines have varying parts of *Tv-1* and *Tv-2* or lack these completely (Table 3). The plants were backcrossed with

cv. Moneymaker to generate five F_2BC_4 families. All F_2BC_4 plants plus parental plants, GCN1.1561 and cv. Moneymaker were genotyped and phenotyped for adult survival, oviposition rate, trichome type and trichome density. Figure 2 shows the distribution for AS and OR and the link to the respective F_2BC_3. Clear differences were seen between cv. Moneymaker and CGN1.1561 for AS and OR ($P<0.01$ and $P<0.01$, respectively). In the studied F_2BC_4 plants, there is mainly segregation for OR with the parents on the extremes of the distribution. In the offspring of PV101088-2 (PV101392) four sibling plants were heterozygous for the region on Chr. 5 and were lacking the *Tv-1* and *Tv-2* region of CGN1.1561. These plants had an OR level comparable to CGN1.1561, but another plant in the same family (PV101392-2), which lacked the CGN1.1561 allele, had a high OR (Figure 3). No differences were found for densities of trichome type VI among the F_2BC_4 plants and cv. Moneymaker.

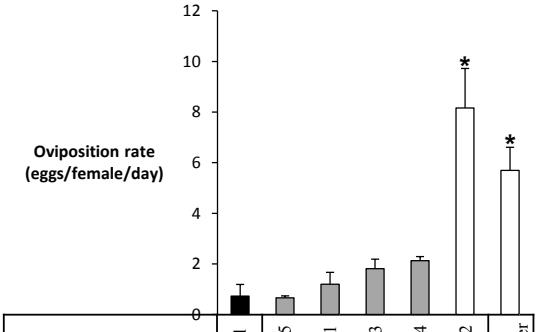
To reduce the size of the introgression on Chr. 5, the four F_2BC_4 plants of Figure 3, with low OR and without *Tv-1* and *Tv-2,* (PV101392-1, PV101392-3, PV101392-4 and PV101392-5) were selfed. Of 275 $F_2BC_4S_1$ plants, 33 recombinants were selected for phenotyping based on differences in the introgression length in heterozygous state. The results grouped by introgression length are shown in Figure 4. To fine map and confirm the introgression on Chr. 5, eight $F_2BC_4S_1$ plants (PV121430-4, PV121430-11, PV121433-30, PV121430-89, PV121432-26, PV121433-29, PV121433-53 and PV121434-57) with low levels of OR and heterozygous for this region were selected for self-pollination. Of, 295 $F_2BC_4S_2$ plants genotyped, 77 recombinants were selected for phenotyping based on differences in the introgression length. The results grouped by introgression length are shown in Figure 5. The $F_2BC_4S_2$ plants with an introgression on top of Chr. 5, between the markers rs2009 (4.76 Mbp) and rs2093 (11.8 Mbp), had an OR similar to the levels of CGN1.1561 and lower than plants with the cv. Moneymaker allele homozygously present in that region (Figure 5). From the $F_2BC_4S_1$ we could narrow down this introgression to a 3.06 Mbp region between the markers rs2009 (4.76 Mbp) and rs2071 (7.83 Mbp).

DISCUSSION

An introgression on Chromosome 5 (*OR-5*) *reduces whitefly fecundity*

Using F_2BC_3 plants, we identified a *S. habrochaites* introgression located on the short arm of Chr. 5 (hereafter called *OR-5*), that is conferring a reduction in *B. tabaci* oviposition rate. By analysing the F_2BC_4, $F_2BC_4S_1$ and $F_2BC_4S_2$ populations, we could confirm that an introgression of 3.06 Mbp on the top of Chr. 5 (*OR-5*) is causing the reduced whitefly fecundity, even in the absence of *Tv-1* and *Tv-2,* which were previously known to cause a reduction of *T. vaporariorum* oviposition (Maliepaard *et al.,* 1995).

The reduction in oviposition caused by the presence of *OR-5* is independent of adult survival and the presence of trichome type IV. Plants were found on which all whiteflies were alive but a reduction in oviposition was observed (Figure 2) and none of the plants had the sticky trichomes type IV. Of the $F_2BC_4S_2$ the plants homozygous for the *S. habrochaites* allele had surprisingly a higher OR compared to the $F_2BC_4S_2$ plants with a heterozygous introgression. This might indicate an interaction between the *S. habrochaites* and the *S. lycopersicum* allele. It would also implicate that the high level of resistance found in CGN1.1561 is the result of a combination of several different mechanisms.



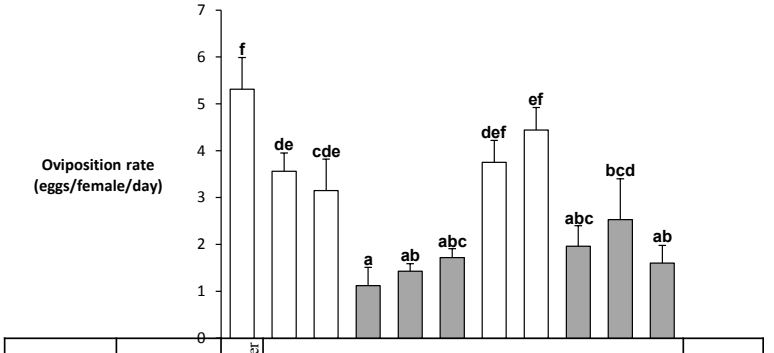
Chr.	Position (bp)	Marker	CGN1.1561	PV101392-5	PV101392-1	PV101392-3	PV101392-4	PV101392-2	cv. Moneymaker
1	809671	rs1675	b	a	a	a	a	a	a
	3676920	rs8273	b	a	a	a	a	a	a
	81791824	rs4624	b	a	a	a	a	a	a
	85528368	rs6450	b	a	a	a	a	a	a
	89401856	rs7797	b	a	a	a	a	a	a
5	2392402	rs7392	b	h	h	h	h	a	a
	2861323	rs2018	b	h	h	h	h	a	a
	5788106	rs7543	b	h	h	h	h	a	a
	22506652	rs7622	b	h	h	h	h	a	a
	41608296	rs1602	b	a	a	a	a	a	a
	60667560	rs6184	b	a	a	a	a	a	a
12	260859	rs7045	b	a	a	a	n.d.	a	a
	1441274	rs7071	b	a	a	a	a	a	a
	2735450	rs3930	b	n.d.	n.d.	a	n.d.	a	a
	14640635	rs7898	b	a	a	a	a	a	a
	62447828	rs7926	b	a	a	a	a	a	a

Figure 3: Role of Chromosome 5 on oviposition rate. Oviposition rate (mean ± standard error) and marker results of 5 F_2BC_4 siblings from the F_2BC_3 PV101088-2. Asterisks indicate statistical significance according LSD (α<0.05). a: homozygous cv. Moneymaker allele. b: homozygous *S. habrochaites* allele h: heterozygous. n.d.: no data.

Selection of the Chromosome 5 region

For the selection of the F_2BC_3 plants, we used markers that are linked to the loci *Tv-1* and *Tv-2* loci, which are located on Chrs. 1 and 12 respectively. It is therefore remarkable that we ended up with an introgression on Chr. 5, which had never actively been selected for. This can be the result of starting with F_2 plants containing the introgression on Chr. 5 either homozygous or heterozygous (3:1 have the introgression) and the chance in the F_2BC_3 that the introgression is still present is 1:4 or 1:8. In total that will lead to a

number of F_2BC_3 plants we found. The fact that Maliepaard *et al.* (1995) did not detect the QTL for OR could be the difference in whitefly species used, namely *T. vaporariorum* vs *B. tabaci*. Different insect species can react differently to the same odour blend giving different behaviours. For example, glucosinolates can confer resistance to some insects, whereas they can be used as host and strong oviposition cues for others (Hopkins *et al.*, 2009).



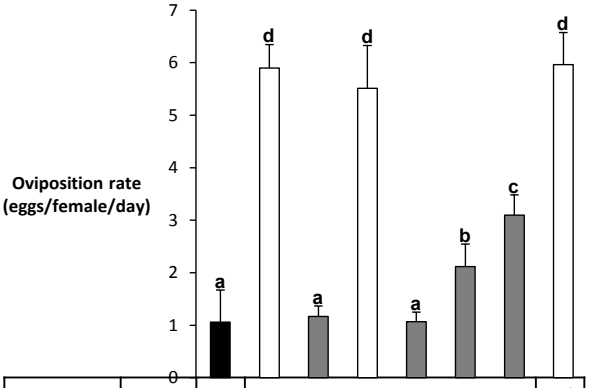
Position (bp)	Marker	cv. Moneymaker	2 (5)	3 (3)	4 (3)	5 (4)	6 (2)	7 (3)	8 (4)	9 (3)	10 (2)	11 (4)	Q-value
2861323	rs2018	a	a	a	a	a	a	h	h	h	h	h	0.756
3184832	rs7366	a	a	a	a	a	h	h	h	h	h	h	0.451
4498192	rs7341	a	a	a	a	h	h	a	h	h	h	h	0.028
4760311	rs2009	a	a	a	a	h	h	a	h	h	h	h	0.024
5788106	rs7543	a	a	a	h	h	h	a	a	h	h	h	<0.001
5887817	rs33	a	a	a	h	h	h	a	a	h	h	h	<0.001
6146671	rs7551	a	a	a	h	h	h	a	a	h	h	h	<0.001
7827671	rs2071	a	a	h	h	h	h	a	a	a	h	h	<0.001
11822165	rs2093	a	a	h	h	h	h	a	a	a	a	h	<0.001
13619865	rs7582	a	a	h	h	h	h	a	a	a	a	h	<0.001
20278680	rs7625	a	a	h	h	h	h	a	a	a	a	h	<0.001
22578908	rs2191	a	a	h	h	h	h	a	a	a	a	h	<0.001
23884988	rs1330	a	a	h	h	h	h	a	a	a	a	h	<0.001
35874804	rs283	a	a	h	h	h	h	a	a	a	a	h	<0.001
36222100	rs7902	a	a	h	h	h	h	a	a	a	a	h	<0.001
37516096	rs7904	a	a	h	h	h	h	a	a	a	a	h	<0.001
41395560	rs1597	a	a	h	h	h	h	a	a	a	a	h	<0.001
41608296	rs1602	a	a	h	h	h	h	a	a	a	a	h	<0.001

Figure 4: Fine mapping of introgression on Chromosome 5. Oviposition rate (mean ± standard error) and marker results of $F_2BC_4S_1$ plants grouped by introgression length. Different letters indicate statistical significance according LSD ($\alpha<0.05$). Between brackets is indicated the number of replicas per introgression length. Q-value: FDR corrected P-value per marker after t-test. a: homozygous cv. Moneymaker allele, h: heterozygous.

Nature of the resistance provided by OR-5

Several QTL related to whitefly resistance have been identified on Chr. 5 (Table 1). Maliepaard *et al.* (1995) found in the region of *OR-5*, a QTL (*TriIV-1*) that increases the density of trichomes type IV. However, we did not detect any type IV trichomes on plants containing the *OR-5* introgression. In a backcross population of potato ((*S. tuberosum* x *S. berthaultii*) x *S. berthaultii*) a region on Chr. 5 was associated with a reduction in the oviposition rate and leaf consumption by the Colorado potato beetle (*Leptinotarsa decemlineata*).This region also had a large effect on the density of the glandular secretory type B trichome (LOD: 19.17, explaining 35.6% of the variance), furthermore

differences in the sucrose ester levels and in the presence of droplet (exudate) on the tip of the trichomes (Bonierbale *et al.*, 1994) were associated with this region on Chr. 5. In *S. pennellii,* two QTLs were described on Chr. 5 that were involved in acyl sugar metabolism, one (*TA5*) related to the total accumulation of acyl sugars and the other (*5*) related to the proportion of 7-methyloctanonate and 9-methyldecanoncate fatty acids that are incorporated into acyl sugars (Blauth *et al.*, 1999, Mutschler *et al.*, 1996).



Position (bp)	Marker	CGN1.1561	1 (15)	2 (15)	3 (2)	4 (15)	5 (15)	6 (15)	cv. Moneymaker
4760311	rs2009	b	a	a	h	h	h	b	a
5788106	rs7543	b	a	h	a	h	h	b	a
5887817	rs33	b	a	h	a	h	h	b	a
6146671	rs7551	b	a	h	a	h	h	b	a
7827671	rs2071	b	a	h	a	h	h	b	a
11822165	rs2093	b	a	h	a	a	h	b	a

Figure 5: Corroboration of functionality of the introgression on Chromosome 5. Oviposition rate (mean ± standard error) and marker results of $F_2BC_4S_2$ plants grouped by introgression length. Different letters indicate statistical significance according LSD (α<0.05). Between brackets is indicated the number of replicas per introgression length. a: homozygous cv. Moneymaker allele. b: homozygous *S. habrochaites* allele, h: heterozygous.

On the 3.06 Mbp introgression of *OR-5,* there are 258 annotated genes including R-genes, transcription factors, genes involved in acyl sugar and terpenoid metabolism amongst others, which can be considered as candidate genes for reducing oviposition. To reduce the list of candidate genes and find the gene(s) responsible for the lower OR further fine mapping and functional analysis, including metabolomics is needed. Due to the low number of recombinants that we got in the $F_2BC_4S_1$ and $F_2BC_4S_2$ populations, and the amount of genes annotated on the *OR-5* region, it will be necessary to increase the number of plants used for recombinant screenings, to ensure enough recombination in the *OR-5* region. A reduction in number of candidate genes is also needed for cloning of the QTL.

Perspectives for breeding for whitefly resistance

Since the late nineties of the 20th century, the efforts to get whitefly resistant tomatoes have increased considerably, but so far they have been unsuccessful since no resistant varieties have been released (Broekgaarden *et al.*, 2011). The screening of genetic resources for novel whitefly resistance mechanisms has increased, going from distant wild relatives of tomato (i.e. *S. pennellii*, *S. habrochaites*) to in depth studies of several accessions of closely related species (i.e. *S. galapagense, S. pimpinellifolium*) (Baldin *et al.*, 2005, Firdaus *et al.*, 2012, Freitas *et al.*, 2002, Lucatti *et al.*, 2013, Muigai *et al.*, 2003, Muigai *et al.*, 2002). These efforts have led to the identification of specific secondary metabolites conferring resistance to whiteflies (methyl ketones, sesquiterpenes, and acyl sugars) (Bleeker *et al.*, 2011, Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012), the identification of QTLs related to resistance (Firdaus *et al.*, 2013a, Maliepaard *et al.*, 1995, Momotaz *et al.*, 2010), and in some cases to the genes responsible for the resistance metabolites (Ben-Israel *et al.*, 2009, Bleeker *et al.*, 2012, Fridman *et al.*, 2005, Schilmiller *et al.*, 2012, Schilmiller *et al.*, 2009). The identification of *OR-5*, affecting specifically whitefly fecundity and independent of the presence of trichome type IV, opens new opportunities for selective breeding. The *OR-5* region is expected to reduce population development of *B. tabaci* severely. As the reduction in oviposition is not linked to the sticky trichomes type I and IV, this resistance will be very suitable in combination with biological control. On varieties containing the *OR-5* region the *B. tabaci* population development will be slowed down giving the natural enemies ample opportunity to keep the population below threshold levels or even to remove the developing whiteflies. Therefore, the gene will in particular be useful in protected tomato production conditions (greenhouse cultivation). For open field production, the resistances based on trichomes type I and IV will be more suitable (Firdaus *et al.*, 2013a).

MATERIALS AND METHODS

Plant materials and growing conditions

The study was carried out using the F_2 offspring population that was created by Maliepaard *et al.* (1995), which was obtained by self-pollination of a single F_1 plant that was derived from a cross between *S. lycopersicum* (cv. Moneymaker) and *S. habrochaites* (CGN1.1561). We have sown more individuals of this F_2 population and selected plants that were homozygous for either one or both QTLs associated to a reduction in oviposition rate using Cleaved Amplified Polymorphisms (CAPs) (Table 2). The selected F_2 plants were backcrossed with *S. lycopersicum* (cv. Moneymaker) for three generations and plants containing the QTLs were selected using markers flanking the

QTLs. The obtained F_2BC_3 and F_2BC_4 families were genotyped and phenotyped for adult survival and oviposition rate. Selected F_2BC_4 plants were selfed to obtain $F_2BC_4S_1$ plants and $F_2BC_4S_2$, which were also genotyped and phenotyped. An overview of the material development is shown in Figure 1.

The tomato plants were grown in a greenhouse in Wageningen, The Netherlands (20 ± 2°C, 70% RH, 16/8 h day/night) in 14 cm diameter pots filled with soil compost. The plants were fertilized twice a week with a tomato standard fertilizer and watered once a day. When the plants were five weeks old, they were transferred to an insect proof greenhouse. The greenhouse temperature was increased slowly from 20 to 27°C to allow plants to adapt to the higher temperature (27 ± 2°C, 70% RH, 16/8 h day/night) used during the infestation (one week after being transferred).

Insect rearing

A non-viruliferous whitefly rearing (*Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I) was maintained on the susceptible tomato cultivar Moneymaker at Wageningen UR Plant Breeding, Wageningen, The Netherlands. The initial inoculum was obtained from a rearing at the Laboratory of Entomology, Wageningen UR, Wageningen, The Netherlands.

No-choice experiment

Whiteflies (four days old) were anesthetized (using CO_2). Five females were selected under a binocular and put in a clip-on cage (2.5 cm diameter and 1.0 cm high). Three cages per plant were attached to the first to third fully expanded leaf counting from the top. Five days after inoculation, the number of living and dead whiteflies was recorded and living whiteflies were removed. The number of eggs was counted, and the Oviposition rate (OR) and Adult survival (AS) were calculated according to Bas *et al.* (1992). For the analysis of AS in the F_2BC_3 population, a Kruskal-Wallis analysis of variance was used (Zar, 2010). A square root transformation was applied to oviposition rate (OR) prior to the data analysis and analysed by one-way ANOVA followed by a least significant difference (LSD) test.

DNA isolation and genotyping

Genomic DNA was extracted from young leaflets using the micro-prep DNA extraction protocol (Fulton *et al.*, 1995). The DNA concentration was adjusted to 50 ng/ul. For molecular marker analysis, three types of marker assays were used: CAPs, a custom made Infinium SNP (Single Nucleotide Polymorphism) bead arrays and KASPar (KBiosciences Competitive Alelle-Specific PCR).

For CAPs the PCR reactions were carried out in a final volume of 20 µl, containing 50 ng of genomic DNA, 0.04 µl of DreamTaq polymerase (Fermentas), 2 µl 10X DreamTaq

buffer (Fermentas), 0.4 µl of dNTP (5mM) and 1 µM of each primer (20 pmol). The cycling profile was: 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Aliquots (5 µl) of the amplified products were digested for at least one hour at 37 °C in a final volume of 15 µl with 0.5 µl of the appropriate restriction enzyme, using the buffer recommended by the supplier. Amplification and digestion products were analysed by agarose gel electrophoresis (1.5% TBE, agarose) and visualized by GelRed® staining. In Table 2 the primer sequences and the restriction enzymes used are shown.

For genome wide SNP marker analysis, an Infinium bead array was used (Viquez-Zamora *et al.*, 2013). On this array, 5528 tomato SNPs are present. Marker analysis was carried out by Service XS Leiden, The Netherlands, according to the Illumina® Infinium HD Ultra Assay protocol (www.illumina.com). After removing missing data and monomorphic markers, 1166 SNP markers were used in the analysis. For fine mapping of the target regions, we developed KASPar assays based on SNP markers that were on the array. The KASPar assays were run by the van Haeringen lab (VHL), Wageningen, the Netherlands.

Trichome description

Trichomes present on the abaxial side of the leaf were classified according to type (Channarayappa *et al.*, 1992). For an estimation of trichome density, the abaxial part of three leaflets was observed under a binocular microscope and a visual scale was used to describe it. The scale used was adapted from Simmons and Gurr (2005) and consisted of four categories: 3, Abundant (>5 per mm2); 2, sparse (5–1 per mm2); 1, very sparse (<1 per mm2), and 0, absent.

Chapter 5

Effects of chemical priming agents on whitefly resistance of different tomato varieties

Alejandro F. Lucatti[1,2], Sjaak van Heusden[1], Richard G. F. Visser[1], Roeland E. Voorrips[1], Jurriaan Ton[3], Ben Vosman[1].

1 Wageningen UR Plant Breeding, Wageningen University and Research Centre. P.O. Box 386, 6700 AJ Wageningen, The Netherlands.

2 Graduate School Experimental Plant Sciences. Wageningen Campus. Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands.

3 Department of Animal and Plant Sciences. University of Sheffield. Western Bank, Sheffield, S10 2TN, UK.

Abstract

Background- Plants can perceive stress signals and respond to them in a specific way through induction of their immune system. They can also induce their immune system in response to exogenous application of hormones, such as Salicylic acid (SA) and jasmonic acid (JA), as well as some xenobiotic chemicals, like benzothiadiazole (BTH), β-aminobutyric acid (BABA), and sugars. These chemicals mimic natural elicitors and prime long-lasting and broad-spectrum resistance by boosting basal resistance. Priming allows plant cells to respond to low levels of stress signals with a faster and/or stronger defence response. Although the use of priming agents was shown in laboratory and field studies, little is known about the effects of priming on partially resistant genotypes. We assessed the effect of selected priming agents on the effectiveness of natural defence in tomato. Our hypothesis is that partial resistance may be enhanced to boost protection against pests/diseases upon treatment with chemical defence priming agents.

Results- A set of no-choice and choice bioassays was conducted using tomato genotypes varying in their level of basal resistance to *Bemisia tabaci* and pathogens. Priming treatment was done by soil drench applications of BABA, JA and fructose. We observed that whitefly survival or oviposition was not affected by priming treatment. Overall, fructose treated plants were more preferred by whiteflies than control plants. A genotype specific effect of priming was seen for the line FCN93-6-2. On this line, priming with BABA and JA reduced the number of whiteflies compared to non-primed plants.

Conclusion- We were able to show that JA and BABA can increase the non-preference to whitefly that was already known for the line FCN93-6-2, thus enhancing its resistance level. Furthermore, we were able to show that priming by fructose soil drench increases whitefly preference, but not whitefly survival or oviposition, which was independent of the genotypes.

Keywords: *Bemisia tabaci,* Jasmonic acid, β-aminobutyric acid, fructose, *S. habrochaites*.

INTRODUCTION

Plants are dynamic organisms that can be affected by a broad range of stresses of biotic (pathogens, herbivores) and abiotic (drought, heat) origin and counteract on them. In the case of herbivores or pathogens, plants perceive stress signals and respond to them in a specific way through inducing their immune system. Plants are able to recognize exogenous molecules known as herbivore or pathogen associated molecular patterns (HAMPs or PAMPs). They can also trigger defence responses upon the recognition of endogenous plant signals (damage associated molecular patterns; DAMPs). In the case of herbivorous insects, plants possess pattern recognition receptors (PRRs) that can detect HAMPs or DAMPs and activate a process known as HAMP-triggered immunity (HTI) (Erb *et al.*, 2012, Hogenhout and Bos, 2011). Successful herbivorous insects have found a way to overcome the HTI response by the use of effector proteins that can block or suppress HTI, resulting in basal levels of resistance that are insufficient to protect the plant against the herbivore. An example is the Mp10 effector from *Myzus persicae* which, when transiently expressed in *Nicotiana benthamiana,* induces chlorosis and local cell death. This effector is also able to suppress the oxidative burst response (PTI) induced by the bacterial PAMP flg22 (Bos *et al.*, 2010). In an evolutionary arms race between plants and herbivores/pathogens, selected plant varieties have evolved a hypersensitive defence reaction that is mediated by *R* proteins, which can recognize the activity of effector proteins. In the case of biotrophic pathogens and some cell/phloem-feeding insects, *R* proteins often mediate a specific cell death response (e.g. hypersensitive response, accumulation of reactive oxygen species, toxins and lipid mobilizations) that provides resistance (Howe and Jander, 2008). For example, more than 30 *R* genes have been identified in wheat, which are involved in the interaction with Hessian fly (*Mayetiola destructor*) (Stuart *et al.*, 2012, Harris *et al.*, 2003). Immunity mediated by *R* proteins is called effector-triggered immunity (ETI) and the model describing the evolutionary arms race between effector triggered susceptibility by the attack (ETS) and ETI in the host plant is commonly referred to as the 'zigzag' model (Jones and Dangl, 2006). In contrast to ETI, HTI and basal resistance are non-specific forms of host resistance and confer broad-spectrum resistance (not specific to a particular pathogen or herbivore) whereas ETI is specific to a pathogen (even race specific) or herbivore (Erb *et al.*, 2012, Stuart *et al.*, 2012, Pieterse *et al.*, 2012, Hogenhout *et al.*, 2008)

Downstream of the initial HTI and ETI responses, several plant hormones (jasmonic acid, salicylic acid, ethylene, abscisic acid, auxin, etc.) play a role in prompting the immune signalling network (Erb *et al.*, 2012). As a general rule, the jasmonic acid (JA) signalling pathway is activated in response to chewing herbivores and the salicylic acid (SA) signalling pathway is activated in response to phloem-feeding herbivores (aphids, whiteflies) and pathogens (Puthoff *et al.*, 2010, Walling, 2008, Zarate *et al.*, 2007). Although the distinction or specialization between pathways is well established,

it is not entirely black and white. It has been shown that these two main signalling pathways interact with each other in different forms and with other pathways (ethylene and abscisic acid) as well (Pieterse *et al.*, 2012, Schweiger *et al.*, 2014). The SA and JA signalling pathways are in general antagonistic to each other. However, synergistic effects have been shown in the case of plants treated with soil borne beneficial microorganisms (van Wees *et al.*, 1999). This so-called crosstalk between pathways depends not only on the pathogen or insect, but also on the ratio between hormones and on the timing and sequence of the attack of herbivores. The crosstalk between JA, SA and other plant hormones is thought to provide plants with regulatory potential to control their inducible defence responses in the most cost-effective manner (Erb *et al.*, 2012, Pieterse *et al.*, 2012).

Plants can develop an enhanced defensive capacity in response to exogenous application of endogenous defence hormones, such as SA and JA, as well as some xenobiotic chemicals, such as benzothiadiazole (BTH), β-aminobutyric acid (BABA), and sugars, such as fructose, sucrose, galactinol amongst others (Bolouri Moghaddam and Van den Ende, 2012, Cohen *et al.*, 1994, Hodge *et al.*, 2005, Nombela *et al.*, 2005, Ton *et al.*, 2005). These chemicals mimic natural elicitors and prime (or induce) long-lasting and broad-spectrum resistance to pests and/or diseases by boosting basal resistance (Ahmad *et al.*, 2011, Luna *et al.*, 2012, Nombela *et al.*, 2005). This process of plant sensitisation by priming has been defined as an augmented capacity of plants to express basal defence mechanisms (Ahmad *et al.*, 2010, Pastor *et al.*, 2013). Priming allows plant cells to respond to low levels of a stimulus (pest or pathogen attack) with a faster and/or stronger defence response (Ahmad *et al.*, 2011, van Hulten *et al.*, 2006, Conrath, 2011, Heil and Ton, 2008). Primed defence responses can affect herbivorous insects directly, affecting the fitness of the insect and indirectly by increasing the effectiveness of natural enemies (Ton *et al.*, 2006).

The use of priming agents to increase resistance to pathogens and insects was shown at the laboratory level as well as in field studies (Kessler *et al.*, 2006, Ton *et al.*, 2006). On tic bean (*Vicia faba* var. minor) it was shown that soil drench applications of BABA can reduce the intrinsic growth rate of *Acyrthosiphon pisum* but did not affect the mortality (Hodge *et al.*, 2005). On tomato (cv. Marmande), spray treatment with BTH results in a delay in whitefly development, which was restricted to the sprayed leaflets (Nombela *et al.*, 2005). Although some studies were performed, little is known about the effects of priming on partially resistant genotypes, i.e. plants expressing relatively high levels of basal resistance. In this work, we assessed the effect of selected priming agents on the effectiveness of natural defence in tomato. Our hypothesis is that relatively high levels of resistance may be enhanced to boost protection against pests/diseases upon treatment with chemical defence priming agents. To test this, a set of no-choice and choice bioassays was conducted using tomato genotypes that vary in their level of basal

resistance to whiteflies and pathogens. We observed a clear genotype specific effect of priming resulting in a decreased preference of whiteflies for the primed plants. The induced resistance by BABA and JA was particularly pronounced in the *S. lycopersicum* genotype FCN93-6-2, which displays relatively high levels of resistance in terms of whitefly preference.

RESULTS

No-choice experiment

A no-choice experiment, using clip-on cages, was carried out to measure long lasting effects of the application of priming agents (jasmonic acid, β-aminobutyric acid and fructose) on the fitness of whiteflies using four tomato genotypes differing in their level of resistance to insects or pathogens (cv. Moneymaker, cv. Motelle, FCN93-6-2 and IL4.1) (Table 1). To assess whitefly resistance two parameters evaluating different stages of the whitefly life cycle were considered; adult survival (AS) and oviposition rate (OR). For AS, no significant genotype or treatment effects were detected ($P=0.92$ and $P=0.60$ respectively) nor an interaction effect of genotype by treatment ($P=0.32$). For OR, statistically significant differences were found among genotypes ($P<0.01$), where the line IL4.1 had on average the lowest oviposition rate (4.0 eggs/female/day) and the line FCN93-6-2 the highest oviposition rate (5.8 eggs/female/day). No significant differences were found for treatments ($P=0.12$) or for the genotype by treatment interaction effect ($P=0.61$) (Table 2).

Table 1: Description of the plant material used.

Genotype	Resistance donor	Characteristic	Reference
IL4.1	*S. habrochaites* (LYC4)	Partially resistant to *Botrytis cinerea* and susceptible to *B. tabaci*	Finkers *et al.*, 2007, Van den Elsen, 2013.
cv. Motelle	*S. peruvianum* (PI128657)	Tomato cultivar partially resistant to *B. tabaci* (*Mi1-2* gene)	Nombela *et al.*, 2003
FCN93-6-2	*S. habrochaites* (FCN3-5)	Pre-breeding line non-preferred by *T. vaporariorum* and *B. tabaci*	Lucatti *et al.*, 2010, Chapter 3.
cv. Moneymaker		Susceptible reference cultivar	

Choice experiment

To measure the effect of priming on whitefly preference a free choice experiment was performed where adult whiteflies were released in a compartment with the different genotypes. The number of whiteflies on the different genotypes was counted 24 hours after the start of the infestation. We detected statistically significant differences in whitefly preference among genotypes ($P<0.01$) and among treatments ($P<0.01$), but

not for their interaction (P=0.13). The line FCN93-6-2 (3.8 insects/plant) was the least preferred by the whiteflies and IL4.1 (12.2 insects/plant) the most preferred. The cultivars Motelle and Moneymaker showed an intermediate preference level (5.7 and 6.5 insects/plant, respectively). Among the treatments, an increase in whitefly preference was observed in plants treated with fructose (10.0 insects/plant) over the other three treatments (Table 3). However, the overall interaction of genotype and treatment was not significant. The line FCN93-6-2 was the only one reacting to the priming treatment (P<0.01) with a clear reduction in the number of insects on the plants treated with JA (1.9 insects/plant) or BABA (2.6 insects/plant) in comparison with the plants treated with fructose (6.1 insects/plant) or water (4.6 insects/plant).

Table 2: No-choice experiment results. Average adult survival and oviposition rate per genotype and treatment.

Genotype	Treatment	Adult Survival	Oviposition rate
IL4.1	H_2O	0.95 (± 0.02)	4.33 (± 0.72)
	Fructose	0.92 (± 0.02)	4.21 (± 0.54)
	BABA	0.90 (± 0.04)	3.92 (± 0.60)
	JA	0.96 (± 0.01)	3.64 (± 0.67)
Average		0.94 (± 0.01)	4.02 (± 0.31) [a]
cv. Moneymaker	H_2O	0.93 (± 0.02)	3.87 (± 0.74)
	Fructose	0.96 (± 0.01)	5.17 (± 0.52)
	BABA	0.91 (± 0.03)	5.26 (± 0.58)
	JA	0.94 (± 0.01)	3.90 (± 0.49)
Average		0.94 (± 0.01)	4.57 (± 0.30) [ab]
cv. Motelle	H_2O	0.94 (± 0.01)	5.10 (± 0.54)
	Fructose	0.97 (± 0.02)	5.19 (± 0.42)
	BABA	0.96 (± 0.02)	4.40 (± 0.46)
	JA	0.82 (± 0.12)	4.56 (± 0.64)
Average		0.92 (± 0.03)	4.81 (± 0.26) [b]
FCN93-6-2	H_2O	0.94 (± 0.02)	4.82 (± 0.39)
	Fructose	0.96 (± 0.01)	6.14 (± 0.69)
	BABA	0.96 (± 0.01)	6.61 (± 0.38)
	JA	0.92 (± 0.03)	5.54 (± 0.41)
Average		0.95 (± 0.01)	5.78 (± 0.26) [c]

Average: mean value per genotype over all treatments.Different letters indicate statistically significant differences according LSD test (P<0.05). When no letters are used, the differences were not significant.

Effect of induced defence on plant growth

To determine the effect of priming on plant growth, the fresh weight of the plants was measured five days after inoculation with the whiteflies. We found significant effects of genotype ($P<0.01$) and treatment ($P<0.01$), but no significant genotype by treatment interaction effect ($P=0.51$). For all genotypes assessed, BABA application and to a lesser extent JA application resulted in a reduction of fresh weight when compared to plants treated with fructose or water (Table 4). To test the weight reduction due to treatment, the fresh weight of each genotype was referred to their weight on water. There was a difference in the weight reduction only for the plants treated with BABA ($P=0.02$). The line FCN93-6-2 (28% reduction in weight) was statistically different from cv. Moneymaker (49% reduction in weight) and IL4.1 (52% reduction in weight), but not from cv. Motelle (36% reduction in weight). This last cultivar was not different from cv. Moneymaker.

Trichome type VI density assay

Three different types of glandular trichomes (Type I, IV and VI) are found among tomato species. Our lines only contain type VI trichomes. To test whether the enhanced whitefly non-preference might be due to an effect of priming on trichome type VI density, the abaxial and adaxial number of type VI trichomes was counted. On the adaxial surface of the leaf, we detected a statistically significant genotype effect on trichome density ($P<0.01$), but not of treatment ($P=0.47$) or interaction ($P=0.88$) effects. The genotypes with lower densities were FCN93-6-2 and cv. Motelle (1.2 and 1.1 trichomes/mm^2 respectively), whereas the higher densities were found in IL4.1 and cv. Moneymaker (2.1 and 1.7 trichomes/mm^2 respectively). On the abaxial surface of the leaf, we detected significant genotype ($P<0.01$) and interaction ($P<0.01$) effects on trichome density, but no significant treatment effects ($P=0.18$). Cultivar Moneymaker had less trichomes than any of the other three genotypes assessed. We observe a clear treatment effect for cv. Moneymaker, where a reduction in the number of trichomes was observed when the plants where treated with JA when compared to fructose, but not when compared to H_2O or BABA (Figure 1).

Table 3: Choice experiment results. Average number of adult whiteflies per plant (mean ± standard error).

Genotype	Treatment				Average per genotype
	H_2O	Fructose	BABA	JA	
IL4.1	10.1 (± 3.72)	19.0 (± 3.84)	11.4 (± 2.58)	8.4 (± 1.32)	12.2 (± 2.86)^{c}
cv. Moneymaker	5.5 (± 1.90)	8.6 (± 1.68)	6.5 (± 1.28)	5.4 (± 0.68)	6.5 (± 1.38)^{b}
cv. Motelle	5.8 (± 0.65)	6.1 (± 0.99)	5.5 (± 0.71)	5.4 (± 0.88)	5.7 (± 0.81)^{b}
FCN93-6-2	4.6 (± 0.86)^{b}	6.1 (± 1.27)^{b}	2.6 (± 0.75)^{a}	1.9 (± 0.40)^{a}	3.8 (± 0.82)^{a}
Average per treatment	6.5 (± 1.78)^{a}	10.0 (± 1.95)^{b}	6.5 (± 1.33)^{a}	5.3 (± 0.82)^{a}	

Different letters indicate statistically significant differences according LSD test (P<0.05).
When no letters are used, the differences were not significant.

DISCUSSION

Whitefly resistance in the lines used

Over all treatments, we did not observe any difference in whitefly resistance among the tomato lines in no-choice assays for adult survival (Table 2). This was as expected for cv. Moneymaker as well as the lines FCN93-6-2 and IL4.1. For cv. Motelle we had expected some level of resistance, as this cultivar carries the *Mi1-2* allele, which has been reported to confer partial resistance to *B. tabaci* (*B* and *Q* biotype) in terms of both adult survival and oviposition rate (Nombela *et al.*, 2003). However, we did not find such resistance in cv. Motelle (homozygous *Mi1-2*), since both adult survival and oviposition rate were not significantly different from cv. Moneymaker (homozygous *mi1-2*). This may be due to the different whitefly strain used in our study (*Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I; former *B*-biotype) (Firdaus *et al.*, 2013) and to the different temperature at which the experiment was done. A transgenic line (143-11-16-36) carrying the *Mi1-2* gene in the cv. Moneymaker background, gave only an effect at lower temperatures (23°C) but not at higher temperatures (27°C) where the insect development is at its optimum (Nombela *et al.*, 2003). However, assessment of oviposition rates revealed differences. Significantly, more eggs were found on FCN93-6-2 than on any of the other lines (Table 2). Cultivar Motelle and the line IL4.1 differed from each other but were not statistically different from cv. Moneymaker. The results obtained for the IL4.1 line were similar to the results obtained by Van den Elsen (2013).

Table 4: Growth costs of priming. Fresh weight (g) per genotype and treatment (mean ± standard error) 24 days after sowing.

Genotype	Treatment				Average per genotype
	H_2O	Fructose	BABA	JA	
IL4.1	16.4 (± 1.42)	16.8 (± 1.1)	8.0 (± 0.94)	12.1 (± 0.93)	13.3 (± 0.84)^{a}
cv. Moneymaker	18.7 (± 0.59)	19.0 (± 0.81)	9.5 (± 1.08)	14.4 (± 0.90)	15.4 (± 0.80)^{b}
cv. Motelle	20.1 (± 0.95)	20.6 (± 0.91)	13.0 (± 0.84)	15.9 (± 0.95)	17.4 (± 0.71)^{c}
FCN93-6-2	21.6 (± 1.01)^{b}	20.7 (± 0.52)^{b}	15.5 (± 1.21)^{a}	15.9 (± 0.81)^{a}	18.4 (± 0.68)^{c}
Average per treatment	19.3 (± 0.59)^{c}	19.3 (± 0.51)^{c}	11.5 (± 0.72)^{a}	14.6 (± 0.51)^{b}	

Different letters indicate statistically significant differences according LSD test ($P<0.05$). When no letters are used, the differences were not different from the average per treament.

The choice assay revealed clear differences between the genotypes. The IL4.1 line was the most preferred by the whiteflies, whereas FCN93-6-2 was the least preferred. The FCN93-6-2 has previously been described as being relatively unattractive to the whitefly *T. vaporariorum* under greenhouse conditions (Lucatti *et al.*, 2010). Interestingly, this line showed the highest oviposition rate in the no-choice assay. This suggests that the mechanisms controlling oviposition resistance and repellence are controlled by antagonistically acting defence signalling pathways, although this hypothesis requires further research.

Treatment specific effects of priming on whitefly preference

Over all genotypes, priming by BABA, JA or fructose did not have an effect on whitefly fitness in terms of adult survival and oviposition rate. However, in our choice experiment we saw a clear effect of long-lasting priming over all genotypes in fructose treated plants. On these plants, we found on average a higher number of whiteflies. This increase in preference (induced susceptibility) may be due to a change in the soil-borne microbes and/or to enrichment in the soluble carbohydrates of the soil. This induced susceptibility by soil-borne microbes has been shown in *Arabidopsis* and tomato, wherein soil inoculation with *Pseudomonas fluorescens* WCS417r had a positive effect on the performance of *Myzus persicae* and *B. tabaci* (Pineda *et al.*, 2012, Shavit *et al.*, 2013). In addition, soil-borne microbes can alter the emission of volatile organic compounds (VOCs). The addition of soil microorganisms on autoclaved soil increased the emissions of VOCs (2-3 bunanediol: 2-3BD) by healthy maize plants. The increased emission of 2-3BD had a positive effect on the caterpillar *Spodoptera littoralis* without affecting plant growth (D'Alessandro *et al.*, 2013). It was seen that the inoculation with *P. fluorescens* WCS417r modifies the blend of plant volatiles emitted by *Arabidopsis* under aphid infestation and reduces the parasitism rate of *Diaeretiella rapae* (Pineda *et al.*, 2013). The induced susceptibility mediated by *P. fluorescens* was associated with a

repression of *ABA1* (ABA-signaling gene) and *MYC2,* and as consequence a suppression of the *MYC2*-branch of the JA pathway (Pineda *et al.*, 2012, Pineda *et al.*, 2013). Also, soil inoculations with *Rhizobium leguminosarum* lead to an increase in growth of nodulating strains of *Trifolium repens* (white clover) and as consequence to an increase on the performance of the generalist caterpillar insect *S. exigua* (Kempel *et al.*, 2009).

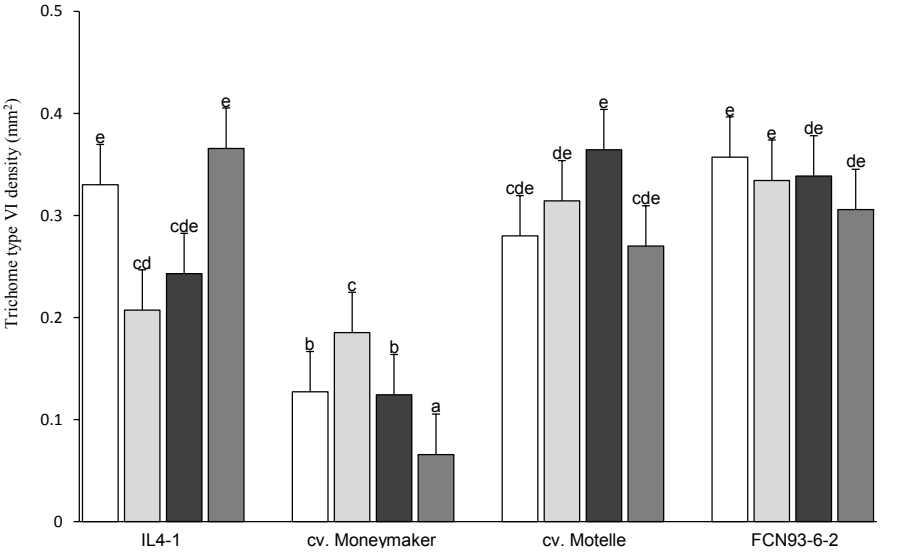


Figure 1: Density of trichomes type VI per genotype and treatment (□ H_2O, ▫ Fructose, ■ BABA and ▪ JA). Different letters indicate statistically significant differences according LSD test (P<0.05).

Genotype specific effects of priming on whitefly preference

A clear genotype specific effect of priming on whitefly preference in tomato was seen for the line FCN93-6-2. The other genotypes did not show such an effect. We saw an enhancement of the non-preference (a reduction in the number of whiteflies) in FCN93-6-2 when treated with BABA and JA. It is known that BABA potentiates SA-induced genes and also primes for callose deposition irrespective of the presence of SA and JA, but requires the presence of ABA (Ton and Mauch-Mani, 2004). Plants treated with BABA had an increased emission of MeSA that was show to be repellent of different species of aphids (Hardie *et al.*, 1994, Walling, 2000, Digilio *et al.*, 2012). On the other hand, several studies have implicated the JA signalling pathway in the emissions of VOCs. In tomato, JA applications trigger the emission of VOCs similar to those after herbivore attack (monoterpenes, MeSA, TMTT, etc.) (Ament *et al.*, 2004, Bruce *et al.*, 2008, Degenhardt *et al.*, 2010). In addition, wild tomato plants (*S. habrochaites* PI127826) treated with JA accumulated higher levels of 7-epizingiberene which is a known repellent of *B. tabaci* (Bleeker *et al.*, 2012). Also, transgenic tomato plants overexpressing Systemin, a known player of the JA-pathway, had an increased emission of volatiles, especially

monoterpenes and MeSA (Degenhardt *et al.*, 2010). The metabolomics profile of the VOCs emitted by FCN93-6-2 after priming will help to elucidate the mechanisms behind the increase in resistance.

Effects of treatments and genotypes on trichomes and plant growth

In tomato, glandular trichomes are the place of synthesis and storage of several types of secondary metabolites (Glas *et al.*, 2012, Schilmiller *et al.*, 2012, Schilmiller *et al.*, 2008, Schilmiller *et al.*, 2009). It has been shown that a foliar spray of tomato leaves with MeJA increases the density of trichome type VI in new apical leaves (Boughton *et al.*, 2005). We did not observe any increase in trichome type VI density in FCN93-6-2 when treated with soil drench applications of JA and can reject the hypothesis that the enhancement of non-preference observed in FCN93-6-2 is a consequence of this, but more likely of a higher synthesis rate and/or emission levels.

We observed that in general, JA and BABA treatments negatively affected plant growth, whereas fructose did not have an effect. One of the mayor disadvantages of induced defence is the negative effect on plant fitness (weight, growth, seed production). For example, SA, BABA and BTH applications reduced seed set and the relative growth rate in *Arabidopsis* (Cipollini, 2002, van Hulten *et al.*, 2006). We found that the fresh weight of FCN93-6-2 was least affected by BABA and the most resistant to whiteflies in the choice experiment. Under disease pressure, it has been shown that the benefits of BABA mediated priming can outweigh its costs (van Hulten *et al.*, 2006). The increased level of whitefly resistance on FCN93-6-2 mediated BABA and JA and its low responsiveness in terms of growth reduction might outweigh the costs of priming under conditions of whitefly infestation, but further research into this is needed.

CONCLUSIONS

Priming of tomatoes may have genotype and treatment specific effects on whitefly preference. We were able to show that JA and BABA can increase the non-preference to whitefly that was already known for the line FCN93-6-2, thus enhancing its resistance level. Such induction was not seen for the other genotypes. Furthermore, we were able to show that priming by fructose soil drench increases whitefly preference independently of the plant genotype, but not whitefly survival or oviposition.

MATERIALS AND METHODS

Plant Material and Growing Conditions

In this study, four tomato (*Solanum lycopersicum*) genotypes were selected based on

their different level of basal resistance to whitefly and botrytis (Table 1). The line IL4.1 was included because this project is part of a larger EU project in which the same genotypes are used to study the effects of priming on botrytis resistance. Finally, cv. Moneymaker was used as susceptible reference.

The seeds were sown in 20 ml pots filled with soil compost and grown in a growth chamber at Wageningen UR, Wageningen, The Netherlands (20 ± 2°C, 70% relative humidity, 16/8 h day/night). When the plants had reached the cotyledon stage (7-8 days after sowing), they were moved to an insect proof greenhouse (22 ± 2°C, 70% relative humidity, 16/8 h day/night). At this point the plants were treated with the priming compound. The plants were watered (50 ml per pot) every other day. After seven days the seedlings were taken out of the soil; the roots rinsed with tap water taking care of not breaking the roots and the plants were re-potted in 500 ml pots containing fresh soil compost. The seedlings were left to grow for ten days and after that the insect bioassays were performed (Figure 2). At the moment of the bioassays, the plants had at least one fully expanded true leaf.

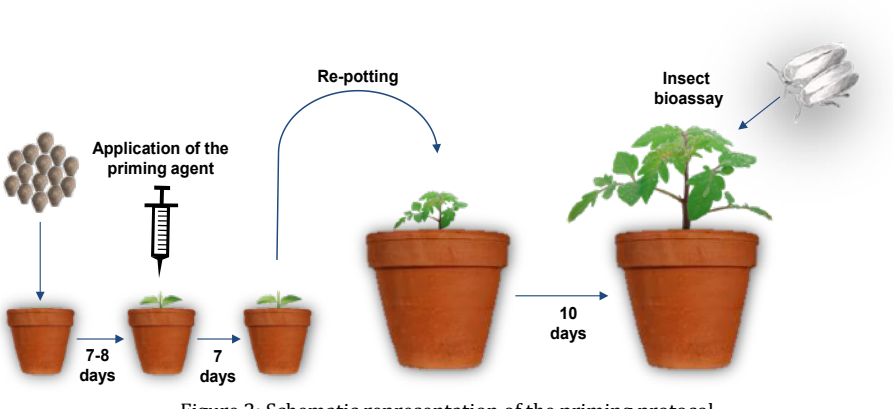


Figure 2: Schematic representation of the priming protocol.

Insect rearing

A non-viruliferous whitefly rearing (*Bemisia tabaci* Group Mediterranean-Middle East-Asia Minor I) (Firdaus *et al.*, 2013) was maintained on the susceptible cv. Moneymaker for several generations at Wageningen UR Plant Breeding, the Netherlands. The initial inoculum was obtained from a permanent rearing at the Laboratory of Entomology, Wageningen UR, Wageningen, the Netherlands.

Priming compounds and concentrations used

In this study, we used three compounds as priming agents: β-aminobutiric acid (BABA, 0.5 mM final concentration), jasmonic acid (JA, 0.05 mM, final concentration) and fructose

(100 ppm, final concentration). Water was used as control. The concentrations used for BABA and JA were the highest concentration that can be applied to cv. Moneymaker with a minimum fitness cost in terms of growth and seed production (Luna, 2012). All priming agents were obtained from Sigma-Aldrich. The priming was done by injecting a concentrated solution in water of the different priming compounds into the soil, to reach the desired concentration in a final volume of 20 ml.

No-choice experiment

Ten days after re-potting (Figure 2), five anesthetized (using CO_2) female whiteflies (four days old) were placed into a clip-on cage (2.5 cm diameter and 1.0 cm high). One clip-on cage per plant and eight plants per genotype/treatment combination were used. All plants were individually randomized in a block design. Five days after inoculation, the number of living and dead whiteflies was recorded and the surviving whiteflies were removed. The number of eggs was counted and the variables Adult survival (AS) and Oviposition rate (OR) were calculated (Bas *et al.*, 1992). The variable AS was transformed to arcsine (sqrt(x)) and the variable OR was transformed to sqrt(x) to achieve an approximately normally distributed residual variation independent of the mean. The data were analysed by two-way ANOVA using the software package Genstat (15^{th} edition).

Choice experiment

For the choice experiment, all plants were individually randomized in a block design with 16 plants per block (2 per genotype-treatment combination) and 8 blocks. Ten days after re-potting (Figure 2), non-sexed whiteflies were evenly released in an insect proof greenhouse (14 m^2) at a density of 20 whiteflies per plant. Twenty-four hours after release, the whiteflies from each plant were re-captured and counted. The number of insects per plant was log (x+0.1) transformed to achieve an approximately normally distributed residual variation independent of the mean. Fresh weight was considered as co-variable and the data were analysed by a two-way ANOVA with blocking using the software package Genstat (15^{th} edition).

Trichome type VI density assay

In order to determine the effect of priming on the induction of glandular trichomes the total number of trichomes type VI was counted on the plants used in the choice experiment. The trichomes were counted ten days after re-potting on the most apical leaflet of the plant (youngest terminal leaflet). On this leaflet, a leaf disc (4 mm in diameter, 12.6 mm^2 in area) was marked and the number of trichomes type VI on the abaxial and adaxial side of the leaflet was counted. In addition, the total leaf area per genotype was measured using the LI-COR® area meter LI-3100C. The number of trichomes type VI on the leaf was log-transformed and analysed by a two-way ANOVA considering the blocks. The statistical

analyses were performed using the software package Genstat (15th edition).

Growth assay

In order to determine the cost in growth of the different genotype-priming combinations, the aerial portion of the plant (leafs, shoots and stems) used in the choice experiment was weighted and the fresh and dry weight were registered. The data was analysed by ANOVA using the software package Genstat (15th edition).

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CHAPTER 6

General discussion

Arthropods are the most widespread and diverse group of animals, with an estimated 4-6 million species worldwide (Schoonhoven *et al.*, 2005). While only a small percentage of arthropods are classified as pests, they cause major damage to crops, destroying around 18% of the world crop production annually, contributing to 20% of losses of stored grains and causing around US$100 billion of damage per year (Nicholson, 2007). Although there is a growing interest in healthy, user and environmentally friendly culture practices (Grygorczyk *et al.*, 2013), crop protection still heavily relies on insecticide application. Host plant resistance is one of the pillars of integrated pest management programs and has been proposed as a promising alternative for insecticide application (Broekgaarden *et al.*, 2011, Schoonhoven *et al.*, 2005). For some insects like aphids and the Hessian fly amongst others, several major genes conferring insect resistance were described and few of them cloned (Yencho *et al.*, 2000, Broekgaarden *et al.*, 2007). In tomato, the *Mi1-2* gene was described as conferring resistance to several species of root-knot nematodes (*Meloidogyne spp.*) (Milligan *et al.*, 1998), some isolates of potato aphid (*Macrosiphum euphorbiae* Thomas) (Rossi *et al.*, 1998) (Kaloshian *et al.*, 1995), the sweet potato whitefly (*B. tabaci*) (Nombela *et al.*, 2003) and the tomato psyllid (*Bactericerca cockerelli*) (Casteel *et al.*, 2006). Although the *Mi1-2* gene is widely present in modern tomato cultivars, the expected whitefly resistance is not always seen (Chapter 5) and may be dependent on the whitefly population. Host plant resistance to insects can also be a complex quantitatively inherited trait. Understanding the genetics behind such complex resistance traits is compulsory for successful breeding of varieties resistant to pests. The study of natural variation coupled to "*omics*" technologies (genomics, metabolomics, transcriptomics) and QTL mapping has the potential to provide valuable insight into the more complex resistance traits and to reach the goal of durable resistant plants based on different resistance mechanisms. By targeting different stages of the insect life cycle and combining resistance traits interfering with these, a more difficult to break and hopefully more durable whitefly resistance will be achieved.

Natural variation, source of resistance traits

In nature, insect resistance is a common phenomenon; these resistances are often not present any more in cultivated crop plants. The loss of genetic variation encoding resistance might be due to selection for high yielding varieties, or due to the emergence of more resistant pests in monocultures because of the high level of selection pressure on insect populations (De Vos and VanDoorn, 2013). Breeders have made use of natural variation present in wild relatives as sources of insect resistance genes (Broekgaarden *et al.*, 2011, De Vos and VanDoorn, 2013, Yencho *et al.*, 2000).

The maintenance and frequency of resistance alleles in a population is shaped by several factors such as selection pressure, co-evolution, spatial variation selection and gene drift. A number of hypotheses were formulated to explain the evolution and maintenance of

plant resistance traits (Chapter 1, Table 1) (Rausher, 2001, Weber and Agrawal, 2012). These hypotheses have in common the concept that mechanisms conferring plant resistance are costly; in other words, there is a trade-off between resistance and plant fitness. Some studies have addressed the factors shaping the population structure of some wild relatives of tomato used as sources of resistance genes. For example, the population structure of *S. pimpinellifolium* was better explained by ecological and climatic variables than by the isolation by distance hypothesis (Zuriaga *et al.*, 2009). For *S. habrochaites*, geographical and climatic variables explain a substantial amount of the variation in terpenoids and acyl sugar concentrations among accessions (Gonzales-Vigil *et al.*, 2012, Kim *et al.*, 2012). However, for *S. galapagense* and *S. cheesmaniae*, the relative concentration of acyl sugars and the resistance level could not be explained by climatically or geographical conditions at the collection sites of the accession, suggesting that other factor(s) might be shaping the differences between the species (Chapter 2).

Host-plant interactions are among the most important selective forces known (Gloss *et al.*, 2013). The evolution of plant-herbivore relationships is ruled by the reciprocal nature of the interaction (Agrawal *et al.*, 2012). The interaction between plant and herbivore populations is likely to maintain non-neutral genetic variation as a result of the spatial variation in the selection pressure imposed by different herbivore communities/species in different environments (Gloss *et al.*, 2013, Hare *et al.*, 2012). Because of the large number of different accessions, environments and genomic data, *Arabidopsis* was shown to be an excellent model to study effects of selection pressure by herbivory on the maintenance of resistance traits. Recently it was shown in two studies that the selection pressure exerted by a single insect herbivore species (either a generalist or a specialist) can be strong enough to shift allele frequencies in a plant population (Agrawal *et al.*, 2012, Züst *et al.*, 2012). It was seen that selection pressure by herbivory has not only a direct selective force on the host, but also affected the ability of a plant population to compete with other populations. In an insect free environment, the *Arabidopsis* accession Sap-0 was the most abundant of 27 accessions. However, under aphid herbivory this accession was not found anymore and which accession was most abundant depended on the aphid species (Züst *et al.*, 2012). Contrarily, when insect herbivory was supressed instead of imposed, a relaxation of plant defences and increase in the competitiveness over generations was seen (Agrawal *et al.*, 2012). Alleles maintained by local selection are likely to contribute significantly to plant fitness, having large phenotypic effects (Gloss *et al.*, 2013). In the case of *S. galapagense*, the presence and maintenance of a trait like the presence of high densities of trichomes types I and IV as well as acyl sugars could be the result of selective pressure by herbivory.

Towards whitefly resistance in tomato based on several mechanisms

Breeding new tomato varieties with an increased and durable resistance against whiteflies depends on the availability of different resistance mechanisms targeting specific stages of the insect's life cycle (Figure 1). Mechanisms to be targeted may include host plant selection by the adult whitefly, its survival, fecundity and nymph survival. In the following paragraphs I will discuss the different options.

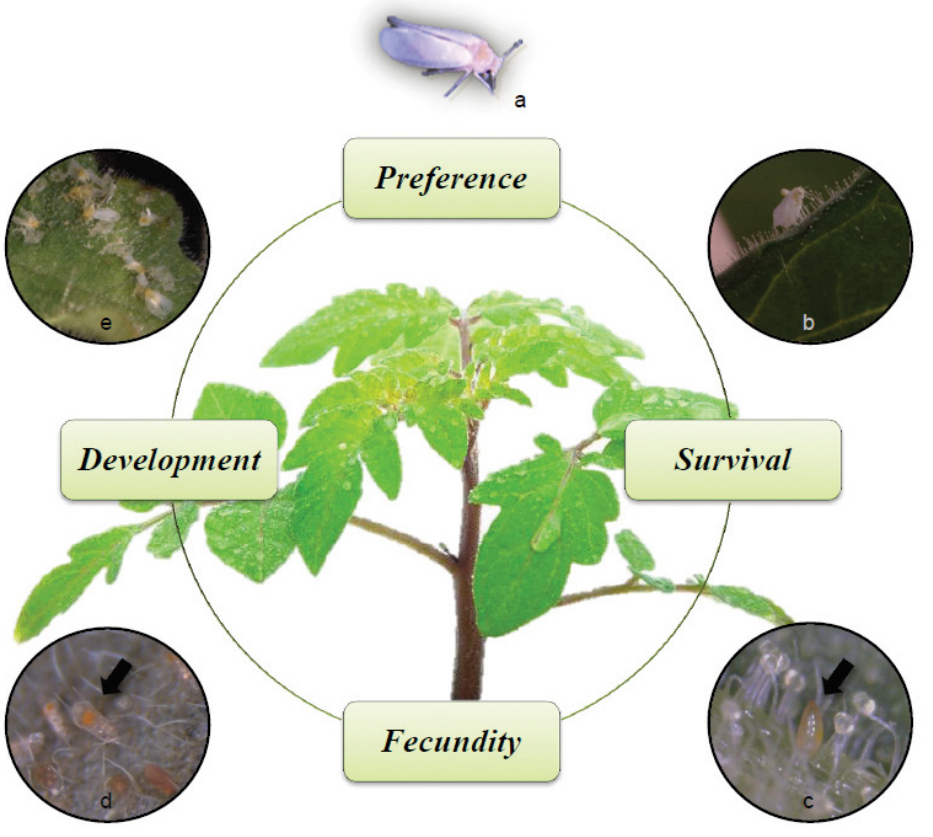

Figure 1: Overview of the tomato resistance mechanisms targeting different stages of the whitefly life cycle. a) *B. tabaci* adult recognizing volatile organic compounds from the tomato plant, b) *B. tabaci* adult trapped by trichomes type IV on a leaf of *S. galapagense*, c) *B. tabaci* egg on a *S. lycopersicum* accession, d) abnormal *B. tabaci* nymph on *S. pimpinellifolium* and e) dead *B. tabaci* adults on *S. galapagense* directly after emerging from the pupae.

Host-plant preference: whiteflies use a set of plant derived cues (visual, olfactory, mechano-sensory and gustatory) during the process of host plant selection (Bleeker *et al.*, 2009, Zhang *et al.*, 2004). The volatiles affecting whitefly preference depend on the wild tomato species. In *Solanum pennellii* (LA2560) and *S. habrochaites* (PI127826) a reduction of up to 60% in the number of whiteflies per plant was observed. In *S. pennellii* this reduction was associated mainly to the emissions of monoterpenes

(*p*-cymene, γ-terpinene and α-phellandrene), whereas in *S. habrochaites* (PI127826) the reduction in number of recaptured whiteflies was correlated to the presence of two sesquiterpenes, 7-epizingiberene and *R*-curcumene (Bleeker *et al.*, 2009, Bleeker *et al.*, 2011). In Chapter 3, we characterized two accessions of *S. habrochaites* (methyl ketone producer), as well as two pre-breeding lines and three tomato cultivars. We have shown that the pre-breeding line FCN93-6-2 was non-preferred by *B. tabaci* when given the choice between this line and cv. Moneymaker. In this line, the preference was not associated to the presence of methyl ketones, but to reduced concentrations of monoterpenes (Chapter 3). Similar results were described in a free choice experiment where the whitefly behaviour was also independent of the presence of methyl ketones (Bleeker *et al.*, 2009). It was also observed that transgenic tomatoes expressing the *ShZIS* gene had not only an increased level of repellence but also a negative impact on the whitefly survival and fecundity (Bleeker *et al.*, 2012). The increase or decrease of the emission of specific secondary metabolites may be used as a breeding target to decrease whitefly infestation. Targeting genes involved in terpenoid metabolism is a promising approach to breed for tomato varieties with increasing emission levels of specific metabolites that could increase the level of repellence and indirect defences.

Adult Survival: several resistance mechanisms affecting whitefly survival on tomato have been described (Table 2, Chapter 1). Most of them are dependent on the presence of glandular trichomes (type I, IV and VI) and on specific secondary metabolites (acyl sugars, sesquiterpenes and methyl ketones) (Table 2, Chapter 1). The wild tomato species *S. pennellii* and *S. habrochaites* were the first to be studied as source of insect resistance (De Ponti *et al.*, 1975, Berlinger, 1986, Romanow *et al.*, 1991). On *S. pennellii,* whitefly resistance was associated to the presence of trichomes type IV and high levels of acyl sugars (Muigai *et al.*, 2002, Muigai *et al.*, 2003, Liedl *et al.*, 1995). On *S. habrochaites*, the resistance mechanism varied depending on the accession used as resistance donor. On some accessions of *S. habrochaites* (i.e. PI134418, CGN1.1561) the resistance was correlated to methyl ketones (i.e. 2-tridecanone, 2-undecanone) and to the presence of trichomes type VI (Bas *et al.*, 1992, Maliepaard *et al.*, 1995, Fridman *et al.*, 2005, Antonious *et al.*, 2005, Firdaus *et al.*, 2012, Antonious, 2001). Whereas, in others (i.e. LA1777, PI127826), resistance was linked to the accumulation of sesquiterpenes (i.e. 7-epizingiberene) and to the presence of trichomes type VI (Bleeker *et al.*, 2009, Bleeker *et al.*, 2011, Bleeker *et al.*, 2012, Freitas *et al.*, 2002, Momotaz, 2005, Momotaz, 2010). Recently, the focus has changed to species more closely related to the cultivated tomato including *S. galapagense S. cheesmaniae* and *S. pimpinellifolium* (Firdaus *et al.*, 2012, Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012). In Chapter 2, we looked into the variation for whitefly resistance, acyl sugar and trichome composition among accessions of *S. galapagense* and *S. cheesmaniae*. Resistance was exclusively found in *S. galapagense*. All resistant accessions had high numbers of trichomes type IV

and high levels of acyl sugars, but not all accessions with high levels of acyl sugars were resistant suggesting the necessity of both mechanisms in order to achieve resistance. However, the presence of an undetected metabolite conferring resistance cannot be excluded. Insect resistance with a less complex genetic background was also found in a *S. pimpinellifolium* (TO-937) that possesses trichomes type IV and acyl sugars. Two QTLs on Chromosome 2 accounting for 40% of the resistance to spider mites (*T. urticae*) were described (Salinas *et al.*, 2013). For whitefly resistance, two major QTLs *Wf-1* and *Wf-2* from *S. galapagense* explained 69% of the variability for adult survival, and 53% for oviposition rate (Firdaus *et al.*, 2013). Due to the relation of trichomes type IV and acyl sugars with resistance to different insects species, it is a logical step to study the effect of the presence of these QTLs on other insect species.

Whitefly fecundity: up to now, most of the resistance mechanisms affecting whitefly fecundity are the result of a strong effect on adult survival and of the presence of high densities of trichomes type I and IV (Table 2, Chapter 1). In 1995, two QTLs (*Tv-1* and *Tv-2*) on Chromosome 1 and 12 respectively, were described reducing the fecundity of the greenhouse whitefly (*T. vaporariorum*) by 23% (Maliepaard *et al.*, 1995). In Chapter 4, we described an additional 3.06 Mbp introgression on Chromosome 5 (*OR-5*) that on its own (without *Tv-1* and *Tv-2*) can reduce the oviposition rate of *B. tabaci* by 80% when compared to a reference tomato cultivar (cv. Moneymaker) without an effect on adult survival and on the presence of trichomes type IV.

Nymph survival: resistance mechanisms affecting nymph survival or development have received much less attention compared to those affecting adult survival and fecundity, most likely because measuring nymph survival is labour intensive. Maliepaard *et al.* (1995) did one of the first QTL mapping studies considering the survival of whiteflies nymphs, but no QTL was identified. It was not until 2013, that a QTL affecting pre-adult survival (*Wf-1*) was identified (Firdaus *et al.*, 2013). Nonetheless, this QTL explained only 13% of the variation for pre-adult survival, was also involved in a reduction of adult survival (54%), oviposition rate (42%) and the density of trichome type IV (66%), thus suggesting that the mechanisms affecting pre-adult survival were the same as the ones affecting adults. In a screening of 59 *S. pimpinellifolium* accessions distributed over the natural growing area, we were able to identify six accessions with a reduced pre-adult survival but with no effects on adult survival and oviposition rate (data not shown in this thesis). These tomato accessions bring a new angle to breeding for whitefly resistance but it is necessary to understand the genetics and the mechanisms behind this reduction in pre-adult survival.

Field vs. protected tomato production; breeding the most suitable varieties

The different resistance mechanisms described in the previous section have their specific advantages and disadvantages. It is important to notice that not all of the resistance mechanisms are equally effective and it is necessary to consider the tomato production system in which the resistant tomatoes will grow. In the following paragraphs I will discuss advantages and disadvantages of the different resistance mechanisms based on the production system.

Open field and mechanized tomato production systems: the production of tomatoes for the industry is mainly done in the open field. Field tomatoes face a wide array of pests of different feeding guilds. Therefore, tomato varieties having broad spectrum resistance to insects are preferred. In this sense, breeding for tomato varieties with trichome based resistance (type I, IV and VI) combined with the production of specific secondary metabolites (acyl sugars, methyl ketones or sesquiterpenes) will be most appropriate. Trichome based resistance was shown to be effective against a large range of insects including phloem feeders (*B. tabaci, M. persicae*) (Rodríguez-López *et al.*, 2012, Firdaus *et al.*, 2012, Rodriguez *et al.*, 1993), cell content feeders (*T. urticae*) (Alba *et al.*, 2009, Fernández-Muñoz *et al.*, 2003) leaf miners (*T. absoluta*, *Liriomyza trifolii*) (Gilardón *et al.*, 2001, Maluf *et al.*, 2010, Moreira *et al.*, 1999) and caterpillars (*Helicoverpa zea*, *Manduca sexta, Spodoptera exigua*) (Farrar and Kennedy, 1988, Farrar and Kennedy, 1987, Eigenbrode *et al.*, 1993). In addition, trichome based resistance will be especially important in open field tomato production where the effectiveness of biological control methods is reduced.

Although the benefits of trichome based resistance in open field tomato productions are clear, one of the potential disadvantages is that in order to achieve an effective resistance level it is necessary to combine QTLs increasing the density of trichomes with genes catalysing the synthesis of active secondary metabolites. For instance a tomato variety might be resistant when it combines QTLs increasing the density of trichomes type IV (for example *TA2A, Wf-1, 3A, TA4, TriIV-1, 6A, 7B, R2/9, TriIV-2, Wf-2, 10A, R1/10, 11A, R3/11a, R4/11b* and D) with genes involved in a higher synthesis of terpenes (*PHS1, NDPS1*, *ShZIS*) (Schilmiller *et al.*, 2009, Bleeker *et al.*, 2012), methyl ketones (*MKS1, MKS2*) (Ben-Israel *et al.*, 2009, Fridman *et al.*, 2005, Yu *et al.*, 2010) or acyl sugars (*BAHD*) (Schilmiller *et al.*, 2012). A potential disadvantage is the dependence of trichomes densities and resistance levels on environmental conditions (Wilkens *et al.*, 1996, Antonious *et al.*, 2005, Firdaus *et al.*, 2013). Trichome density is a highly variable trait, responding to changes in the environment like CO_2 concentration, seasonal variation, temperature, drought and day length (Snyder and Antonious, 2009). In *S. habrochaites*, differences in the density of glandular trichomes and metabolites were correlated with differences in the day/night ratio and temperature. For example,

under short day conditions the zingiberene concentrations were decreased by about 50% when the temperature was either raised or lowered by 5 °C from an optimum of 25/20 °C (Gianfagna *et al.*, 1992). In addition, the evaluation of an F_2 population derived from *S. galapagense* in two different environments, showed a lower number of trichomes type IV in hotter conditions, however these results have to be considered carefully due to the lack of information about the exact environmental conditions in one of the locations (Firdaus *et al.*, 2013). The effectiveness of trichome-based resistance could be compromised in the open field, where the environmental conditions are much more variable.

Protected tomato production systems: in this type of cultivation, biological control is broadly used and a very effective method of pest control. In these systems, trichome based resistance mechanisms are not really preferred because they have the potential disadvantage of reducing predator and parasitoid effectiveness (Farrar *et al.*, 1992, Kashyap *et al.*, 1991, Simmons and Gurr, 2005, Kennedy, 2003). In protected cultivation, traits that give natural enemies more opportunities to deal with the pest insects would be beneficial. In that sense, resistance mechanisms affecting whitefly preference (i.e. sesquiterpenes, monoterpenes), fecundity (*OR-5*), nymph development and mechanisms increasing the efficiency with which the natural enemies can locate the pest insect (indirect defences) would be more appropriated. It is to be expected that on tomato varieties carrying *OR-5* (Chapter 4) in combination with a reduced pre-adult survival will have a big impact on the whitefly population dynamics. A reduction in the increase rate of the whitefly population growth will give to the parasitoids and predators more chances to keep the whitefly population below the damage threshold. In addition, plants release volatile compounds (mainly terpenes) that are recognized by predators and parasitoids helping them to locate herbivore-infested plants. Thus, breeding tomato varieties for an increased level of indirect defences (higher emissions of specific volatiles) would be a suitable approach for protected growth.

Priming as an additional measure in pest control

Plants are dynamic organisms and under attack, they can defend themselves by inducible resistance mechanisms. These inducible resistance mechanisms are not only induced in response to pathogen/herbivore attack, but they can also be triggered in response to exogenous application of endogenous defence hormones (SA, JA and their methyl derivate), some volatile organic compounds (VOCs), as well as in response to some xenobiotic chemicals (benzothiadiazole BTH, β-aminobutyric acid BABA) and sugars (fructose, sucrose, galactinol) (Cohen *et al.*, 1994, Hodge *et al.*, 2005, Nombela *et al.*, 2005, Ton *et al.*, 2005, Bolouri Moghaddam and Van den Ende, 2012). The state of induction or plant sensitization is known as priming. Priming allows plant cells to respond to low levels of a stimulus in a more rapid and robust manner displaying faster

and/or stronger, activation of defence responses when subsequently challenged by pathogens, insects, or abiotic stresses. Not only that, but priming was shown to have a trans-generational effect. *Arabidopsis* and tomato plants infected by pathogens or insects produce offspring with an increased level of resistance (Slaughter *et al.*, 2011, Rasmann *et al.*, 2012).

The use of priming agents to increase resistance to insects was shown at the laboratory level as well as in field studies (Kessler *et al.*, 2006, Ton *et al.*, 2006). On tic bean (*Vicia faba* var. minor) it was shown that soil drench applications of BABA can reduce the intrinsic growth rate of the specialist aphid *Acyrthosiphon pisum* but did not affect the mortality (Hodge *et al.*, 2005). On tomato (cv. Marmande), spray treatment with BTH resulted in a delay in whitefly development on the sprayed leaflets (Nombela *et al.*, 2005). In our experiments, soil drench treatment with fructose resulted in an increased level of susceptibility (more whiteflies per plant). The phenomenon of increased susceptibility was described before in *Arabidopsis* and tomato. On these plants, the inoculation with *Pseudomonas fluorescens* WCS417r had a positive effect on the performance of two generalist sap-feeder insects (*Myzus persicae* and *B. tabaci*) (Pineda *et al.*, 2012, Shavit *et al.*, 2013). In addition, we have seen a specific priming by genotype effect (Chapter 5). When primed with BABA or JA, the line FCN93-6-2 showed an increased level of non-preference (reduction in the number of adults) compared to control plants. The detection of tomato genotypes that after priming react to a pathogen/insect attack with stronger defence responses will bring new options for breeding tomato varieties with increased levels of induced defences. Nonetheless, at the moment of considering priming as a tool in pest control it is not only important to take into account the plant genotype, but also the insect feeding mode (piercing-sucking or chewing) and host range (generalists or specialists). So far, priming for insect resistance was successful for specialist insect species (i.e. *Brevicoryne brassicae, A. pisum*) (Hodge *et al.*, 2005, Hodge *et al.*, 2006), but not so much for generalists (i.e. *Myzus persicae, B. tabaci*) (Pineda *et al.*, 2012, Shavit *et al.*, 2013).

Impact of host plant resistance on virus transmission

Bemisia tabaci affects tomato production not only by harming the plant directly, but also by its ability to vector a wide array of *Begomo* viruses. Although some *R*-genes for virus resistance were found for the control of major tomato viruses like Tomato yellow leaf curl virus (TYLCV)(Verlaan *et al.*, 2013), the control of the vector is important as well as it will reduce the selective pressure imposed by the virus and leading to lower incidences of the viruses. Tomato genotypes with an increased level of resistance to whiteflies can have a big impact on the virus transmission, not only on TYLCV but also on the other viruses transmitted by this insect. On the breeding line ABL 14-8 (derived from *S. pimpinellifolium* TO-937) the presence of trichomes type IV and the synthesis

of acyl sugars affected whitefly preference, and shifted settling and feeding site to the adaxial surface of the leaf and did reduce primary and secondary virus transmission. On this accession, whiteflies did spend more time searching and scouting the plant surface without making a probe and as a consequence showing a reduced primary and secondary transmission of the TYLCV (Rodriguez-Lopez *et al.*, 2011, Rodríguez-López *et al.*, 2012). On the other hand, resistance mechanisms that affect whitefly preference without an effect on whitefly survival (Chapter 3) might increase the transmission of non-circulative viruses by increasing searching times and the number of short probes. In addition, mechanisms that only affect whitefly fecundity (Chapter 4) are not expected to influence the whitefly ability as vector of viruses, however they might reduce the virus incidence by slowing down the whitefly population build up. In any situation, the effect of the different resistance mechanisms described in this thesis in relation to virus acquisition times, transmission efficiency and performance of virouliferus whiteflies still has to be addressed.

Transgenic methods for whitefly control

Genetically modified (GM) plants for pest management are available. GM crops expressing a "Cry" toxin from *Bacillus thuringiensis* provide resistance to Lepidoptera and Coleoptera. However, Hemipterans insects are either insensitive or only mildly affected by Cry toxins (Chougule and Bonning, 2012, Whitfield *et al.*, 2014). This brings the necessity of exploring other alternatives for whitefly control, like defence proteins, protein inhibitors, viral coating of toxins and RNAi. Chougule and Bonning (2012) extensively reviewed the use of plant defence proteins and protease inhibitor technologies for the control of Hemipteran pests. In short, defence proteins like lectins are in general carbohydrate-binding proteins, which act as defence against pests by binding to glycoproteins in the insect gut epithelium. In the same manner, protease inhibitors act by binding to digestive proteases in the insect gut impairing digestion and consequently insect growth. The applicability of these technologies depends on the presence of proteolytic enzymes in the digestive tract of the insect. Regrettably, it was shown for *B. argentifolli* that it does not have gut proteolytic activity (Salvucci *et al.*, 1998), which makes it unlikely that this technology will be very effective for whitefly control.

Recently, the development of a recombinant coat protein from a circulative virus, with a proline-rich motif and an insect specific peptide toxin (Hv1a) proved to be an effective method to bypass the barrier imposed by the insect gut and deliver the toxins directly in the aphids hemocoel (Bonning *et al.*, 2014). When *Arabidopsis* plants constitutively expressed this recombinant coat protein, the aphid population growth was severely affected (Bonning *et al.*, 2014). The exploration of this type of technologies opens doors for the development of whitefly resistant transgenic plants.

Finally, the use of RNA interference (RNAi) for crop protection is considered as a new-generation "insecticide" for insect pest control. RNAi refers to double-stranded RNA (dsRNA)-mediated gene silencing. If plant mediated RNAi can protect plants from piercing-sucking insects, it would be a revolution in plant protection (Burand and Hunter, 2013). The use of RNAi to control whiteflies is under development. So far, it has been proven that microinjection of a RNAi construct could reduce the expression of tissue specific *B. tabaci* genes by 70% (BtCG5885, BtGATAd, and BtSnap) resulting in an increased whitefly mortality (Ghanim *et al.*, 2007). It was also shown that it is possible to silence whitefly genes by feeding them artificial diets containing dsRNAs and siRNAs. By this method, two genes where identified (*Ribosomal protein L9* and *V-ATPase A*), which when silenced increase the whitefly mortality up to a 98% after 6 days of continuous feeding (Upadhyay *et al.*, 2011). It was shown that silencing the whitefly homologue gene of the *Drosophila chickadee* gene produces malformations on the follicular cells surrounding the eggs stopping whitefly development (Ghanim *et al.*, 2007). Although the potential of RNAi for whitefly control is there, some points need further consideration. First, the selection of target genes is a crucial step for successful RNAi. The availability of the whitefly genome (adults and nymphs) (Leshkowitz *et al.*, 2006) , of tissue and developmental stage specific transcriptomic data (Wang *et al.*, 2010, Wang *et al.*, 2012, Su *et al.*, 2012), as well as the genome sequence of primary (Jiang *et al.*, 2012) and secondary whitefly endosymbionts (Rao *et al.*, 2012a, Rao *et al.*, 2012b) will bring plenty of options for target gene selection. Secondly, once the target genes are selected, it is necessary to have a high-throughput screening method that allows the screening of large numbers of target genes at a relative low cost and high speed. Third, one of the major drawbacks of RNAi for the control sap feeders (whiteflies, aphids, and leafhoppers) is the uptake of the dsRNA into the cell. For the effective use of RNAi it is necessary to possess dsRNA transgenic plants in which the dsRNA is expressed in the phloem under the control of tissue specific promoters. Finally, this leads to the last and one of the biggest limitations of the technology; the public refusal of transgenic crops. It does not matter how safe, target specific and environmentally friendly the technology can be, it will not be applied until a change is made on the public opinion about the use of GMO technologies.

Future directions

We observed that in *Solanum galapagense* there is a clear correlation between whitefly resistance, acyl sugars and trichomes type IV. However, we cannot be completely sure that these metabolites are responsible for conferring whitefly resistance in this species and if they are, which ones are contributing to the resistance. To solve these questions it is necessary to split the contribution of acyl sugars and trichomes type IV in whitefly resistance. Once again, nature has provided the material to address this problem. We have identified accessions of *S. cheesmaniae* that accumulate high levels of acyl sugars

without having trichomes type IV. By producing a segregating population between an accession of *S. cheesmaniae* that accumulates acyl sugars and one that does not, it would be possible to know and understand the genetics behind acyl sugar metabolism independent of the presence of trichomes type IV. A similar experiment was done using *S. pennellii*, with the exception that both accessions used to produce the segregating population had trichomes type IV (Blauth *et al.*, 1999). In addition, intraspecific crosses between accessions of *S. pimpinellifolium* with and without trichomes type IV could be made to understand the genetics behind trichome development. These experiments will complement each other providing further understanding of the resistance mechanisms against whiteflies in tomato.

In this thesis, we have described a tomato line that was not preferred by *B. tabaci* and also an introgression line from *S. habrochaites* (*OR-5*) capable to reduce whitefly fecundity. These two mechanisms are promising tools to be used in combination with biological control. However, there is still gap in the relation between resistance mechanisms, biological control and virus transmission. It will be necessary to test the effectiveness of whitefly parasitoids and predators on resistant tomato lines and also the effect of the resistance mechanisms in relation to the effectiveness of the whiteflies as virus vectors.

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Summary

Tomato (*Solanum lycopersicum*) is affected by a wide range of biotic stresses, of which *Bemisia tabaci* is one of the most important. *Bemisia tabaci* affects tomato directly through phloem sap feeding, and indirectly through its ability to be the vector of a large number of viruses. Different methods are available for whitefly control, and although several biological control agents are used against whiteflies in greenhouse cultivation, chemical control still is an essential component in open field tomato production. Breeding for host plant resistance is considered as one of the most promising methods in insect pest control in crop plants, and especially it is a promising alternative in whitefly control. Resistance to whiteflies was found in several wild relatives of tomato like *Solanum peruvianum, S. pennellii*, *S. habrochaites, S. lycopersicum var. cerasiforme, S. pimpinellifolium* and *S. galapagense*. In spite of previous breeding efforts, whiteflies are still a problem in tomato cultivation. The aim of my research was to identify and understand resistance mechanisms targeting specific stages of the whitefly life cycle in order to provide breeders with tools for developing whitefly resistant varieties.

I assessed the natural variation and whitefly resistance in *Solanum galapagense* and *S. cheesmaniae*, two wild tomato species endemic to the Galapagos Islands. Previously, *Solanum galapagense* and *S. cheesmaniae* were classified as two species based on a morphological species concept, but with molecular markers no clear separation could be made. So far, only a limited number of accessions/populations of *S. galapagense* and *S. cheesmaniae* have been evaluated for insect resistance and therefore it was unknown if the insect resistance coincides with the morphological species boundaries. Neither was there any knowledge about the relation between geographical and climatic conditions today on the Galapagos and the occurrence of the two species. We characterized twelve accessions of *S. galapagense*, 22 of *S. cheesmaniae*, and as reference one of *S. lycopersicum* for whitefly resistance using no-choice experiments. Whitefly resistance was found in *S. galapagense* only and was associated with the presence of relatively high levels of acyl sugars and the presence of glandular trichomes of type I and IV. It is likely that a minimum level of acyl sugars and the presence of glandular trichomes type IV are needed to achieve an effective level of resistance. Genetic fingerprinting using 3316 polymorphic SNP markers did not show a clear differentiation between the two species endemic to the Galapagos. Acyl sugar accumulation as well as the climatic and geographical conditions at the collection sites of the accessions did not follow the morphological species boundaries. Altogether, our results suggest that *S. galapagense* and *S. cheesmaniae* might be considered as morphotypes rather than two species and that their co-existence is likely the result of selective pressure.

Plants possess several resistance mechanisms acting at different time points during the interaction with herbivorous insect. Before any contact with the insects, plants emit an array of volatile organic compounds that can act as attractant or repellent of insects. *Bemisia tabaci* use a set of plant-derived cues in the process of host plant selection. It recognizes mainly monoterpenes (p-cymene, γ-terpinene and β-myrcene, α-phellandrene) and sesquiterpenes (7-epizingiberene and *R*-curcumene). Previously the line FCN93-6-2, which was derived from a cross between a susceptible tomato cultivar (Uco Plata INTA) and *S. habrochaites* (FCN3-5) was proved to be non-preferred by the greenhouse whitefly *Trialeurodes vaporariorum*. We identified chemical cues produced by FCN93-6-2 and *S. habrochaites* that can affect the preference of the whitefly *B. tabaci* as well as the potential chromosomal region(s) of *S. habrochaites* harbouring the genes involved in the preference. Two *S. habrochaites* accessions (CGN1.1561 and in FCN3-5) and the line FCN93-6-2 were non-preferred by *B. tabaci* when the whiteflies could get in direct contact with the plant and also when the whiteflies were offered olfactory cues only. The non-preference was independent of trichome type IV and of the presence of methyl-ketones but associated to the presence of monoterpenes in lower concentrations. Functional validation of the candidate metabolites and of the different introgressions is still needed.

Once the insect has landed on a plant, another set of resistance mechanisms enter into action. We have described a 3.06 Mbp introgression on top of Chromosome 5 (*OR-5*) from the wild tomato species *S. habrochaites* (CGN1.1561). For the identification of *OR-5*, we went from the selection of specific F_2 plants to the development of $F_2BC_4S_1$ and $F_2BC_4S_2$ families. This introgression was sufficient to reduce whitefly fecundity without an evident effect on whitefly survival. The identification of mechanisms exclusively affecting whitefly fecundity and independent of trichomes type IV opens new doors for resistance breeding to whiteflies that may be especially interesting in greenhouse cultivation combination with natural enemies of the whitefly.

As an additional layer of defences, plants can perceive stress signals and respond to them in a specific way through induction of their immune system. This induction can also be triggered by exposing the plants to priming agents like hormones, some xenobiotic chemicals, like benzothiadiazole (BTH), β-aminobutyric acid (BABA), and sugars. Although the effect of priming agents was shown in laboratory and field studies, little is known about the effect of the genetic background of tomato on the extent of the priming, e.g. do genotypes varying in their level of resistance to insects and pathogens respond in the same way to a priming agent. We assessed the effect of selected priming agents on the effectiveness of natural defence in tomato. A set of no-choice and choice bioassays was conducted using tomato genotypes varying in their level of basal resistance to *Bemisia tabaci* and pathogens. We observed that whitefly survival and

oviposition were not affected by the priming treatment in no-choice assays. Overall, in choice assays, fructose treated plants were more preferred by whiteflies than control plants. A genotype specific effect of priming was seen for the line FCN93-6-2. On this tomato line, JA and BABA applications decreased the number of whiteflies, e.g. making them less preferred.

In this thesis, I have gone from the screening of wild relatives of tomatoes to in depth characterization of resistance mechanisms. I have identified resistance mechanisms targeting specific stages of the whitefly life cycle, thus providing new tools for breeding durable whitefly resistance in tomato.

Samenvatting

Tomaat (*Solanum lycopersicum*) heeft last van een groot aantal ziekten en plagen, waarvan de wittevlieg *Bemisia tabaci* een van de belangrijkste is. Dit insect beïnvloedt de tomaat rechtstreeks omdat het zich voedt met floëem sap en indirect omdat het een groot aantal virussen kan verspreiden. Er zijn verschillende manieren om wittevlieg te bestrijden. In de glastuinbouw wordt vooral gebruik gemaakt van biologische bestrijding, terwijl in de open veld teelt vooral chemische bestrijding wordt gebruikt. Veredeling op waardplantresistentie wordt beschouwd als een veelbelovende methode in het bestrijden van insectproblemen in gewassen, en het is vooral een veelbelovend alternatief in de bestrijding van wittevlieg. Resistentie tegen wittevlieg is gevonden in verschillende wilde verwanten van tomaat zoals *Solanum peruvianum*, *S. pennellii*, *S. habrochaites*, *S. lycopersicum* var. *cerasiforme*, *S. pimpinellifolium* en *S. galapagense*. Ondanks veredelingsinspanningen in het verleden is wittevlieg nog steeds een probleem in de tomatenteelt. Het doel van mijn onderzoek was om resistentiemechanismen gericht op specifieke ontwikkelingsstadia van de wittevlieg te identificeren en daarmee veredelaars te voorzien van nieuwe mogelijkheden voor het ontwikkelen van wittevlieg resistente rassen.

Ik heb de natuurlijke variatie en wittevlieg resistentie in *S. galapagense* en *S. cheesmaniae*, twee wilde tomaatsoorten die endemisch zijn op de Galapagos eilanden bestudeerd. In het verleden werden *S. galapagense* en *S. cheesmaniae* op basis van een morfologische soortsconcept als twee soorten beschouwd, maar met moleculaire merkers kon dit niet bevestigd worden. Tot nu toe zijn slechts een beperkt aantal accessies/populaties van *S. galapagense* en *S. cheesmaniae* geëvalueerd op resistentie tegen insecten en daarom was ook niet bekend of de resistentie tegen insecten samenvalt met de morfologische soortgrenzen. Evenmin was er kennis over de relatie tussen de hedendaagse geografische en klimatologische omstandigheden op de Galapagos en het voorkomen van de twee soorten. We hebben twaalf accessies van *S. galapagense*, 22 van S*. cheesmaniae*, en als referentie een accessie van *S. lycopersicum,* gebruikt om de resistentie tegen wittevlieg te meten m.b.v. geen keuze experimenten. Alleen in *S. galapagense* was resistentie tegen wittevlieg aanwezig en die bleek geassocieerd met de aanwezigheid van relatief hoge concentraties acyl suikers en met de aanwezigheid van glandulaire trichomen type I en IV. Het is waarschijnlijk dat een minimumniveau van acyl suikers en de aanwezigheid van type IV trichomen nodig zijn om een effectief niveau van resistentie te bereiken. Een genetische vingerafdruk gemaakt met 3316 polymorfe SNP merkers kon geen duidelijk onderscheid tussen de twee soorten maken. Acyl suiker accumulatie, evenals de klimatologische en geografische omstandigheden op de plekken waar de accessies verzameld werden volgen ook niet de morfologische soortgrenzen. Samenvattend

suggereren onze resultaten dat *S. galapagense* en *S. cheesmaniae* beter kunnen worden beschouwd als morphotypen in plaats van als twee soorten. Hun co-existentie is waarschijnlijk het gevolg van selectiedruk door insecten.

Planten beschikken over verschillende resistentiemechanismen die actief zijn op verschillende tijdstippen tijdens de interactie met herbivore insecten. Al voordat er contact met de insecten is scheiden planten een hele reeks van vluchtige organische stoffen uit die kunnen fungeren als attractant of repellent van insecten. *Bemisia tabaci* gebruikt een set van deze plantaardige signalen bij de waardplantselectie. Het herkent voornamelijk monoterpenen (p-cymeen, γ-terpineen, β-myrceen en α-fellandreen) en sesquiterpenen (7-epizingiberene en R- curcumene). In het verleden was al gebleken dat de tomatenlijn FCN93-6-2, die is afgeleid van een hybride tussen een vatbare tomaat cultivar (Uco Plata INTA) en een *S. habrochaites* accessie (FCN3-5), niet geprefereerd wordt door de kaswittevlieg *Trialeurodes vaporariorum*. Wij hebben de chemische signalen geïdentificeerd die door FCN93-6-2 en *S. habrochaites* worden geproduceerd en die tevens de voorkeur van de wittevlieg *B. tabaci* beïnvloeden, alsmede de mogelijke chromosomale gebieden van *S. habrochaites* waar de verantwoordelijke genen betrokken bij de preferentie gelokaliseerd zijn. Twee *S. habrochaites* accessies (CGN1.1561 en FCN3-5) en de lijn FCN93 6-2 werden niet geprefereerd door de wittevlieg wanneer deze in direct contact met de plant konden komen en ook niet wanneer de wittevliegen alleen olfactorische signalen kregen. De niet-preferentie is geassocieerd met de aanwezigheid in lagere concentraties van monoterpenen maar is onafhankelijk van het trichoom type IV en de aanwezigheid van methylketonen. Functionele validatie van de kandidaat metabolieten en van de verschillende locaties van kandidaat genen zijn nog steeds nodig.

Zodra het insect is geland op een plant, komt een andere set van resistentie mechanismen in actie. We hebben een 3,06 Mbp introgressie aan de top van Chromosoom 5 (OR-5) geïdentificeerd die afkomstig is uit de wilde verwant *S. habrochaites* (CGN1.1561). Voor de identificatie van OR-5, hebben we F2BC4S1 en F2BC4S2 families ontwikkeld uitgaande van specifieke F2 planten die afkomstig waren uit een kruising tussen CGN1.1561 en de cultuurtomaat. Deze introgressie was voldoende om de eileg van wittevlieg sterk te verminderen zonder dat deze een duidelijk effect had op de overleving van de wittevlieg. De identificatie van resistentiemechanismen die uitsluitend van invloed zijn op de vermeerdering van de wittevlieg en onafhankelijk van het type trichomen IV opent nieuwe deuren voor resistentieveredeling en is met name interessant voor teelt in kassen in een combinatie met natuurlijke vijanden van de witte vlieg.

Als een extra afweerlaag kunnen planten stresssignalen waarnemen en daar specifiek op reageren via inductie van hun immuunsysteem. Dit type resistentie kan ook

opgewekt (*geprimed*) worden door het blootstellen van de planten aan hormonen en sommige xenobiotica, zoals benzothiadiazool (BTH), β-aminoboterzuur (BABA) en suikers. Alhoewel het effect van *priming agents* is aangetoond in zowel laboratorium als in veldstudies, is er weinig bekend over het effect van de genetische achtergrond van tomaatgenotypen op de werkzaamheid van de *priming*. Met andere woorden reageren genotypen die verschillen in resistentie tegen ziekten en plagen op dezelfde manier op de *priming agents*. Wij hebben het effect van een aantal *priming agents* op de effectiviteit van de natuurlijke verdediging van tomaat onderzocht. Een set van geen keuze en keuze *bioassays* werd uitgevoerd op tomatengenotypes met een verschillend niveau van basale resistentie tegen *B. tabaci* en ziekteverwekkers. We zagen dat wittevlieg overleving en eileg niet werd beïnvloed door een *priming* behandeling in geen keuze testen. In keuze-toetsen vonden we na een fructose behandeling op alle genotypen meer wittevliegen dan op controle planten. Een genotype-specifiek effect van *priming* werd gezien op de lijn FCN9-6-2. Op deze lijn daalde het aantal wittevliegen nadat ze behandeld waren met JA en BABA. Behandelde lijnen werden niet geprefereerd in de keuze toets.

In dit proefschrift heb ik het gehele traject van de screening van wilde verwanten van tomaten voor resistentie tegen wittevlieg tot een diepgaande karakterisering van resistentiemechanismen beschreven. Ik heb resistentiemechanismen geïdentificeerd die gericht zijn op specifieke stadia van de wittevlieg levenscyclus en daarmee nieuwe mogelijkheden ontwikkeld voor veredeling van tomaat variëteiten met een duurzame resistentie tegen witte vlieg.

Resumen

El tomate (*Solanum lycopersicum*) es afectado por un gran número de estreses bióticos, entre los cuales *Bemisia tabaci* es uno de los más importantes. Este insecto, comúnmente conocido como mosca blanca del tabaco, afecta al cultivo del tomate en forma directa a través del consumo de savia del floema, e indirectamente debido a su capacidad como vector de un amplio número de virus. Existen diferentes métodos para el control de las moscas blancas, y aunque en tomate crecido en invernáculos diversos controladores biológicos son efectivamente utilizados, en condiciones de campo el uso de insecticidas sigue siendo un componente esencial para el control de esta plaga. En cultivos, el fitomejoramiento enfocado en la resistencia de la planta huésped es considerado como uno de los métodos más promisorios para el control de plagas en general y de moscas blancas en particular. Varias especies silvestres de tomate fueron descriptas como resistentes a mosca blanca, entre las cuales se puede nombrar *Solanum peruvianum*, *S. pennellii*, *S. habrochaites*, *S. lycopersicum var. cerasiforme*, *S. pimpinellifolium* and *S. galapagense*. Sin embargo, a pesar de los esfuerzos realizados, la plaga en cuestión continúa siendo un grave problema en las producciones de tomate. El objetivo de esta tesis fue el de identificar y entender mecanismos de resistencia que afectan estadios específicos del desarrollo de la mosca blanca, con la finalidad de proporcionar nuevas herramientas para el desarrollo de variedades resistentes a la mosca blanca.

En el presente trabajo, se estudió la variación natural en el nivel de resistencia en dos especies silvestres de tomate endémicas de las Islas Galápagos, *Solanum galapagense* y *S. cheesmaniae*. Anteriormente, *S. galapagense* y *S. cheesmaniae* fueron clasificadas como dos especies usando el concepto morfológico de especie, pero dicha distinción no pudo ser validada mediante el uso de marcadores moleculares. Hasta el momento, un número limitado de poblaciones/accesiones de *S. galapagense* y *S. cheesmaniae* fueron evaluadas como fuentes de resistencia a insectos, por lo cual no se conocía si la resistencia a insectos coincidía con los límites de las dos especies. Tampoco se conocía la relación entre las condiciones climáticas y geográficas actuales en las Islas Galápagos y la presencia de las dos especies. Caracterizamos la resistencia a mosca blanca usando ensayos de no elección en doce accesiones de *S. galapagense*, 22 accesiones de *S. cheesmaniae* y, como referencia, en una accesión de *S. lycopersicum*. Las accesiones de *S. galapagense* fueron las únicas resistentes a la mosca blanca, y la resistencia estuvo asociada con la presencia de elevadas concentraciones de acil azúcares y de tricomas glandulares tipo I y IV. Es probable que un nivel mínimo de acil azucares conjuntamente con la presencia de tricomas glandulares del tipo IV son necesarios para obtener un efectivo nivel de resistencia. La caracterización genética mediante el uso de 3316 marcadores SNP no mostro una clara diferenciación entre las dos especies endémicas de

las Islas Galápagos. La acumulación de acil azucares así como las condiciones climáticas en el punto de colección de las accesiones no siguieron la diferenciación entre especies. En su conjunto, estos resultados sugieren que *S. galapagense* y *S. cheesmaniae* podrían ser considerados como morfo-tipos en vez de como dos especies y que su coexistencia podría ser el resultado de presión de selección.

Las plantas poseen diversos mecanismos de defensa que actuan a diferentes momentos durante la interacción con un insecto herbívoro. Antes de cualquier tipo de contacto, las plantas emiten un conjunto de compuestos orgánicos volátiles que pueden funcionar como atrayentes o repelentes de insectos. *Bemisia tabaci* también emplea volátiles emitidos por las plantas como señales en el proceso de selección de una planta huésped. Esta mosca blanca reconoce principalmente monoterpenos (p-cimeno, γ-terpineno and β-mirceno, α-felandreno) y sesquiterpenos (7-epizingibereno and R-curcumeno). Anteriormente, la línea FCN93-6-2, que procede de un cruzamiento entre un cultivar de tomate susceptible (Uco Plata INTA) y *S. habrochaites* (FCN3-5), fue identificada como no preferida por la mosca blanca de los invernaderos *Trialeurodes vaporariorum*. Identificamos metabolitos producidos por FCN93-6-2 y *S. habrochaites* que pueden afectar la preferencia de *B. tabaci*, así como también las regiones cromosómicas en *S. habrochaites* conteniendo los genes involucrados en la resistencia. Dos accesiones de *S. habrochaites* (CGN1.1561 y FCN3-5) y la línea FCN93-6-2 fueron no preferidas por *B. tabaci*, cuando las moscas blancas fueron puestas en contacto directo con las plantas y también cuando las moscas fueron ofrecidas con señales químicas exclusivamente. La no preferencia fue independiente de la presencia de tricomas tipo IV y de la presencia de metil-cetonas, pero asociada a la presencia en bajas concentraciones de monoterpenos. La caracterización y validación funcional de los metabolitos candidatos así como de las diferentes introgresiones son necesarias.

Una vez que un insecto se posa en una planta, un conjunto diferente de mecanismos de resistencia se pone en acción. Hemos descripto una introgression de 3.06 Mbp. al comienzo del Cromosoma 5 (*OR-5*) procedente de la especie silvestre *S. habrochaites* (CGN1.1561). Para la identificación de *OR-5*, fuimos desde la selección de plantas F_2 hasta el desarrollo de familias $F_2BC_4S_1$ y $F_2BC_4S_2$. Esta introgresion fue suficiente para reducir la fecundidad de las moscas blancas sin un evidente efecto en la supervivencia. La identificación de mecanismos de resistencia que afectan específicamente la fecundidad de las moscas blancas independientemente de la presencia de tricomas tipo IV abre nuevas puertas para el mejoramiento genético de plantas resistentes y a su vez, puede ser de especial interés en combinación con el uso de controladores biológicos.

Como una barrera adicional de defensa, las plantas tiene la capacidad de percibir señales de estrés y de responder a estas de una forma específica a través de la inducción de su sistema inmune. Esta inducción, también puede ser iniciada exponiendo las plantas a

compuestos cebadores "priming agents" como hormonas vegetales, algunos compuestos xenobioticos como Benzotiadiasol (BTH), ácido β-amino butírico (BABA) y azucares. A pesar de que el uso de compuestos cebadores fue probado en estudios de laboratorio y de campo, poco se conoce sobre el efecto del ambiente genético del diferentes variedades de tomate en el nivel de inducción "priming". Por ejemplo, si genotipos de tomate que varían en el nivel de resistencia a insectos y patógenos responden de la misma manera al tratamiento con un compuesto cebador. Se evaluó el efecto de algunos compuestos cebadores en la resistencia a mosca blanca en tomate. Un conjunto de bio-ensayos de elección y de no elección fueron realizados usando genotipos de tomate con diferentes niveles basales de resistencia a *B. tabaci* y a patógenos. Observamos que tanto la supervivencia como la ovoposición de las moscas blancas no fue afectada por la aplicación de los compuestos cebadores en los ensayos de no elección. En general, las plantas tratadas con fructosa fueron más preferidas por las moscas blancas que las plantas control. Un efecto específico entre genotipo y compuesto cebador fue detectado para la línea FCN93-6-2. En esta línea de tomate, la aplicación de ácido jasmónico (JA) y BABA disminuyeron el número de moscas blancas haciéndolas menos preferidas en los experimentos de elección.

En esta tesis, se partió desde el análisis de especies silvestres de tomate a la profunda caracterización de los mecanismos de resistencia. Se identificaron mecanismos enfocados en diferentes estadios del desarrollo de la mosca blanca, brindando nuevas herramientas para una durable resistencia a mosca blanca en tomate.

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Curriculum vitae

Alejandro Francisco Lucatti was born on May 29th, 1985, in Salta, Argentina. In 2008, he graduated as Biologist from the National University of Salta. In March 2009, he received a doctoral fellowship from CONICET to work under the supervision of Dr. Adriana Alvarez on the effect of induced senescence on the interaction between *Myzus persicae* and *Solanum tuberosum*. In March 2010, he got a scholarship from the Fundacion CAPACIT-AR del NOA to start a PhD in the Department of Plant Breeding WUR under the supervision of Dr. Ben Vosman and Dr. Sjaak van Heusden. In July 2014, he will start a new job as Scientist in Entomology at Bayer CropScience Vegetable Seeds.

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Date: 30 June 2014
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Oral presentation at Laboratory of Plant Breeding and Plant Research International	Oct 12, 2010
► **Writing or rewriting a project proposal**	
Resistance to whitefly in Solanum habrochaites L.	Nov 30, 2010
► **Writing a review or book chapter**	
► **MSc courses**	
Insect Plant Interactions (ENT-50806)	Apr 26-May 21, 2010
Breeding for Quality and Resistance (PBR-30306)	Mar 07- Apr 25, 2011
Advanced Statistics (MAT-20306)	Sep 02-Oct 21, 2013
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student days, Utrecht University	Jun 01, 2010
EPS PhD student days, University of Amsterdam	Nov 30, 2012
5th European Retreat of PhD Students in Plant Sciences, Ghent, Belgium	Jul 23-26, 2013
► **EPS theme symposia**	
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents, Wageningen University	Feb 03, 2011
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents, Utrecht University	Feb 25, 2013
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Experimental Plant Science-Lunteren	Apr 19-20, 2010
ALW Meeting Experimental Plant Science-Lunteren	Apr 02-03, 2012
ALW Meeting Experimental Plant Science-Lunteren	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
5th Plant-Insect Interaction Workshop - Wageningen University	Nov 11, 2010
6th Plant-Insect Interaction Workshop - University of Amsterdam	Nov 23, 2011
7th Plant-Insect Interaction Workshop - Leiden University	Nov 28, 2012
Symposium 'Intraspecific pathogen variation, implications and opportunities'	Jan 18, 2013
EPS Flying seminar by Detlef Weigel: '*Arabidopsis thaliana* as a model for the study of evolutionary questions'	Feb 27, 2013
Invited seminar by Alain Tissier: 'Glandular trichomes of tomato: from terpene biosynthesis to trichome differentiation'	May 03, 2013
Plant sciences seminars by Robert Hall and Nicole van Dam: 'Plant metabolomics in the lab and in the wild'	Oct 10, 2013
► **Seminar plus**	
► **International symposia and congresses**	
EUSOL meeting, Natal, Brazil	Nov 15-16, 2010
Hemipteran-Plant Interactions Symposium, Piracicaba-SP, Brazil	Jul 11-14, 2011
PURE kick-off meeting, L'Isle-sur-la-Sorgue, France	Mar 28-30, 2011
Conference Next Generation Breeding, Ede, NL	Nov 11-14, 2012
Future IPM in Europe:Pesticide use and Risk reduction, Riva del Garda, Italy	Mar 19-21, 2013
► **Presentations**	
Poster: EUSOL meeting, Natal, Brazil	Nov 15-16, 2010
Poster: Hemipteran-Plant Interactions Symposium, Piracicaba, Brazil	Jul 11-14, 2011
Poster: ALW Meeting EPS, Lunteren, NL	Apr 02-03, 2012
Oral: Next Generation Breeding conference, Ede, NL	Nov 12, 2012
Oral: 7th Plant-Insect Interaction Workshop, Leiden, NL	Nov 28, 2013
Oral: Future IPM Europe, Riva del Garda, Italy	Mar 19-21, 2013
Oral: 5th European PhD Retreat, Ghent, Belgium	Jul 23-26, 2013
► **IAB interview**	
Meeting with a member of the Interantional Advisory Board	Nov 15, 2012
► **Excursions**	
Subtotal Scientific Exposure	*17.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Postgraduate course 'Introduction Bioinformatics - a User's Approach'	Aug 30-Sep 03, 2010
Postgraduate course 'Introduction to R for statistical Analysis'	Jun 11-12, 2012
Postgraduate course 'Systems Biology: Statistical Analysis of ~Omics Data'	Dec 10-14, 2012
► **Journal club**	
Literature discussion WUR Plant Breeding	2010-2014
► **Individual research training**	
Subtotal In-Depth Studies	*5.1 credits**

4) Personal development	*date*
► **Skill training courses**	
WGS course 'Presentations skills'	Oct 14-28, 2011
WGS course 'Techniques for Writing and Presenting Scientific Papers'	Jul 03-06, 2012
WGS course 'Scientific Writing'	Oct-Dec, 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.3**
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.*	

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