Identification of whitefly resistance in tomato and hot pepper

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Thesis

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Chapter I

General Introduction

Problems related to whitefly infestation in tomato and pepper cultivation

The silverleaf or sweetpotato whitefly (*Bemisia tabaci* Genn, syn *B. argentifolii* Bellows and Perring) belongs to the order Hemiptera of the family Aleyrodidae. Economically it is one of the most important pests in tomato and pepper (Brown *et al.* 1995b; Morales 2007). It causes direct damage through feeding on plant phloem sap and indirect damage through virus transmission and sooty mold growth. Whitefly related damage reduces crop quality and quantity (Morales 2007). Whitefly control is costly, especially in open cultivation systems commonly found in tropical countries such as Indonesia (Morales 2007; Hidayat *et al.* 2008).

Being a phloem-feeding insect, whiteflies are different from many other herbivores which cause extensive external damages. The whitefly carefully maneuvers its stylet through the epicuticular layer, epidermis and sponge tissue to reach the phloem (Freeman *et al.* 2001). The stylet is wrapped with a gel-like substance that is suggested to reduce plant defense responses (Freeman *et al.* 2001; Kaloshian and Walling 2005). A heavy whitefly infestation can cause a reduction of plant vigor and yield, early wilting, leaf chlorosis and defoliation (Gerling *et al.* 1980; Berlinger *et al.* 1986; Schuster *et al.* 1990a; Summers and Estrada 1996). Whitefly infestations may also result in leaf silvering of tomato, squash and cotton (Yokomi *et al.* 1990; Cohen *et al.* 1992; Costa *et al.* 1993b).

Whiteflies also produce honeydew (Figure 1), which contains sugars and amino acids (Byrne and Miller 1990; Blua and Toscano 1994). Honeydew is a good substrate for sooty mold growth (Figure 1) (McCollum *et al.* 2004) that can cover the plant's surface including leaves, stems and fruits. Heavy sooty mold growth reduces photosynthesis and results in physiological disorders such as irregular fruit ripening (Schuster *et al.* 1990b; Brown and Bird 1992; McCollum *et al.* 2004; Filho and Paiva 2006; Morales 2007).

Virus transmission is the main problem caused by whiteflies. Whiteflies are vectors of more than 200 plant viruses (Morales and Jones 2004). Of the transferred viruses, over 90% are Begomoviruses such as Tomato yellow leaf curl virus (TYLCV), Tomato chlorotic mottle virus (ToCMoV), Tomato crumple

mottle virus (TCMV), Tomato curly stunt virus (ToCSV), Tomato golden mosaic virus (TGMV), Tomato golden mottle virus (ToGMoV), Tomato mottle virus (ToMoV), Tomato yellow leaf curl Sardinia virus (TYLCSV), Pepper yellow leaf curl virus (PYLCV), Chino del tomate virus (CdTV) and Pepper huasteco virus (PHV) (Brown and Bird 1992; Moriones *et al.* 1993; Bedford *et al.* 1994; Blua and Toscano 1994; Brown 1994; Polston *et al.* 1996; Morales and Jones 2004; Tahiri *et al.* 2006; Hidayat and Rahmayani 2007; Morales 2007). These viruses cause serious damages in tomato and pepper (Figure 1).

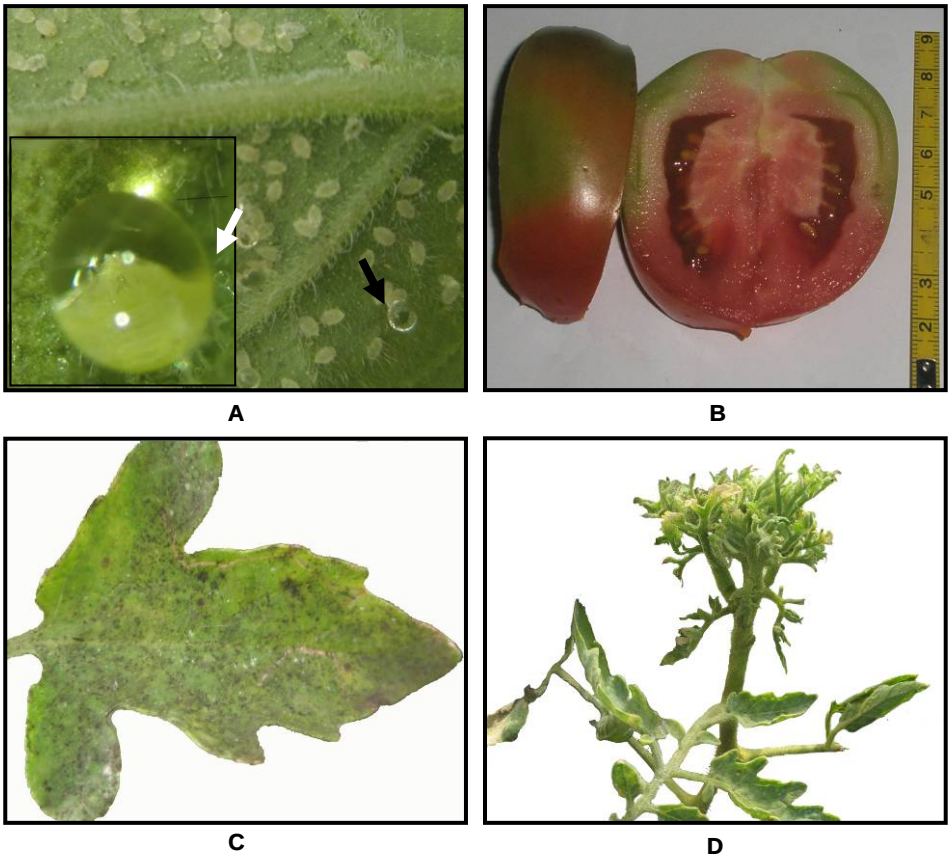

Figure 1. Symptoms due to whitefly infestation in tomato; A. Honeydew produced by a whitefly nymph (black arrow) with an insert of a magnified honeydew (white arrow), B. Irregular ripening of tomato fruit, C. Sooty mold covering a tomato leaflet, D. A tomato plant infected by geminivirus.

Problems in controlling whitefly infestation

Whitefly is a multivoltine (many broods and generations per year), hemimetabolous insect with six life stages: egg, four nymphal instars and adult (Figure 2) (McAuslane *et al.* 2004; Freeman *et al.* 2001). A whitefly female can

reproduce with or without mating (arrhenotokous parthenogenesis) and lays up to 400 eggs (Byrne and Bellows 1991). Eggs hatch in four days to a few months depending on ambient temperature. The first instar, also called crawler, has a size of about 0.26 mm in length and can move on the leaf surface (Freeman *et al.* 2001). Once they feed, they will be immobile and their body will flatten and changes into the second instar (~ 0.33 mm). Afterwards their legs and antennae get atrophied and the third instar stage is reached (~ 0.46 mm in length). The fourth instar is marked by reddening and enlarging of the eyes and reaching a body length of about 0.71 mm. The fourth instars molt to become adults with wings. Overall developmental time between egg to adults highly depends on host plant and ambient temperature (Butler *et al.* 1983; Horowitz 1986). So far there are no methods to eliminate eggs, meaning that a heavy whitefly infested crop has to be sprayed several times with insecticide to control the whitefly population.

Pesticides such as neonicotinoid, pyrethroid, organochlorines, organophosphate and insect growth regulator compounds are widely used and are an effective way to control whiteflies (Sharaf 1986). However, continuous application of insecticides results in the development of whiteflies that are resistant to those compounds (Dittrich *et al.* 1990; Omer *et al.* 1993; Cahill *et al.* 1995; Cahill *et al.* 1996; Crowder *et al.* 2006; Erdogan *et al.* 2008). Subsequently, a more frequent application of pesticides or the development of more toxic insecticides is needed (Dittrich *et al.* 1990). The effectiveness of pesticides is influenced by the fact that whiteflies prefer to live on the underside of a leaf, making it difficult to reach them with the chemicals. Also they are less directly exposed to the insecticides as they feed directly from the phloem and cover their body with prolific waxy layers. A disadvantage is also that pesticides are harmful for human health, environment and non-target organisms such as beneficial insects (Abudulai *et al.* 2001).

Alternative pesticides that might be more safe and environmentally friendly are biological pesticides such as entomopathogenic fungi and plant extracts. Entomopathogenic fungi that can be applied for whitefly control are *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, *Aschersonia aleyrodis* Webber and *Verticillium lecanii* (Osborne and Landa 1992; Davidson *et*

al. 1996). Extracts (most are essential oils) of some plants such as Petunia (Kays *et al.* 1994), neem (*Azadirachta indica*) (Kumar and Poehling 2007; Lynn *et al.* 2010), harmal (*Peganum harmala* L.) (Al-mazra'awi and Ateyyat 2009) Solanaceae (Carter *et al.* 1989; Goffreda *et al.* 1989; Tunc and Sahinkaya 1998) can be used as natural pesticides or deterrents. However, the application of biological pesticides is expensive and has some limitations such as a weak effect, short shelf-life of the product and toxicity to beneficial insects (Stephenson *et al.* 1984; Faria and Wraight 2001). Whitefly predators such as *Amblyseius* spp, *Chrysoperla* spp and *Euseius* spp and whitefly parasitoids such as *Encarsia* spp, *Eretmocerus* spp, *Amitus bennetti* are commercially produced to control whiteflies in greenhouses (Meyerdirk and Coudriet 1986; Legaspi *et al.* 1994; Drost *et al.* 1999; Manzano *et al.* 2002; Urbaneja *et al.* 2007; Calvo *et al.* 2008; Takahashi *et al.* 2008). Successful biological control depends on the environment and the host plant (van Lenteren *et al.* 1997; Drost *et al.* 1998). Furthermore, the application of biological controls has to coincide with the time of whitefly infestation and development, which are unpredictable. So clearly alternatives and complementary measures are needed. Natural resistance of cultivated plants may be a much more effective and sustainable solution (Broekgaarden *et al.* 2011).

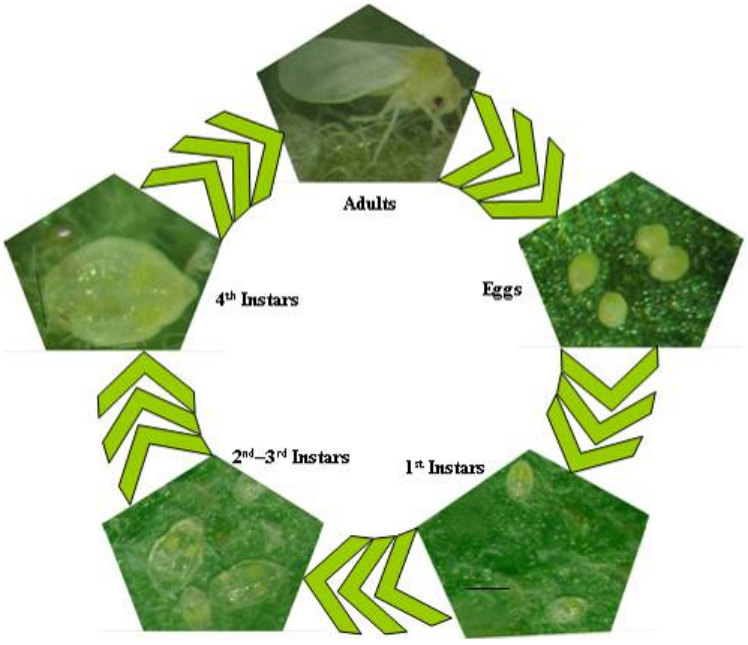

Figure 2. The whitefly life cycle; adults start producing eggs when they are two to ten days old, eggs hatch within five to seven days, development from 1st instars to adults takes about 11 days. All depending on the temperature.

The taxonomic status of *B. tabaci*

In 1978, twenty five species that were separated based on minor differences such as host plant were synonymized as *Bemisia tabaci* (Gennadius). No morphological differences were found between the 25 species (Perring 2001). Therefore, the differences among them were considered as variants within the species. Later, identification and taxonomical status of *B. tabaci* as a species complex has resulted in many studies, papers and reviews (Brown *et al.* 1995a; Gerling 1996; Oliveira *et al.* 2001; Perring 2001; De Barro *et al.* 2005; De Barro *et al.* 2011).

Host races of *Bemisia* strains have been previously determined based on the host plants, especially strains which were nearly monophagous (Bird 1957; Wool *et al.* 1994; Brown *et al.* 1995a). Another way of discriminating strains was to look at specificity in transmitted viruses (Robertson 1987). However, most *B. tabaci* strains, can live on many hosts and cannot be identified/separated based on host plant (Perring 2001). When the genetic structure of the *B. tabaci* complex was characterized by isozymes and/or DNA markers the results did not agree with the results based on the host race designation (De Barro *et al.* 2005). Biotype designation of *Bemisia* populations has been done with isozymes, induction of phytotoxic reactions and differences in insecticide resistance (Burban *et al.* 1992; Perring *et al.* 1992; Byrne and Devonshire 1993; Costa *et al.* 1993a; Bedford *et al.* 1994; Bergh *et al.* 1995). The B-biotype is the most prominent biotype with a worldwide distribution and a wide host range (Bedford *et al.* 1994; Byrne *et al.* 1995; Rosell *et al.* 1997; Frohlich *et al.* 1999; De Barro *et al.* 2000; Brown 2000).

DNA-based molecular markers such as random amplified polymorphic DNA (RAPD) (Guirao *et al.* 1997; Perring 2001; Lima *et al.* 2002; De Barro and Khan 2007), amplified fragment length polymorphism (AFLP) (Cervera *et al.* 2000; Zhang *et al.* 2005), sequencing of the internal transcribed spacer 1 (*ITS1*) of ribosomal DNA (rDNA) (De Barro *et al.* 2000), 16s rDNA subunit (Frohlich *et al.* 1999), partial sequencing of the mitochondrial cytochrome oxidase sub unit 1 (*mtCOI*) gene (Kirk *et al.* 1993; Frohlich *et al.* 1999; Brown 2000) and microsatellites (De Barro *et al.* 2003; Tsagkarakou and Roditakis 2003) divided the worldwide collected *Bemisia* strains in several biotypes. These techniques

didn't solve all problems because some strains couldn't be clearly classified (Perring 2001; Boykin *et al.* 2007). The use of sequence information of ribosomal *ITS1* and *mtCOI* (Frohlich *et al.* 1999; De Barro *et al.* 2000; Abdullahi *et al.* 2003; Viscarret *et al.* 2003; Berry *et al.* 2004) made it possible to distinguish six major groups based on their geographical origin: Asia, Bali, Australia, Sub-Saharan Africa, Mediterranean/Asia-Africa, and the New World. By using more samples, twelve groups were identified based on the geographical origin (Boykin *et al.* 2007), with the exception of Mediterranean/Asia-Africa biotypes (including Q, J and L) and Mediterranean (B and B2) biotypes that can be found worldwide.

The species status of *B. tabaci* is difficult due to intraspecific variation (Rosell *et al.* 1997; Frohlich *et al.* 1999; Brown 2000; De Barro *et al.* 2005). The genetic variation among *B. tabaci* populations did not support *B. tabaci* as a single complex species but more as a number of sibling species (De Barro and Hart 2000; Maruthi *et al.* 2004; De Barro *et al.* 2005; Boykin *et al.* 2007; Dinsdale *et al.* 2010). The partial *mtCOI* dataset of Boykin *et al.* (2007) included pseudogenes, various sizes of partial *mtCOI* genes and duplicated haplotypes which affect the result of a phylogenetic analysis (Dinsdale *et al.* 2010). Therefore, Dinsdale *et al.* (2010) reconstructed the phylogenetic tree based on 202 haplotypes without pseudogenes and found twenty four groups with at least 3.5% divergence among groups. There were 11 distinct groups with more than 11% genetic divergence (Dinsdale *et al.* 2010) and between distinct groups also mating incompatibilities were observed (Byrne *et al.* 1995; De Barro and Hart 2000; Maruthi *et al.* 2004; Liu *et al.* 2007; Wang *et al.* 2011). De Barro *et al.* (2011) described the species status of *B. tabaci* as a morphologically cryptic species complex. As only 202 haplotypes were included the phylogenic picture of the *Bemisia* complex is probably still far from complete.

Whitefly resistance in tomato and pepper

Tomato (*Solanum lycopersicum*) and pepper (*Capsicum* spp.) belong to the night shade family (Solanaceous). Tomato is classified in *Solanum* sect. *Lycopersicon* which contains 13 species as shown in Figure 3 (Perralta *et al.* 2008; Rodriguez *et al.* 2009). *Solanum lycopersicum* is the cultivated tomato,

whereas the other 12 species are wild relatives. The genus *Capsicum* has twenty-five species of which five are cultivated, including *C. annuum, C. chinense, C. baccatum, C. pubescens* and *C. frutescens* (Pickersgill 1971; Mcleod *et al.* 1982). The domestication of tomato and pepper resulted in loss of genetic diversity which makes them prone to abiotic and biotic stresses such as pest attacks (Bai and Lindhout 2007). To date, no cultivated tomato and chili peppers are resistant to whiteflies.

Wild relatives of the cultivated tomato have been reported to be more resistant to whiteflies. Examples are the *S. pennellii* accessions LA716, LA1674, LA1735, LA1340, LA2560 and IVT 72160 (de Ponti *et al.* 1975; Erb *et al.* 1994; Toscano *et al.* 2002; Muigai *et al.* 2002; Oriani and Vendramim 2010), *S. habrochaites* f. *glabratum* Pl134417 and Pl134418 (Toscano *et al.* 2002; Fancelli and Vendramim 2002; Baldin *et al.* 2005; Oriani *et al.* 2011), *S. habrochaites* f. *typicum* LA1777, LA1353 and IVT 70826 (de Ponti *et al.* 1975; Muigai *et al.* 2003; Maruthi *et al.* 2003), *S. peruvianum* LA1739 (Fancelli and Vendramim 2002), *S. pimpinellifolium* LA1584 and IVT 66064 (de Ponti *et al.* 1975; Fancelli and Vendramim 2002). In pepper, resistance screenings have not been as extensive as in tomato (Laska *et al.* 1982) studied whitefly preference in sweet pepper cultivars (*C. annuum*) and found some that were less preferred. No screenings have been done for chili pepper germplasm which include *C. annuum, C. chinense* and *C. frutescens*.

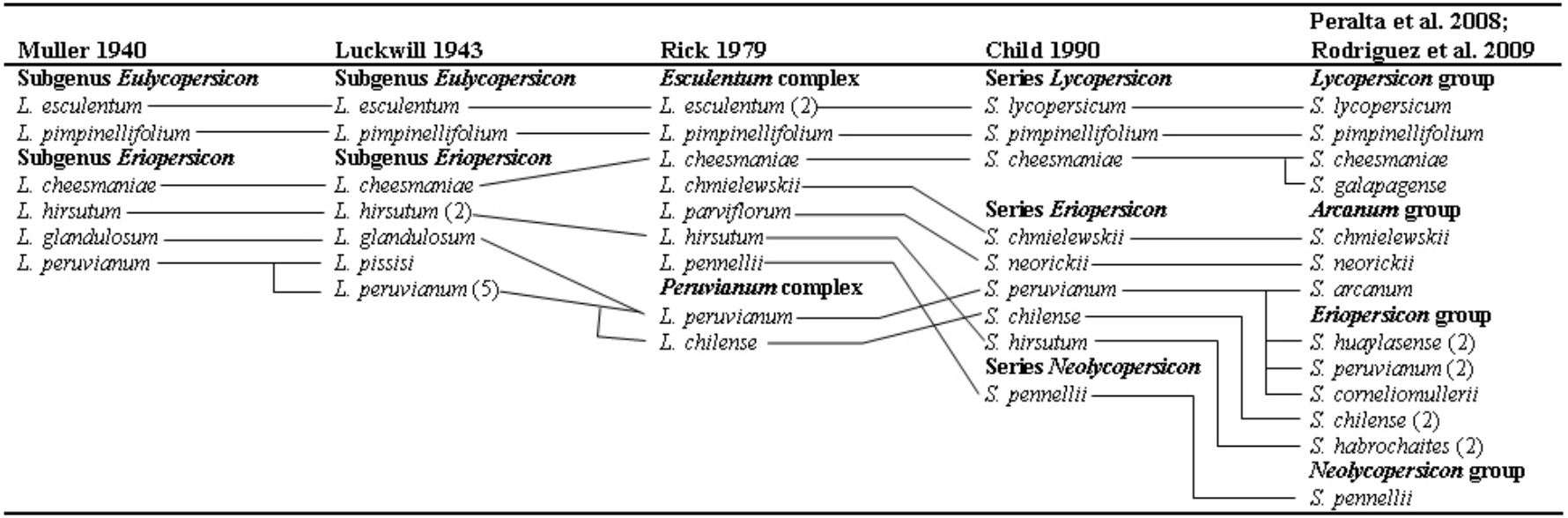

Figure 3. Chronological flow chart of hypothetical species boundaries and relationships in *Solanum* sect. *Lycopersicon*, as proposed by Müller (1940), Luckwill (1943), Rick (1979), Child (1990) in Peralta *et al.* (2008) and Rodriguez *et al.* (2009). The figure is adapted from Peralta *et al.* (2008) and ordered based on the relationship of species with the cultivated tomato (*S. lycopersicum*) (Rodriguez *et al.* 2009). The numbers in parentheses represent the number of intraspecific taxa recognized by these authors.

Components of whitefly resistance in tomato and pepper

Whitefly resistance assessment

The two major methods to evaluate the level of whitefly resistance are the free-choice test and the no-choice test (de Ponti *et al.* 1975; Muigai *et al.* 2003). Free-choice test is a preference test and is used to identify presence of plant attractants and/or repellents (van Emden 2002) and to a lesser degree it looks at the presence of antibiotic components (Toscano *et al.* 2002). Parameters that can be evaluated in a free-choice test are density of whitefly adults, eggs, nymphs and pupal cases (Toscano *et al.* 2002; Fancelli and Vendramim 2002; Muigai *et al.* 2003; Oriani and Vendramim 2010; Oriani *et al.* 2011). In a no-choice test insects are forced to feed from a plant, which for instance can be accomplished using clip-on cages (Figure 4) (de Ponti *et al.* 1975). This test identifies antibiosis factor(s) and investigates the suitability of a plant as a host (Lei *et al.* 1999; van Emden 2002). Antibiosis-based resistance results in increased mortality, reduced reproduction and/or shorter life span of the whiteflies (Painter 1958). The parameters which were assessed in a no-choice test were mortality or survival of whitefly adults and nymphs (pre-adults) (de Ponti *et al.* 1975; Bas *et al.* 1992; Erb *et al.* 1994; Maliepaard *et al.* 1995; Muigai *et al.* 2002). The developmental period from egg to adult can also be measured in no-choice tests (Bas *et al.* 1992; Maliepaard *et al.* 1995). Without a reliable assessment of whitefly resistance the genetic basis of the resistance can never be unravelled.

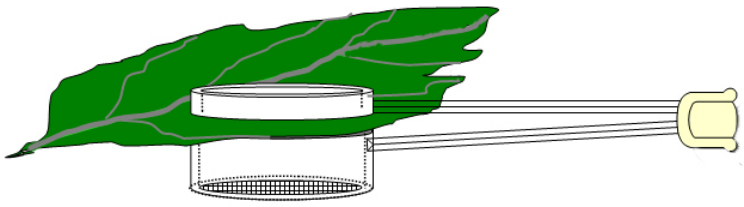

Figure 4. A clip-on cage that is used in no-choice tests to evaluate whitefly resistance.

Plant characters related to whitefly preference

Host plant recognition is the first step in a whitefly infestation. Whiteflies recognize and choose a target host plant mainly based on visual and olfactory cues

(Shibuya *et al.* 2010). Examples of visual cues are leaf characters such as color, shape, pubescence and architecture (Sippell *et al.* 1987). In tomato, sweet pepper and cotton, whiteflies prefer light yellow-green leaves (Vaishampayan *et al.* 1975; Liu and Stansly 1998). This color is believed to be the most important factor for whitefly host recognition (Berlinger 1980). A less open plant canopy is also preferred by whiteflies (McAuslane *et al.* 2004; Srinivasan and Uthamasamy 2005).

Whiteflies are able to recognize plant headspace compounds (Bleeker *et al.* 2009). Olfactory cues are very important in locating hosts and allow whiteflies to make a choice from a distance. Most compounds that were studied in cultivated tomato and its relatives were non-preference compounds (repellants) (Maluf *et al.* 2001; Freitas *et al.* 2002; Bleeker *et al.* 2009). Whitefly repellence of a tomato is thought to be caused by compounds such as monoterpenes and sesquiterpenes produced by type IV and VI trichomes (Maluf *et al.* 2001; Freitas *et al.* 2002; Bleeker *et al.* 2009). These compounds are also repellant to other herbivores (Eigenbrode *et al.* 1996; Maluf *et al.* 1997; Leite *et al.* 1999; Frelichowski and Juvik 2001; Maluf *et al.* 2007) and can be selected for in breeding programs of tomato (Rahimi and Carter 1993; Antonious and Kochhar 2003; Frelichowski and Juvik 2005). There is no information in tomato and pepper about plant compounds that act as whitefly attractant. Therefore, preference in many studies is more due to avoidance from non-preferred plants.

After landing on a plant, whiteflies evaluate suitability for feeding by probing using their stylet (Rosell *et al.* 1999). They prefer to live on the abaxial side of leaves to avoid direct light and natural enemies. Also the phloem vessels are closer to the abaxial leaf surface (Chu *et al.* 1995). Whiteflies prefer thin leaves with a thin cuticle layer (Cohen *et al.* 1996). Thick cuticles prevent the stylet from being inserted into the epidermis and reaching the phloem sieves (Janssen *et al.* 1989). Such thicker cuticles have been reported to play a role in resistance to different kinds of phloem-feeding/piercing insects in vegetables such as tomato, cotton and cassava (Eigenbrode and Espelie 1995; Bellotti and Arias 2001). Chemical make-up and plant age (Walker and Perring 1994), nutritional value (Blackmer and Lee 2008), leaf pubescence and trichome density (van

Lenteren and Noldus 1990) also play a role in whitefly preference and host suitability.

Characters that effect whitefly feeding also effect oviposition. It was suggested that whiteflies choose the most suitable host for oviposition not only because they can feed on it, but also because the offspring should be able to survive (Nomikou *et al.* 2003). Host plant selection has a profound effect on the fitness of the offspring (van Lenteren and Noldus 1990). Whiteflies prefer to lay eggs on tomato leaves with a high density of non-glandular trichomes (Heinz and Zalom 1995; Toscano *et al.* 2002; Sanchez-Pena *et al.* 2006), cotton (Butter and Vir 1989; Flint and Parks 1990), and soybean (McAuslane 1996). The non-glandular trichomes provide suitable microclimate and shelter (Berlinger and Lehmannsigura 1986; Butter and Vir 1989).

Plant characters related to whitefly antibiosis

Antibiosis is one of the resistance mechanisms in which a plant exerts an adverse influence on the growth and survival of the insect (Painter 1958). The most prominent tomato characters that contribute to whitefly antibiosis are glandular trichomes (Channarayappa *et al.* 1992a; Muigai *et al.* 2002). Tomato and its wild relatives have six types of trichomes, the presence of the different types varies (Figure 5; Table 1). Type III and V are non-glandular trichomes and type I, IV, VI and VII are glandular trichomes.

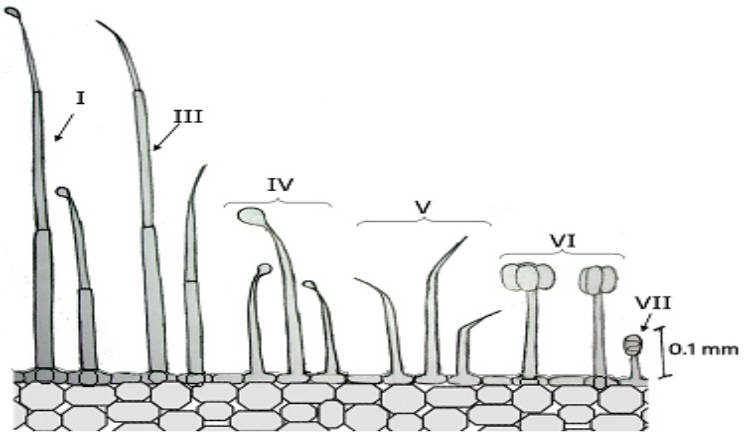

Figure 5. Trichomes which are found on tomato. Numbers show the type of trichomes according to Luckwill (1943).

The wild relatives of tomato that were reported resistant to whiteflies had an abundance of glandular trichomes, especially type IV (Table 1). In genetic studies a high correlation was found between presence of type IV trichomes and whitefly resistance (Channarayappa *et al.* 1992a; Erb *et al.* 1994; Mutschler *et al.* 1996; Snyder *et al.* 1998; Fancelli and Vendramim 2002; Muigai *et al.* 2003; Oriani and Vendramim 2010; Rodriguez-Lopez *et al.* 2011). In *S. habrochaites* f. *glabratum* type VI trichomes were associated with resistance (Dimock and Kennedy 1983; Chatzivasileiadis and Sabelis 1997; Antonious 2001; Fridman *et al.* 2005). Type I, IV and VI glandular trichomes are also associated with tomato resistance to other pests such as tobacco hornworm (*Manduca sexta* L.), tomato fruitworm/corn earworm (*Helicoverpa zea* Boddie), aphids (*Aphis gossypii* Glover and *Myzus persicae*), Colorado potato beetle (*Leptinotarsa decemlineata* Say); pinworm (*Tuta absoluta*), spider mite (*Tetranychus urticae* Koch), tomato pinworm (*Keiferia lycopersicella* Walsingham) and beet armyworm (*Spodoptera exigua* Hubner) (Williams *et al.* 1980; Carter and Snyder 1986; Lin *et al.* 1987; Coates *et al.* 1988; Kennedy *et al.* 1991; Gianfagna *et al.* 1992; Barbour *et al.* 1993; Eigenbrode *et al.* 1996; Simmons *et al.* 2005).

Glandular trichomes might act as a physical barrier, interfering with whitefly landing, feeding and oviposition (Dimock and Kennedy 1983; Snyder and Carter 1984; Channarayappa *et al.* 1992a). The viscous exudates of trichomes are a sticky trap for whiteflies (Toscano *et al.* 2002) and the glandular trichomes produce phytochemicals that play a role as repellents or antibiotics (Gentile *et al.* 1968; Goffreda *et al.* 1989; Chermenskaya *et al.* 2009). Trichome exudates which are associated with whitefly resistance in tomato are terpenoids, methyl ketones and acyl sugars.

High levels of monoterpenes and sesquiterpenes are produced in *S. habrochaites* f. *typicum* and *S. pennellii* (Fridman *et al.* 2005; Bleeker *et al.* 2009) and are associated with a reduced whitefly preference and survival (Freitas *et al.* 2002; Antonious *et al.* 2005; Bleeker *et al.* 2009). Examples of these monoterpenes are p-cymene, γ-terpinene, α-terpinene and α-phellandrenes (Martin-Luengo *et al.* 2010). Other terpenoid compounds toxic to whiteflies are sesquiterpenes, such as zingiberene and curcumene (Eigenbrode *et al.* 1994; de

Azevedo *et al.* 2003). Besides being toxic, sesquiterpenes also play a role as repellant (Coates *et al.* 1988; Carter *et al.* 1989; Freitas *et al.* 2002; Frelichowski and Juvik 2005; Bleeker *et al.* 2009).

Table 1. Abundances (based on density) of trichome types in tomatoes

Species	Type of trichome						References
	non glandular trichomes		glandular trichomes				
	III	V	I	IV	VI	VII	
S. lycopersicum	VS	A	VS	-	A	-	(Simmons and Gurr 2004)
S. pimpinellifolium$^{\Theta)}$	VS	A	-	-	S	-	(Perralta *et al.* 2008; Oriani *et al.* 2011)
S. cheesmanii	-	A	-	-	VS	VS**	(Simmons and Gurr 2004)
S. galapagense$^{\Delta}$	-	VS**	VS**	A	S**	VS**	(Simmons and Gurr 2004)
S. chmielewskii	VS	A	-	-	S	VS	(Perralta *et al.* 2008)
S. neorickii	VS	A	-	-	S	VS	(Perralta *et al.* 2008)
S. arcanum	VS	A	-	-	S	VS	(Perralta *et al.* 2008; McDowell *et al.* 2011)
S. huaylasense	VS	A	-	-	S	VS	(Peralta *et al.* 2008)
S. peruvianum	VS	A	-	-	VS	VS	(Peralta *et al.* 2008; Oriani *et al.* 2011)
S. corneliomulleri	VS	A	VS	-	S	VS	(Perralta *et al.* 2008)
S. chilense	-	A	-	-	VS	VS	(Perralta *et al.* 2008; Oriani *et al.* 2011)
S. habrochaites f. *typicum*$^{*)}$	VS**	S***	VS**	A	A	VS**	(Simmons and Gurr 2004)
S. habrochaites f. *glabratum*$^{*)}$	VS	S***	VS	A	A	VS**	(Eigenbrode and Trumble 1993; Simmons and Gurr 2004)
S. pennellii$^{*)}$	VS**	A**	VS	A	VS	VS**	(Simmons *et al.* 2005)

* Some accessions of these species are reported resistant to whiteflies (Muigai *et al.* 2003; Oriani *et al.* 2011), ** Absent from some individuals (Simmons and Gurr 2004, 2005; Simmons *et al.* 2005), *** also reported abundant (Simmons *et al.* 2005), $^{\Theta}$ one accession of this species was reported to have abundant type IV trichomes and resistance to whiteflies and spider mite (Alba *et al.* 2009; Rodriguez-Lopez *et al.* 2011), $^{\Delta}$ reported resistant to aphid (Simmons *et al.* 2005), A = abundant (>5 per mm^2); S = sparse (1 to 5 per mm^2); VS = very sparse (<1 per mm^2); - = not found

Methyl ketones such as 2-undecanone and 2-tridecanone are produced abundantly (between 2700 and 5500 µg per g fresh weight) in type VI trichomes of *S. habrochaites* f. *glabratum* (Dimock and Kennedy 1983; Chatzivasileiadis and Sabelis 1997; Antonious 2001; Fridman *et al.* 2005). These compounds are very toxic to tomato pinworm, beet armyworm (Lin *et al.* 1987) and spider mite (Chatzivasileiadis and Sabelis 1997). Methyl ketone production is controlled by many genes (Zamir and Tadmor 1986; Barbosa and Maluf 1996; Ben-Israel *et al.* 2009). The methylketone synthases *MKS1*and *MKS2* have been identified as an

important enzymes in methylketone production (Fridman *et al.* 2005; Ben-Israel *et al.* 2009). *MKS2* catalyzes the hydrolysis of 3-keto acyl-ACP thioester bond, and *MKS1* catalyzes the subsequent decarboxylation of the released 3-keto fatty acid, during methylketone biosynthesis (Yu *et al.* 2010).

Acyl sugars are the trichome (type I and IV) exudates that cause resistance in *S. pennellii* (Gentile and Stoner 1968; Burke *et al.* 1987; Goffreda *et al.* 1989; Heinz and Zalom 1995; Freitas *et al.* 2002; Resende *et al.* 2002; Fancelli and Vendramim 2002; Fancelli *et al.* 2005). Approximately 90% of type IV trichome exudates of *S. pennellii* LA716 are acyl sugars (Burke *et al.* 1987). These acyl sugars are sticky substances that act as a glue trap (Toscano *et al.* 2002) and are also toxic for whiteflies (Goffreda *et al.* 1989; Liedl *et al.* 1995; Rodriguez-Lopez *et al.* 2011). The type IV trichomes of *S. pimpinellifolium* accession TO-937 contain also acyl sugars which are associated with resistance to whiteflies (Rodriguez-Lopez *et al.* 2011) and two-spotted spider mite (*T. urticae*; Alba *et al.* 2009). The acyl sugars produced in *S. pennellii* and *S. pimpinellifolium* are acyl sucroses and acyl glucoses with different combinations of esters (Blauth *et al.* 1999; Slocombe *et al.* 2008; Schilmiller *et al.* 2009; Rodriguez-Lopez *et al.* 2011).

Genetic basis of whitefly resistance

The analysis of an F2 population based on the cross *S. lycopersicum* x *S. habrochaites* f. *typicum* LA1777 showed that four regions were involved in whitefly resistance (Momotaz *et al.* 2010) and QTLs for oviposition rate were found on chromosomes 9, 10 and 11. The individual QTLs explained only a small part of variation between the parents. The presence of Type IV trichomes co-localized with these QTLs. Monforte and Tanksley (2000) also concluded that the presence of type IV trichomes is controlled by several genes. Monoterpenes and/or sesquiterpenes (including α-santalene and α-bergamotene and β-bergamotene) are involved in whitefly resistance in *S. habrochaites* f. *typicum* PI127826 (Freitas *et al.* 2002; Bleeker *et al.* 2009). The presence of these compounds is highly associated with the presence of type I and IV trichomes (Maluf *et al.* 2001; Freitas *et al.* 2002; Bleeker *et al.* 2009). Genetic studies in

progeny plants of the cross *S. habrochaites* LA1333 (does not produce sesquiterpenes) x *S. habrochaites* LA 1777 (produces sesquiterpenes) showed that the level of sesquiterpenes is controlled by several genes (Frelichowski and Juvik 2005). In another study the inheritance of sesquiterpenes and type IV, VI and VII trichomes could be explained by one single major gene (Freitas *et al.* 2002). In *S. habrochaites* LA1777 a single locus, named Z, controls sesquiterpene production (Rahimi and Carter 1993). Van der Hoeven *et al.* (2000) described another locus (*sst2*) controlling the level of sesquiterpenes on chromosome 8.

In an F2 population of *S. lycopersicum* x *S. habrochaites* f. *glabratum* two QTLs (on Chromosome 1 and 12) explained partly the differences in oviposition rate of the greenhouse whitefly (*Trialeurodes vaporariorum*) (Maliepaard *et al.* 1995). Since not all variation was explained more loci might be involved. Although the presence of type IV and VI trichomes highly correlates with whitefly resistance in *S. habrochaites* f. *glabratum* (Erb *et al.* 1994; Muigai *et al.* 2002; Muigai *et al.* 2003), Maliepaard *et al.* (1995) found the trichome QTLs on different chromosomes (Chromosome 5 and 9 for type IV).

In the whitefly resistance of *S. pennellii* (Freitas *et al.* 2002; Toscano *et al.* 2002; Blauth *et al.* 2008) type IV trichomes play an important role. In an interspecific cross of *S. lycopersicum* x *S. pennellii* LA716 the inheritance of type IV trichomes was suggested to be controlled by two unlinked genes (Lenke and Mutschler 1984). It was suggested that inheritance of acyl sugar production is controlled by a single recessive allele of *S. pennellii* (Resende *et al.* 2002; Saeidi *et al.* 2007). However other studies using a similar interspecific cross suggested that more than one locus was involved, two on chromosome 2 and one on chromosomes 3, 4 and 11 (Mutschler *et al.* 1996). Alleles of *S. pennellii* LA716 have to be present homozygously to get a high production of acyl sugars. QTLs for trichome density and acyl sugar production were also identified in the intraspecific cross of *S. pennellii* LA1912 (low density of type IV trichomes and low acyl sugar production) x *S. pennellii* LA716 (high density of type IV trichomes and high acyl sugar production). Inheritance of type IV trichome density and acyl sugar composition and acyl sucrose production showed the involvement of several genes (Blauth *et al.* 2008). One locus on chromosome 10

was associated with acyl sugar production and type IV trichome density. Nine additional QTLs were described; four QTLs for both trichome density and acyl sugar production, three QTLs specifically for trichome density and two QTL specifically for acyl sugar production.

Based on all these studies it is clear that most probably more than one pathway is involved in resistance, as is the presence of type IV trichomes. Which pathway exactly depends on the (sub)species and even accession studied. Introduction of the resistance trait into the cultivated tomato has so far not been successful, which may at least partly be caused by the fact that most of the resistant sources are distantly related to the cultivated tomato (Covey *et al.* 2010) (Covey *et al.* 2010). Therefore, using taxonomically more close relatives, such as *S. galapagense* and *S. pimpinellifolium* as donors for whitefly resistance study may make the introgression of the resistance trait more feasible (Zamir and Tadmor 1986; Blauth *et al.* 2008).

Scope and outline of the thesis

Chapter 1. A general introduction is given.

Chapter 2. *Bemisia tabaci* is a morphologically cryptic species complex which contains many sibling species. The current genetic structure of this species complex is not yet completely resolved due to incomplete worldwide sampling. *Bemisia* populations collected in Asia showed a lot of variation. Therefor additional sampling of *B. tabaci* collected from some islands of Indonesia and from China, Thailand and India will contribute to a better knowledge of the *B. tabaci* phylogeny. Chapter 2 updates the global picture of the *B. tabaci* species complex by analyzing new samples from the Asian countries mentioned as well as all sequences currently present in the database, resulting in the most complete picture to date. Knowledge about the diversity within *B. tabaci* is important for evaluating the broadness of whitefly resistance in tomato and pepper.

Chapter 3 and 4. During the last three decades, several studies have been performed to find whitefly resistance in wild relatives of tomato and sweet pepper. A number of these wild relatives were more resistant (antibiosis-based resistance) than the cultivated material. However, efforts of introducing whitefly

resistance in the cultivated tomato were not successful. New approaches and resistant sources should be considered. Therefore, large screenings for whitefly resistance in tomato and pepper were carried out to identify the most promising sources of resistance. Chapter 3 and 4 describe these whitefly resistance screenings for pepper and tomato accessions. Whitefly resistance was evaluated under free-choice and no-choice circumstances in Indonesia and the Netherlands. Plant characters such as cuticle thickness, glabrousness, types and density of trichomes were identified. In these chapters, whitefly resistance assessments were also compared.

Chapter 5. So far, the genetic studies on whitefly resistances were in *S. habrochaites* and *S. pennellii* which are distantly related to the cultivated tomato (Figure 3). In this chapter, a genetic study is described based on a cross with a close wild relative that shows the highest level of resistance: *S. galapagense* LA1401. QTLs for whitefly resistance, types and properties of trichomes as well as metabolites related to whitefly resistance were found in an F2 population of *S. lycopersicum* x *S. galapagense*. The results were confirmed in a set of F3 plants resulting from selfed F2's. The results obtained show one major QTL which will facilitate introgression into cultivated germplasm. The QTL is in a region with a high recombination frequency which is convenient for map based cloning of the gene(s) underlying the major QTL.

Chapter 6. A general overview of the results in this thesis are presented and their relation to other studies. Strategies and possibilities of introducing the identified QTLs in cultured varieties are discussed.

Chapter 2

The *Bemisia tabaci* species complex: additions from different parts of the world

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Abstract

Bemisia tabaci is one of the most threatening pests in many crops with a large diversity. We sequenced part of the gene mitochondrial cytochrome oxidase 1 from fifty whitefly populations collected in Indonesia, Thailand, India and China. Nineteen unique sequences (haplotypes) of the cytochrome oxidase 1 were identified in these populations. They were combined with sequences available in databases, resulting in a total of 405 haplotypes and analyzed. A phylogenetic tree was calculated using maximum likelihood and maximum parsimony methods. The trees showed that all groups that were found in a previous study were also found in our study. Additionally, seven new groups were identified based on the new haplotypes. Most *Bemisia tabaci* haplotypes grouped based on geographical distribution. Two groups were found to have a world-wide distribution. Our results indicate that our knowledge on the species complex around *Bemisia tabaci* is still far from complete.

Key words: whitefly, *mtCOI*, dendrogram, haplotype, species complex.

Introduction

Bemisia tabaci (Gennadius), commonly known as silverleaf whitefly or sweet potato whitefly, is one of the most threatening pests in many crops (De Barro *et al.* 2011). Its wide distribution is believed to be the result of human activities and its ability to adapt to diverse host plants (Dalton 2006). The identification of morphologically indistinguishable *B. tabaci* biotypes originating from many parts of the world was originally based on different traits, such as host range (Perring 2001), adaptability to new host plants (Burban *et al.* 1992), induction of phytotoxic reactions and physiological changes (Costa *et al.* 1993c), insecticide resistance (Erdogan *et al.* 2008), plant virus transmission capabilities (Bedford *et al.* 1994) and the rate of development (Bird 1957; Mound 1967; Bird and Maramorosch 1978; Byrne *et al.* 1995). The differences between the "A biotype" (now New World Group) and "B biotype" (now Middle East Asia Minor 1) were considered substantial enough to separate them into different species. The B biotype was named *B. argentifolii* by Bellows *et al.* (1994). However, the genetic structure and phylogenetic relationships between the worldwide collected *B. tabaci* populations could not be resolved well (Rosell *et al.* 1997).

To solve this problem, many different techniques, such as allozymes RAPDs, PCR-RFLP, SSRs, SCARs, as well as sequencing of the *ITS1* and 16s ribosomal DNA have been used (Perring 2001; De Barro *et al.* 2011). Although many differences among *B. tabaci* populations were revealed, the *B. tabaci* phylogeny still could not be resolved (De Barro *et al.* 2011). Nowadays, sequencing of the mitochondrial gene *mtCOI* is frequently used for reconstruction of evolutionary relationships in animals and plants (Lunt *et al.* 1996), as is also the case for whiteflies (Frohlich *et al.* 1999; Boykin *et al.* 2007; Ueda *et al.* 2009). Data on the sequence of *mtCOI* of worldwide collected whitefly strains are stored in databases (www.ebi.ac.uk). Analysis of these sequences has shown that the genetic differentiation of *B. tabaci* populations corresponded to the geographical distribution, except for the biotypes B and Q which can be found all over the world (Guirao *et al.* 1997; De la Rua *et al.* 2006; De Barro *et al.* 2005; De Barro *et al.* 2011; Hu *et al.* 2011; Alemandri *et al.* 2012; De Barro, 2012). The strong

geographical association of populations with hardly any gene flow between them suggests that *B. tabaci* may be a species complex consisting of numerous morphologically cryptic species. Further studies have also shown mating incompatibility (Brown *et al.* 1995a; Frohlich *et al.* 1999; De Barro *et al.* 2005; Xu *et al.* 2010; Wang *et al.* 2011).

The global genetic structure and phylogeny of *B. tabaci* have been nicely reconstructed (Boykin *et al.* 2007; De Barro *et al.* 2011; Dinsdale *et al.* 2010; De Barro, 2012). A cladistic analysis of *B. tabaci* populations using the *mtCOI* gene and Bayesian calculations showed that *B. tabaci* consisted of eleven well-defined high level groups with more than eleven percent genetic divergence (Dinsdale *et al.* 2010). At the lower level (3.5% genetic divergence) 24 groups could be identified, suggesting that *B. tabaci* is a cryptic species complex consisting of at least 24 distinct species. Since the Dinsdale *et al.* (2010) publication four new groups have been identified in China (Hu *et al.* 2011) and one (New World 2) in Argentina (Alemandri *et al.* 2012). The number of *mtCOI* accessions from *B. tabaci* that is currently available in databases is three times higher than at the time of the study by Dinsdale *et al.* (2010), including accessions from new locations. For our study on whitefly resistance in tomato, we collected *B. tabaci* populations from regions in Indonesia, Thailand, China and India. We wanted to see how these strains fit in the recent phylogenetic tree of *B. tabaci.* A good recognition of whitefly species in term of their specificities such as susceptibility towards insecticides, biological control efficacy, invasiveness and ability in virus transmission is important in the choice of management decisions (Chu *et al.* 2006; De Barro 2012).

Material and Methods

Whitefly collection

In 2009 fifty populations were sampled from several locations in Indonesia (30), Thailand (3), India (3) and China (14) (Table 1). Adults of *B. tabaci* were collected from every location using an aspirator and stored in 1 ml of 70% ethanol (EtOH) at 4^{o}C. All strains were analyzed at Wageningen UR Plant Breeding, The Netherlands.

Partial *mtCOI gene* amplification and sequencing

Part of the COI gene was amplified directly from a single whitefly (two samples per location) with the specific primer pair (C1-J-2195 TTGATTTTTTGGTCATCCAGAAGT and L2N3014 TCCAATGCACTAAT CTGCCATATTA) (Frohlich *et al.* 1999). These primers amplify the region between position 725 and position 1560 of the *mtCOI* gene. Stored whiteflies were taken out of the Eppendorf tube with alcohol and dried on a paper towel for a few minutes. After drying, the whitefly was divided in two parts and one part was directly used in a PCR reaction without prior DNA extraction whereas the other part was stored as a backup. The whitefly half was mixed with 10 µl PCR solution, which was prepared according to the standard manual of Qiagen Inc (5 µl Multiplex PCR kit, 0.25 µl for each 10 nM primer C1-J-2195 and L2N3014 and 4.5 µl ddH2O). The whitefly was incubated for 15 minutes at 95^{o}C, which results in lysis. The PCR reaction was performed in the same tube for 45 cycles (95^{o}C for 30 seconds, 50^{o}C for 30 seconds and 72^{o}C for 45 seconds). A final DNA extension step of 10 minutes at 72^{o}C was used. After the PCR reaction 10 µl ddH_2O was added and five µl of this mixture was loaded together with 1 µl 6x loading dye (10 mM Tris-HCl (pH 7.6), 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA) on an 1% agarose gels containing Ethidium Bromide (0.5µg.ml^{-1}) and run at 80 Volt for 30 minutes. PCR products (single band of about 800 base pairs) were directly sequenced by Greenomics Inc. (www.greenomics.nl) using the DETT® sequencing kit. The forward and reverse DNA sequences were combined using SeqMan Pro, Lasergene DNASTAR Inc. version 8.1.2. All sequences were trimmed to the same size (657bp) and position (781 to 1438) as used by Dinsdale *et al.* (2010).

Retrieving and processing *mtCOI* sequences from database

All *mtCOI* sequences of *B. tabaci* which were available in the database on November, 30th 2010 were retrieved (www.srs.ebi.ac.uk). Of the 2400 accessions present in the database, 1406 accessions were selected because the sequences contained the same region (from position 781 to 1438) of the *mtCOI* gene as used by Dinsdale *et al.* (2010). Furthermore, to avoid possible pseudogenes (Dinsdale *et al.* 2010) we only selected sequences that contained one open reading frame

(ORF) and no insertion-deletions (*indels*) by using the translate tool of MEGA version 5.05 (Tamura *et al.* 2011). This resulted in the exclusion of 317 accessions leaving 1089 accessions. Next, from all sequences that were present more than once, one sequence was selected. Finally, 387 accessions of *B. tabaci* from database and three other accessions (two *Bemisia atriplex* and one *Bemisia afer*) that were used in Dinsdale *et al.* (2010) were included in phylogeny reconstruction (supplementary data 2.1). All available information including groups based on Dinsdale *et al.* (2010), host plant from which the whitefly sample was collected, location where it was collected, country of origin, accession number, year of collection or submission in data base were recorded and incorporated in the accession name. These data were also analyzed in order to identify haplotype and/or group characteristics such as distribution width and host range.

Prior to multiple sequence alignment analyses, all sequences were renamed according to: *Bemisia tabaci* = Bt(haplotype)(group), host plant, place, country, accession number and year of collection or submission in the data base. The name of the group was only mentioned for the accessions which were in the dataset of Dinsdale *et al.* (2010). Missing data were denoted as xxx. All 387 unique sequences from the database search, the three sequences of the accessions that were used as outgroups and the 15 new unique sequences from our own whitefly collection, were used for pairwise multiple sequence alignment using the ClustalW program (Thompson *et al.* 1994), which is part of the MEGA 5.05 package (Tamura *et al.* 2011).

Phylogenetic reconstruction of *B. tabaci* populations

The final dataset which was obtained from multiple sequence alignment analysis was used for tree reconstruction by using maximum parsimony (MP) and maximum likelihood (ML) methods that are available in MEGA 5.05 (Tamura *et al.* 2011). In the ML method we used the Tamura-Nei model (TN93) and Gamma distributed with invariant site (G+I) for substitution model among sites. This model was the best because it showed the lowest Bayesian Information Criterion (BIC) score and a high Maximum Likelihood value (*inL*) (Tamura *et al.* 2011, Felsenstein, 1981). We used 1000 bootstraps to test the robustness of the

phylogeny (Felsenstein, 1981). Divergence between and within groups were also calculated by using the numbers of differences method of MEGA 5.05 package (Tamura *et al.* 2011).

Results

Bemisia tabaci haplotypes obtained from the database

Sequences of the *mtCOI* gene of about 2400 accessions of *B. tabaci* were retrieved from the database. Over 50% of them were not used because the sequenced fragment did not cover our target area or they may be pseudogenes. The 1089 selected accessions, contained 390 unique *mtCOI* gene sequences (haplotypes) that were used for reconstruction of the phylogenetic tree (supplementary data 2.1). The data originated from collection sites in 73 countries in Africa, Asia, America, Australia and Europe.

Bemisia tabaci haplotypes obtained from own collection trips

An additional fifty partial *mtCOI* sequences were obtained from whiteflies collected in Indonesia, Thailand, China and India. The sequences of all samples from India and one from Indonesia (Indo#17_S.melo_W.Borneo_Indonesia) were identical to the sequence that was already in the database (Bt2Asia1_S.melo_ Karnataka_India_AJ748361_2004). The sequence of all samples from Thailand, 21 samples from Indonesia and one from China were identical to Bt3Asia1_G.hirsu_Singapore_AY686095_2004. Of our collected samples Indo#18_C.sati_W.Borneo_Indonesia and Bt22Asia2.7_C.luna_Karnataka_India_ AJ748378_2004 were identical and Chi#12_C.sati_China was identical to Bt37Med_xxx_xxx_China_GU086329_2009. Five new sequences were identified from the eight samples from Indonesia. Also in the strains from China new sequences were identified (in 10 out of the 14 samples; Table 1). In total, we could add 15 new sequences as new haplotypes to be used in the construction of the phylogenetic tree.

Table 1. Collection of *Bemisia tabaci* from Thailand, India, Indonesia and China

No	Collection name	Host plant	Haplotype*	Group	Accession number
1	Tha#01_S.lyco_Chiangmai_Thailand	*S. lycopersicum*	3	Asia I	HE653676
2	Tha#02_S.lyco_Pongyang_Thailand	*S. lycopersicum*	3	Asia I	HE653677
3	Tha#03_S.lyco_Suphanbure_Thailand	*S. lycopersicum*	3	Asia I	HE653678
4	Indo#19_S.lyco_C.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653700
5	Indo#21_S.lyco_C.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653702
6	Indo#27_S.lyco_C.Sulawesi_Indonesia	*S. lycopersicum*	3	Asia I	HE653708
7	Indo#20_S.melo_E.Java_Indonesia	*S. melongena*	3	Asia I	HE653701
8	Indo#01_S.melo_W.Borneo_Indonesia	*S. melongena*	3	Asia I	HE653682
9	Indo#03_S.lyco_W.Borneo_Indonesia	*S. lycopersicum*	3	Asia I	HE653684
10	Indo#04_C.sati_W.Borneo_Indonesia	*C. sativus*	3	Asia I	HE653685
11	Indo#16_S.lyco_W.Borneo_Indonesia	*S. lycopersicum*	3	Asia I	HE653697
12	Indo#05_C.annuum_W.Java_Indonesia	*C. annuum*	3	Asia I	HE653686
13	Indo#06_S.lyco_W.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653687
14	Indo#07_S.melo_W.Java_Indonesia	*S. melongena*	3	Asia I	HE653688
15	Indo#08_Amaranthus.sp_W.Java_Indonesia	*Amaranthus sp*	3	Asia I	HE653689
16	Indo#09_S.lyco_W.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653690
17	Indo#10_S.lyco_W.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653691
18	Indo#11_S.lyco_W.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653692
19	Indo#12_S.melo_W.Java_Indonesia	*S. melongena*	3	Asia I	HE653693
20	Indo#14_S.melo_W.Java_Indonesia	*S. melongena*	3	Asia I	HE653695
21	Indo#22_S.lyco_W.Java_Indonesia	*S. lycopersicum*	3	Asia I	HE653703
22	Indo#30_C.annuum_W.Java_Indonesia	*C. annuum*	3	Asia I	HE653711
23	Indo#28_S.lyco_W.Sumatera_Indonesia	*S. lycopersicum*	3	Asia I	HE653709
24	Chi#10_S.lyco_S.lyco	*S. lycopersicum*	3	Asia I	HE653721
25	Indi#01_S.lyco_Aurangabad_India	*S. lycopersicum*	2	Asia I	HE653679
26	Indi#02_S.lyco_Bangalore_India	*S. lycopersicum*	2	Asia I	HE653680
27	Indi#03_S.lyco_Tamilnadu_India	*S. lycopersicum*	2	Asia I	HE653681
28	Indo#17_S.melo_W.Borneo_Indonesia	*S. melongena*	2	Asia I	HE653698
29	Indo#24_S.lyco_C.Sulawesi_Indonesia	*S. lycopersicum*	76	Asia I	HE653705
30	Indo#26_S.lyco_C.Sulawesi_Indonesia	*S. lycopersicum*	76	Asia I	HE653707
31	Indo#15_S.lyco_W.Borneo_Indonesia	*S. lycopersicum*	76	Asia I	HE653696
32	Indo#29_S.lyco_W.Java_Indonesia	*S. lycopersicum*	76	Asia I	HE653710
33	Indo#25_S.lyco_C.Sulawesi_Indonesia	*S. lycopersicum*	121	Asia I	HE653706
34	Indo#02_S.melo_W.Borneo_Indonesia	*S. melongena*	119	Asia I	HE653683
35	Indo#23_S.lyco_W.Java_Indonesia	*S. lycopersicum*	120	Asia I	HE653704
36	Chi#06_S.lyco_Naning_China	*S. lycopersicum*	77	Asia IV	HE653717
37	Chi#02_S.lyco_Putian_China	*S. lycopersicum*	77	Asia IV	HE653713
38	Chi#09_S.lyco_Dinganhainan_China	*S. lycopersicum*	128	Asia IV	HE653720
39	Chi#01_S.lyco_Guangdong_China	*S. lycopersicum*	127	Asia IV	HE653712
40	Chi#04_S.lyco_Tiangyang_China	*S. lycopersicum*	128	Asia IV	HE653715
41	Chi#11_C.annuum_China	*C. annuum*	130	Asia IV	HE653722
42	Indo#18_C.sati_W.Borneo_Indonesia	*C. sativus*	22	Asia II 7	HE653699
43	Indo#13_S.melo_W.Java_Indonesia	*S. melongena*	156	Asia II 12	HE653694
44	Chi#14_S.lyco_China	*S. lycopersicum*	78	Mediterranean	HE653725
45	Chi#12_C.sati_China	*C. sativus*	37	Mediterranean	HE653723
46	Chi#13_S.lyco_China	*S. lycopersicum*	78	Mediterranean	HE653724
47	Chi#03_S.lyco_Putian_China	*S. lycopersicum*	343	MEAM1	HE653714
48	Chi#05_S.lyco_Tiamyang_China	*S. lycopersicum*	344	MEAM1	HE653716
49	Chi#08_S.lyco_Yuanmntil_China	*S. lycopersicum*	346	MEAM1	HE653719
50	Chi#07_S.lyco_Yuanmou_China	*S. lycopersicum*	345	MEAM1	HE653718

* Haplotype names were made based on the group list that can be found in detail in supplementary data 2.1; MEAM1 is Middle East-Asia minor 1

Phylogenetic relationship among *B. tabaci* haplotypes

The phylogenetic trees based on two calculation methods (MP and ML) showed similar topology. We choose to show only the condensed MP tree (Figure 1). The complete MP tree as well as the ML tree can be found as supplementary figure 2.1 and 2.2. A difference between the MP and ML was in the position of some groups within the Asia clade notably China3 and Asia2.12. A large genetic distance (19% difference) was found between the Uganda group and its closest group (New World). We identified 33 groups which were at least 3.5% (23 out of the 657 nucleotides) different (Figure 1). The dataset obtained by Dinsdale *et al.* (2010) showed 24 of our 33 groups. Only a few minor changes were observed (the position of the Sub-Sahara-Africa (SubSahAf) clade and the Mediterranean-Middle East-Asia Minor (MEAM) clade).

Our results lead to the addition of seven groups to the previous reported phylogenies (Dinsdale *et al.* 2010, Hu *et al.* 2011, Alemandri *et al.* 2012). Two new groups (Asia IV, and Japan1) were placed in the same clade with Asia I. Another two of the new groups (Asia II 11 and Asia II 12) were placed in the clade Asia II. The fifth new group was Africa which is located close to the Asia II in the Asian clade (see Figure 1). The remaining two new groups were Japan2, which is located close to the *B. atriplex* and SubSahAf5 which was located in the SubSahAf clade (SubSahAf1 to SubSahAf4). Based on this data, of 33 groups, 19 groups belong to Asian clade.

Genetic diversity among samples collected from Indonesia, Thailand, China and India

Our fifty newly collected samples were classified in six groups (Table 1). Thirty five of the strains collected in Indonesia (28), Thailand (3), India (3) and China (1) were classified as Asia I 1. Additionally six strains from China were classified as Asia III and two strains from Indonesia were classified as Asia II 7 and Asia II 12. Finally, three strains from China were classified in the group Mediterranean and four in the MEAM1 group.

Diversity and distribution of *B. tabaci* groups

Haplotype composition and distribution of groups are presented in Figure 1. Mediterranean and MEAM1 are the groups with a very wide distribution.

MEAM1 had 83 haplotypes with a mean divergence of 1.2% and these have been collected in 35 countries of Asia, Africa, Europe, America and Australia. The Mediterranean group contains 69 haplotypes with 1.6% divergence mean and has been collected in 28 countries in Asia, Africa, Europe and America. Asia1.1 was the most widely distributed group in Asia with 55 haplotypes and 1.5% divergence mean. Several groups such as Asia1.2, Italy, SubSahAf3 (Figure 1) originate from specific countries. A high number of haplotypes and groups were found in Asian countries such as China, Japan and India (Table 2).

Discussion

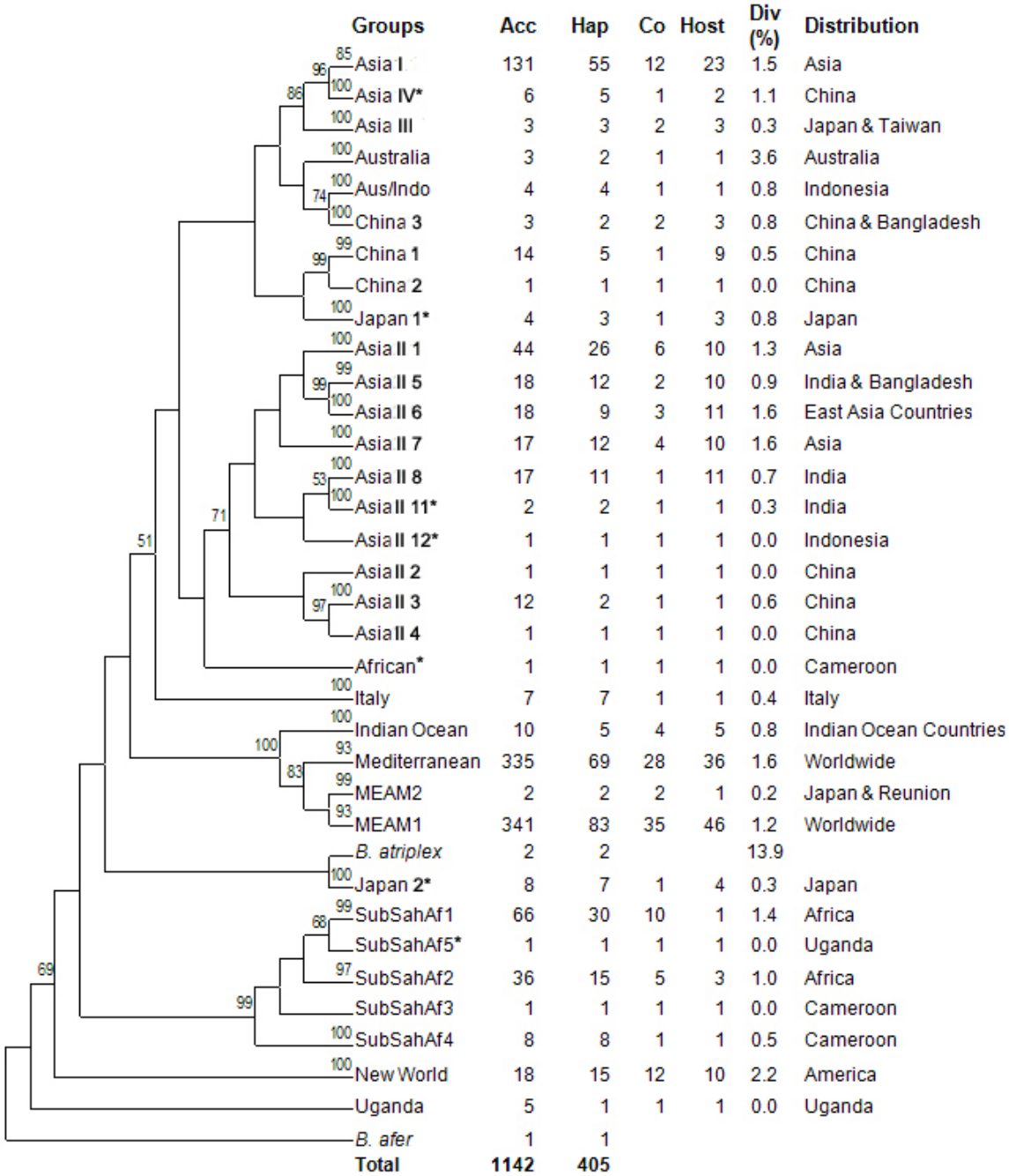
mtCOI haplotyping and genetic diversity among *B. tabaci* collected in Indonesia, Thailand, India and China

A partial *mtCOI* sequence was used to identify variation (haplotypes) among *B. tabaci* populations. The same approach was used by McKenzie *et al.* (2009) to identify genetic diversity within the *B. tabaci* Q biotype in Florida. Nineteen haplotypes were identified among the 50 samples that we collected. Four of these haplotypes were already present in the database. One haplotype (Table 1, no. 3) was found in 20 samples from Indonesia including Java, West Sumatra, West Borneo and Central Sulawesi, all three strains from Thailand and one sample from China. This haplotype is also known to be present in Taiwan (Hsieh *et al.* 2006), Cambodia (Fleury *et al.* 2010), Japan (Ueda *et al.* 2009) and Pakistan (Ahmed *et al.* 2009). It was suggested to be the *B. tabaci* haplotype that originally invaded Indonesia and Central Thailand between 1994 and 1999 (De Barro *et al.* 2005). Another haplotype (no. 2) was found in all samples from India and one sample from West Borneo, Indonesia. This one also has a wide distribution in Asia including India, Bangladesh, Pakistan and China (Rekha *et al.* 2005, Ahmed *et al.* 2009, Simon *et al.* 2003, Ellango *et al.* Unpublished, Maruthi, unpublished) and Turkey (De la Rua *et al.* 2006). Haplotype no. 22 was found in West Borneo, Indonesia. It was reported earlier from Indonesia (Hsieh *et al.* 2006) and Jiangsu and Fujian, China (Hsieh *et al.* 2006, Rao *et al.* 2011). Haplotype no. 37 was found in some parts of China such as Hubei, Jiangzhou, Hangzhou, and Wuhan (Teng *et al.* 2010; Luo *et al.* Unpublished; Rao *et al.* 2011). This haplotype was

also found in Japan, South Korea, Greece, France, Portugal, Spain, Marocco, Tunisia, and USA (Dinsdale *et al.* 2010; Lee *et al.* 2008; Shatters *et al.* 2009; Gueguen *et al.* 2010; McKenzie *et al.* 2009; Dalmon *et al.* 2008; Tsagkarakou *et al.* 2007; Boykin *et al.* 2007; Ueda and Brown; 2006). In previous studies, seven additional unique haplotypes were identified in Indonesia (Dinsdale *et al.* 2010; Hidayat *et al.* 2008). One of these was a member of the MEAM1, two were classified as Asia1.1 and the other four haplotypes as Australia/Indonesia. In Indonesia, we found five new haplotypes. One of them (haplotype no. 76) was found in four regions, the other four were each restricted to one region. Finding the new haplotypes in Indonesia and China showed that both countries have a large diversity of *B. tabaci* that might have resulted from local adaptations.

Phylogenetic relationship among *B. tabaci* haplotypes

Since the study of Dinsdale *et al.* (2010) more than 1200 new *B. tabaci* sequences had been added to the database. To find out whether they would affect the previously reconstructed phylogeny, we analyzed all data again. More than 65% (752 of 1142) of the useful partial *mtCOI* sequences that were retrieved from the database were duplicates and excluded from further analysis. Of the haplotypes that remained (390), 201 haplotypes were also included in the analysis of Dinsdale *et al.* (2010). These haplotypes regrouped in the same groups as in the study of Dinsdale *et al.* (2010). This shows that using a functional part of the *mtCOI* gene is a good way to resolve phylogenetic relationship among *B. tabaci* populations. Our phylogenetic tree is also highly similar to the ones produced in other studies (Ueda *et al.* 2009, Boykin *et al.* 2007), indicating that the topology of the tree is very robust. The haplotypes that made new groups, such as Japan2, might have caused the small differences in topology between our study and the study of Dinsdale *et al.* (2010). Similar changes were also found after the addition of *B. atriplex* in the phylogenetic reconstruction of Dinsdale *et al.* (2010) when compared to the previous reconstruction of Boykin *et al.* (2007). *Bemisia atriplex* is placed within *B. tabaci* clade and close to Japan2 which was placed outside the Asian clade (Figure 1).



Groups	Acc	Hap	Co	Host	Div (%)	Distribution
Asia I	131	55	12	23	1.5	Asia
Asia IV*	6	5	1	2	1.1	China
Asia III	3	3	2	3	0.3	Japan & Taiwan
Australia	3	2	1	1	3.6	Australia
Aus/Indo	4	4	1	1	0.8	Indonesia
China 3	3	2	2	3	0.8	China & Bangladesh
China 1	14	5	1	9	0.5	China
China 2	1	1	1	1	0.0	China
Japan 1*	4	3	1	3	0.8	Japan
Asia II 1	44	26	6	10	1.3	Asia
Asia II 5	18	12	2	10	0.9	India & Bangladesh
Asia II 6	18	9	3	11	1.6	East Asia Countries
Asia II 7	17	12	4	10	1.6	Asia
Asia II 8	17	11	1	11	0.7	India
Asia II 11*	2	2	1	1	0.3	India
Asia II 12*	1	1	1	1	0.0	Indonesia
Asia II 2	1	1	1	1	0.0	China
Asia II 3	12	2	1	1	0.6	China
Asia II 4	1	1	1	1	0.0	China
African*	1	1	1	1	0.0	Cameroon
Italy	7	7	1	1	0.4	Italy
Indian Ocean	10	5	4	5	0.8	Indian Ocean Countries
Mediterranean	335	69	28	36	1.6	Worldwide
MEAM2	2	2	2	1	0.2	Japan & Reunion
MEAM1	341	83	35	46	1.2	Worldwide
B. atriplex	2	2			13.9	
Japan 2*	8	7	1	4	0.3	Japan
SubSahAf1	66	30	10	1	1.4	Africa
SubSahAf5*	1	1	1	1	0.0	Uganda
SubSahAf2	36	15	5	3	1.0	Africa
SubSahAf3	1	1	1	1	0.0	Cameroon
SubSahAf4	8	8	1	1	0.5	Cameroon
New World	18	15	12	10	2.2	America
Uganda	5	1	1	1	0.0	Uganda
B. afer	1	1				
Total	**1142**	**405**				

Figure 1. Phylogenetic tree of global *Bemisia tabaci* populations estimated by using Maximum Parsimony. Numbers above branches represent the percentage of 1000 bootstraps. The groups are followed with number of accessions (Acc), haplotypes (Hap), countries (Co), host plants (Host) and mean of genetic divergence percentage (Div). Aus/Ind is Australia/Indonesia, MEAM is Middle East-Asia Minor and SubSahAf is Sub Sahara-Africa. Notation of * is the identified new groups compared to previous study of Dinsdale *et al.* (2010).

Table 2. Countries with a high number of groups, haplotypes and accessions of *Bemisia tabaci*

No	Country	Number of Groups	Haplotypes	Accessions
1	China's mainland	13	51	226
2	Japan	9	27	42
3	India	7	88	122
4	Taiwan	7	13	17
5	Uganda	5	33	83
6	Indonesia	5	15	45
7	Cameroon	5	14	17
8	Bangladesh	4	15	21
9	Pakistan	3	21	25
10	Spain	3	9	14
11	Reunion	3	8	12
12	USA	3	7	160
13	Turkey	3	5	6

The position of *B. atriplex* was not expected in the *B. tabaci* group. However, its position may have been affected by the number and type of outgroup species used in the phylogeny reconstruction (Boykin *et al.* 2007). *Bemisia atriplex* was excluded from *B tabaci* clade by including a number of close sister species such as *B. emiliae, B. afer, B. berbericola, B. subdecipiens, B. tuberculata* in the phylogenetic reconstruction (Hu *et al.* 2011; De Barro *et al.* 2011). Japan2 was previously called the honeysuckle (*Lonicera japonica*) race population, which is adapted to low temperatures and dominant in Kyushu and Honshu Islands (Ueda *et al.* 2009). The Uganda group was also genetically very different from the other *B. tabaci* populations. The Uganda group was collected on sweet potato (*Ipomoea batatas*) and was also different from collections made on cassava and cassava weeds (Legg *et al.* 2002; Maruthi *et al.* 2002; Maruthi *et al.* 2004). It is considered as species distinct from *B. tabaci* (De Barro *et al.* 2011). Furthermore, mating incompatibilities were found between the Uganda and *B. tabaci* collected on cassava and *Euphorbia geniculata* (Maruthi *et al.* 2004) and amongst *B. tabaci* groups (Xu *et al.* 2010; Wang *et al.* 2011; Liu *et al.* 2012). These differences are also evidence that *B. tabaci* is a species complex. That it is a species complex can also be seen from the multimodal distribution of pairwise nucleotide differences among the 402 haplotypes (Figure 2). A single species with continuous gene flow would produce a unimodal distribution of pairwise difference (Highton, 1998).

The new groups in the global *B. tabaci* phylogenetic tree indicate that there is still a lot of unsampled variation. It is likely that several more groups exist,

especially in countries that are not or hardly sampled such as Chile, Peru, Vietnam, islands in east part of Indonesia, Philippines, Papua New Guinea and some parts in Africa and Australia. The distribution of *B. tabaci* groups was wide spread (Figure 1). Mediterranean and MEAM1 group were found in tropical and subtropical countries around the world and are believed to be the most invasive groups (Chu *et al.* 2010, Boykin *et al.* 2007, De Barro *et al.* 2011, De Barro, 2012). Other groups were found in a specific continent or region such as Asia I, Asia II 1, New World, SubSahAf1 and SubSahAf2 or were restricted in a specific country or area such as Japan1, Japan2, China1 and China2. However, this may also be caused by under sampling. Human activities such as plant trading and agriculture play a very important role in the spread of *B. tabaci*, other factors such as geographical and behavioral barriers are the decisive factors in the survival of newly introduced *B. tabaci* strains in a new area (De Barro *et al.* 2005, De Barro *et al.* 2011).

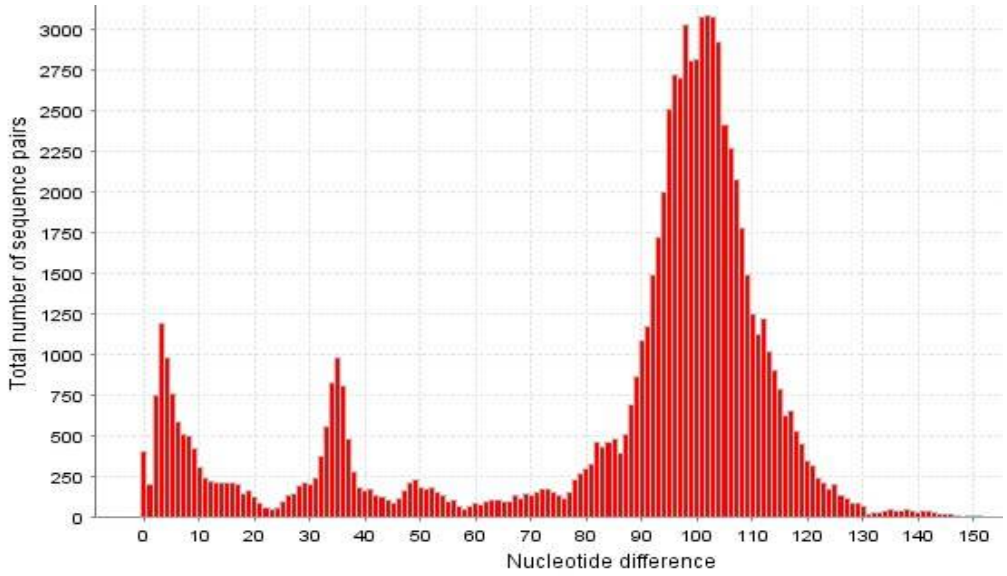


Figure 2. Frequency of pairwise nucleotide differences comparison among the 402 *Bemisia tabaci* haplotypes estimated by using CLUSTERER (Klepac-Ceraj *et al.* 2006). The total number of nucleotides compared was 657 bases.

Accessions from the same region often grouped together, which indicates that geographical barriers are the main factor effecting differentiation and speciation of *B. tabaci*. Historical events, specific climate, host plant diversity and

availability in each geography separation are believed to be the geographical separation factors (De Barro *et al.* 2005).

In a recent study (Hu *et al.* 2011) analyzed new whitefly samples collected from China and they introduced 4 new groups compared to Dinsdale *et al.* (2010). Sequences that were included in two new groups (Asia III and China 3) of Hu *et al.* (2011) were also found in our study and grouped in Asia III and China 3. The other two groups (Asia II 9 and Asia II 10) were more than 3.5% divergent from any of the 33 group we found. Sampling in Argentina also resulted in the identification of a group with more than 3.5% different from the New World group (Alemandri *et al.* 2012), bringing the total number of groups with a more than 3.5% divergence to 36. As mentioned it is very likely that this number will increase when more areas are sampled. Another point is that the 3.5% threshold for species delimitation is still under debate as reproductive incompatibilities were also shown between *B. tabaci* populations with less than 3.5% divergence (De Barro and Ahmed 2011; De Barro 2012). Uncertainty of species delimitation based on mtCOI divergence was also shown from different levels of species distinctiveness of *B. tabaci* populations by using Kimura two-parameters (K2P) and four more stringent measures (Boykin *et al.* 2012). These results indicate that more genes should be included in the analysis to resolve species delimitation among *B. tabaci* populations (Boykin *et al.* 2012; De Barro 2012). Also, more sequences of *B. tabaci* populations representing other habitats and various host plants are needed, as are more studies to better establish host specificity, effects of different responses to pest control measures and differences in virus transmission. Such information is essential for host plant resistance breeding programs in crops such as tomato and pepper.

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Chapter 3

Identification of Silverleaf Whitefly Resistance in Pepper

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Abstract

Whitefly is economically one of the most threatening pests of pepper worldwide, which is mainly caused by its ability to transmit many different viruses. In this research, we characterized pepper germplasm to identify whitefly-resistant accessions that will form the basis for future resistance breeding. Forty-four pepper accessions representing four species (*Capsicum annuum, C. frutescens, C. chinense, C. baccatum*) were screened for resistance to whiteflies. Screening parameters were adult survival (AS) and oviposition rate (OR) in a no-choice test and whitefly, egg and nymphal density in free-choice tests. To combine parameters in free-choice tests, a plant resistance value was calculated. The results show that AS and OR were significantly different among accessions and were positively correlated, which was also the case for the parameters in the free-choice tests. Accessions identified as highly resistant in no-choice and free-choice tests generally were *C. annuum*. Whitefly density and OR correlated positively with trichome density and negatively with cuticle thickness of leaves.

Key words: *Capsicum, Bemisia tabaci,* trichome density, cuticle thickness

Introduction

Pepper (*Capsicum* spp.) is one of the most important vegetable crops worldwide. The cultivation and yield of pepper are hampered during the long and hot season because of pests and diseases. Whiteflies and the viruses they transmit play an important role in the disease pressure in the field (Hidayat and Rahmayani 2007). The silverleaf whitefly (group Mediterranean-Middle East-Asia Minor I of *Bemisia tabaci* Genn), also known as *B. argentifolii* Bellows and Perring (Bellows *et al.* 1994), is worldwide the most harmful and invasive pest insect in tomato and pepper cultivation (Morales 2007). *Bemisia tabaci* transmits more than 200 plant viruses efficiently (Morales and Jones 2004); 90% of them are begomoviruses (Morales 2007). The visible, direct damage caused by whiteflies are leaf deformation and honeydew production on which sooty moulds can grow as well as physiological disorders and irregular ripening of the fruits (McCollum *et al.* 2004).

To reduce damage, it is necessary to reduce the population growth of the whiteflies. This can be carried out by using pesticides. However, pesticides are costly and hazardous to the environment, growers and consumers. Frequent use of pesticides can also quickly result in resistant whiteflies (Dittrich *et al.* 1990, Erdogan *et al.* 2008). Alternative approaches are the use of resistant varieties or biological control using pathogens, parasitoids and/or predators of the whitefly or a combination of them (van Lenteren and Martin 1999). To explore the possibilities of developing whitefly resistant varieties, it is essential to identify resistance in pepper germplasm.

In tomato, whitefly resistance has been characterized by recording life-history parameters such as adult survival (AS), oviposition rate (OR) and pre-AS (Bas *et al.* 1992). Whitefly mortality on resistant plants can be caused by starvation resulting from physical barriers (thickness of cuticle layers) and/or chemical compounds. The cuticle thickness of leaflets may prevent the stylet from reaching the phloem (Janssen *et al.* 1989). Such a resistance mechanism to different kinds of phloem feeding/piercing insects has been reported in tomato, cotton and cassava (Heinz and Zalom 1995, Bellotti and Arias 2001, Jindal *et al.* 2008). Glandular trichomes may cause high whitefly mortality, as compounds,

such as acyl sugars can act as a glue and/or toxin and consequently work as a trap and/or natural insecticide for the whiteflies (Liedl *et al.* 1995, Mutschler *et al.* 1996). Other plant secondary metabolites such as methyl-ketones and derivates of sesquiterpene carboxylic acid can have negative effects on population development (Williams *et al.* 1980, Eigenbrode *et al.* 1994). These compounds can be present in the leaf mesophyll or they can be released as volatiles that could play a role as an attractant, repellent or antibiotic substance (Antonious and Kochhar 2003, Chermenskaya *et al.* 2009).

Compared with tomato or cotton, there have been only a few studies on whitefly resistance in pepper. Whitefly resistance has been investigated in sweet pepper cultivars by observing Tomato leaf curl Bangalore virus (ToLCBV) incidence after viruliferous-whitefly infestation (Maruthi *et al.* 2003) and counting the black puparia resulting from parasitizing by Encarsia formosa (Laska *et al.* 1986). Lei *et al.* (1999) used electrical penetration graphs (EPG) to identify possible resistance factors in sweet pepper leaves. Until now, there is no information available on whitefly resistance in commercial pepper cultivars and their close relatives. This research was carried out to investigate the level of resistance to silverleaf whitefly in a number of commercial pepper varieties and wild relatives of the cultivated pepper using different types of assays. Trichome density and cuticle thickness were measured and correlated with whitefly resistance to get information on the possible mechanism(s) of resistance.

Materials and Methods

Plant and whitefly material

Thirty-two accessions, including hot pepper, sweet pepper and wild relatives of pepper (four species), were screened at Wageningen UR Plant Breeding, the Netherlands in 2008. Thirty-two accessions, including 20 accessions from the 32 that were used in The Netherlands and 12 new accessions were screened for whitefly resistance under tropical conditions at PT East West Seed Indonesia, Purwakarta, West Java, Indonesia in October 2008. All 44 accessions were obtained from Centre for Genetic Resources – the Netherlands (CGN). Silverleaf whiteflies (*B. tabaci*, group Mediterranean-Middle East-Asia Minor I) were

obtained from the Department of Entomology (Wageningen University) and the Plant Pathology Department of Bogor Agricultural University. Sequence analysis was carried out on the of the mitochondrial cytochrome oxidase c subunit 1 (mtCOI) gene fragment (for details, see Frohlich *et al.* 1999) of individuals from the whitefly population used in the Netherlands and the one used in Indonesia. Although some sequence differences were found, they clearly were both biotype B (results not shown). The whitefly populations were reared on susceptible genotypes of tomato (the Netherlands) and eggplant (Indonesia).

No-choice test

Plant accessions were grown in an insect-proof glass house at 25 ºC, 16/8 h day/night and 60% humidity at Wageningen University and Research Centre, the Netherlands. There were two randomized blocks with two plants per accession in each block. After 3 months, young leaves at the 3rd or 4th node from the apex of each plant were cut at the petiole base and embedded in wet floral foam (Oasis). A clip-on cage containing five young female whiteflies was placed on the leaf (Maliepaard *et al.* 1995). Four days after inoculation, the clip-on cages were removed and the number of living females was counted. The number of eggs was counted under a stereo microscope. AS and OR were calculated per cage according to van Giessen *et al.* (1995) and subjected to an analysis of variance and Duncan's Multiple Range Test (DMRT), implemented in the software package SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). The equations of AS and OR are as follows:

$$AS = \left(\frac{m}{n}\right)^{1/d} \text{ day}^{-1} \qquad \text{and} \qquad OR = \frac{2e}{d(m+n)} \text{ eggs female}^{-1} \text{ day}^{-1}$$

where *e*, the number of eggs; *d*, the number of days (4 days); *n*, the number of females in leaf cages (five females); *m*, the number females alive after *d* days.

Free-choice tests

Free-choice tests were conducted at PT East West Seed Indonesia, Purwakarta and Lembang, West Java, Indonesia. Two kinds of free-choice test were used.

Detached-branch test. Pepper accessions were grown without the use of insecticides in an insect-proof screen house in the highlands (Lembang, West

Java, Indonesia). There were four plants per accession which were placed randomly. Three months after sowing, the pepper plants were cut, put in a cool container with 12–15 ºC and 60–70% humidity, and transferred to Purwakarta (West Java, Indonesia), where the conditions are suitable for a good bioassay. On the same day, a branch containing four young leaves in different stages were taken from each plant, embedded vertically in wet floral foam (Oasis), and subsequently placed in a water-containing glass jar. The branches of all accessions were placed randomly in two insect-proof screen cages, so there were two branches per accession in each cage. On the next day, they were infested by shaking heavily infested eggplants into the cages (at least 2000 whiteflies per cage). Five days after infestation, the whiteflies on the leaf undersides were counted directly. The leaves were taken to the laboratory to count the eggs. Leaf area was measured and every leaf area was divided by the smallest leaf area to be one unit leaf area. A pilot experiment was performed to determine the best place to count the whitefly eggs. We compared different parts of the leaf (tip, middle and basis). The results showed that *B. tabaci* prefers to deposit eggs at the base of the leaf, which is in line with literature (de Lima and Campos 2008). Therefore, we counted eggs at that place (three 1-cm^2). From the data obtained, we calculated the whitefly density (WD, number of whiteflies per leaf) and oviposition (ovi, number of eggs per square cm of leaf).

Screen house test. At least 15 seeds of each pepper accession were sown in trays in potting soil. Five weeks after sowing, pepper accessions were transplanted into a screen house and grown in soil in two randomized blocks with six plants per accession in each block. Plants were arranged 75 cm between rows and 45 cm between plants in a row. No pesticides were used and the plants were infested by releasing whiteflies by shaking heavily infested eggplant in between accessions (about 600–1000 whiteflies) 6 weeks after planting. The mesh used for the screenhouse was thought to be impermeable for whiteflies, but still some whiteflies were present in the screenhouse before infestation. One week after release of the whiteflies, three leaflets of the third, fourth and fifth leaves from the top were randomly collected from three plants in each block. Eggs and nymphs

were counted on each leaf under the stereo microscope. Leaf area was also measured.

Trichome counting and cuticle thickness measurement

Two leaflets of the fourth leaf from the apex in the detached branch test were used for trichome counting and cuticle measurement. The number of trichomes present on 1 cm^2 was counted at two sites at the basis of the abaxial leaf surface using a stereo microscope (40x). For cuticle thickness measurement, the leaf blade was inserted in polystyrene foam and excised as thin as possible with a razor. The sections were put on a glass slide and coloured with 0.1% safranin in glycerin-water (1 : 1) and then covered. The sections were observed and cuticle thickness was measured under a binocular microscope equipped with a micrometer.

Data analysis

To compare accessions, data on the number of adult, eggs and nymphs were transformed ($\sqrt{x+0.5}$) before the analysis of variance and means separation by DMRT (Steel and Torrie 1980) using SPSS 16.0 for Windows (SPSS Inc.). Prior to statistical analysis of the screen house data, the total number of eggs, the total number of nymphs and nymphal survival were calculated. The value for the total number of eggs was the sum of the number of eggs present, plus the number of nymphs. Nymphal survival was the total number of nymphs divided by the total number of empty eggs. To describe general preference of each accession in free-choice tests, scoring was done from 0 to 2 based on the average and significance of the individual parameters. The accession with the lowest average (the most resistant) and other accessions that were not statistically different from this accession were scored as 2. The susceptible control, *C. frutescens* Tabasco, and the accessions which were not significantly different from it were scored as 0. Accessions in between the two extremes were scored as 1. Scores of each accession for each parameter were used to compute the resistance value (R) according to the equation below:

$$R = \sum_{j=1}^{m} \sum_{i=1}^{n_j} \frac{X_{ij}}{X\max_{ij} .n_j .m}$$

where *R*, Resistance value; *Xij*, Score for parameter *i* using evaluation method *j* (in our case the results from the no-choice test, the detached branch test and the screenhouse test); *Xmaxij*, Maximum score for parameter *i* using method *j*; *nj*, Total number of parameters in method *j*; *m*, Total number of methods used; *i*, Parameters in the row of the matrix; *j*, Methods in the column of the matrix.

Resistance classification was based on the R value where 0–0.20 was classified as susceptible, 0.21–0.50 was classified as moderately susceptible, 0.51–0.80 was classified as moderately resistant, and 0.81–1.0 was classified as resistant. Pearson's correlation coefficient (Steel and Torrie 1980) between resistance parameters in no-choice and free-choice tests, between the resistance parameter (R) and trichome density and between the resistance parameter and cuticle thickness were calculated using linear correlation and regression program of SPSS 16.0 for Windows (SPSS Inc.).

Results

No-choice and free-choice test

The variance analysis of all parameters observed in the no-choice and free-choice tests showed that there were significant differences in whitefly preference between the pepper accessions (Tables 1 and 2). Large differences between accessions were observed for each parameter using DMRT (Tables 1 and 2).

The lowest AS in the no-choice test was 0.75 (one of four whiteflies dies each day) and was found on *C. annuum* California Wonder 300 and *C. chinense* No1720. However, this AS was not significantly different from several other accessions (Table 1). The OR on all accessions with low AS was also low with the exception of *C. annuum* Yolo Wonder L. Other accessions with a low OR were *C. annuum* Sweet Chocolate, *C. annuum* de Arbol, *C. annuum* Serrano, *C. baccatum* Aji Blanco and *C. baccatum* RU72-51. *Capsicum annuum* Serrano and *C. chinense* No 4661 had the highest AS and OR, respectively.

Table 1. Mean of whitefly adult survival (AS, surviving whiteflies.day^{-1}) and oviposition rate (OR = eggs.female^{-1}.day^{-1}) on pepper accessions in no-choice experiment

No	Acc No.	Acc Name	Clip-on Cage Test	
			AS*	OR*
1	CGN16922	*C. annuum* Sweet Chocolate	0.89 ± 0.06b-f	2.5 ± 1.1a-f
2	CGN16975	*C. annuum* AC 1979	0.92 ± 0.04c-f	3.5 ± 1.8c-i
3	CGN20503	*C. annuum* Bisbas	0.83 ± 0.05a-d	1.6 ± 1.1ab
4	CGN21534	*C. annuum* de Arbol	0.87 ± 0.08b-f	3.0 ± 1.2a-h
5	CGN22173	*C. annuum* Sweet Banana	0.92 ± 0.04c-f	3.8 ± 1.0d-j
6	CGN22830	*C. annuum* Serrano	0.96 ± 0.02f	3.2 ± 1.8a-h
7	CGN23222	*C. annuum* Keystone Res. Giant	0.90 ± 0.09b-f	4.0 ± 2.2e-j
8	CGN23289	*C. annuum* Long Sweet	0.84 ± 0.09a-e	2.3 ± 0.5a-e
9	CGN23765	*C. annuum* CM 331	0.86 ± 0.04b-f	1.3 ± 0.6a
10	PRI1996048	*C. annuum* Tit super	0.91 ± 0.04b-f	4.8 ± 1.3hij
11	PRI1996236	*C. annuum* Laris HS	0.82 ± 0.09abc	3.0 ± 1.1a-h
12	PRI1999049	*C. annuum* Jatilaba	0.93 ± 0.05def	4.1 ± 0.7e-j
13	PRI2004001	*C. annuum* (Bruinsma Wonder)	0.87 ± 0.08b-f	4.3 ± 1.0f-j
14	PRI2007007	*C. annuum* PBC 473	0.92 ± 0.05c-f	3.7 ± 1.4d-j
15	PRI2007008	*C. annuum* PBC 535	0.92 ± 0.05c-f	5.3 ± 0.8ij
16	CGN19189	*C. annuum* California W. 300	0.75 ± 0.07a	1.7 ± 1.7abc
17	CGN23098	*C. annuum* Yolo Wonder L	0.85 ± 0.05a-e	3.5 ± 1.0b-i
18	CGN17028	*C. baccatum*	0.91 ± 0.08c-f	4.1 ± 0.9e-j
19	CGN17042	*C. baccatum* No. 1553	0.80 ± 0.09ab	2.0 ± 0.4a-d
20	CGN21470	*C. baccatum* Aji Blanco	0.86 ± 0.06b-f	2.5 ± 1.7a-g
21	CGN23206	*C. baccatum* RU 72-51	0.91 ± 0.09c-f	2.0 ± 0.4a-d
22	CGN16994	*C. chinense* RU 72-194	0.92 ± 0.09c-f	4.5 ± 1.0g-j
23	CGN16995	*C. chinense* RU 72-241	0.94 ± 0.06ef	3.7 ± 1.3d-j
24	CGN17219	*C. chinense* No.4661 Selection	0.89 ± 0.03b-f	4.4 ± 0.6d-j
25	CGN21469	*C. chinense* AC 2212	0.86 ± 0.13b-f	4.1 ± 1.9e-j
26	CGN21557	*C. chinense* No.4661	0.88 ± 0.00b-f	5.6 ± 2.5j
27	CGN22829	*C. chinense* Miscucho Colorado	0.89 ± 0.06b-f	3.8 ± 2.3d-j
28	CGN22862	*C. chinense* No.1720	0.75 ± 0.07a	2.0 ± 0.8a-d
29	PRI1996108	*C. chinense* PI 281428	0.95 ± 0.05f	4.6 ± 0.6hij
30	PRI1996112	*C. chinense* PI 315023	0.90 ± 0.08b-f	4.2 ± 1.2e-j
31	CGN22817	*C. frutescens* L. Lombok	0.88 ± 0.05 b-f	3.5 ± 0.7c-i
32	CGN21546	*C. frutescens* L. Tabasco	0.93 ± 0.05 def	4.7 ± 1.5hij

Whitefly numbers, oviposition and nymphal density were measured in choice tests. In the detached branch test, the averages for WD and egg density and OR were lowest for *C. annuum* CM331, but it was not significantly different from several others (see Table 2). *C. annuum* CM331 also had the lowest egg density in the screenhouse test, but again it was not significantly different from several others. *Capsicum annuum* California Wonder 300 showed the lowest nymphal density and nymphal survival in the screenhouse test, but also it was not significantly different from several others. Based on the resistance value, *C. annuum* Serrano, *C. annuum* California W 300, *C. annuum* CM 331, *C. annuum* Gold California W and *C. chinense* AC 2212 were resistant. Most *C. annuum* species were moderately resistant, whereas most *C. chinense* and all species of *C. baccatum* and *C. frutescens* in this screening were classified as moderately susceptible or susceptible.

The parameters observed in the no-choice and free-choice tests were positively correlated and in most cases significant (Table 3). AS was moderately correlated with OR in the clipon cage test and the parameters in screenhouse test; however, there was no correlation with the parameters in detached-branch test. The OR observed in the clip-on cage test strongly correlated with oviposition in detached-branch test and oviposition and nymphal density in the screenhouse test. High correlations were also found between parameters in detached-branch test and screenhouse test. Even though positive correlations were found between parameters in no-choice and choice test, several accessions showed differences between the no-choice and free-choice test (Tables 1 and 2). *Capsicum annuum* Serrano and *C. chinense* AC 2212 were susceptible in no-choice test but not preferred in the choice test.

Trichome density and cuticle thickness

Trichome density and cuticle thickness were measured because these factors might play a role in the resistance of pepper to whiteflies. Significant differences in trichome density and type, as well as cuticle thickness, were found among the pepper accessions (Table 2). Trichome density ranged widely from eight trichomes.cm^{-2} (*C. annuum* Gold California Wonder) to 507 trichomes.cm^{-2} (*C. chinense* PI 315023). The thinnest cuticle averaged 3.18 μm (*C. chinense* PI 315023), and the thickest cuticle averaged 7.82 μm (*C. annuum* Gold California W.; Table 2). Glandular trichome density was negatively correlated with adult WD, eggs density and OR, whereas non-glandular trichome density was positively correlated with them (Table 4). Cuticle thickness was negatively correlated with WD, egg density and OR (Table 4).

Table 2. Mean of whitefly resistance parameters, value of resistance index (RI), resistance level (R class) and leaf characteristics for pepper accessions.

No	Acc No.	Acc Name	Detached Branch Test			Screenhouse Test			RI	R Class	Trichome Density (trichomes.cm^{-2})	Cuticle Thickness (µm)
			WD	Ovi	OR	Ovi	ND	NS				
1	CGN16922	*C. annuum* Sweet Chocolate	3.0±0.8 ef	0.6±0.4 b-e	0.21±0.16 abc	4.5±1.1 g	3.7±0.7 h	0.83±0.07 de	0.50	MS	47.3± 5.6 c-g Φ	5.9±0.1 l
2	CGN16975	*C. annuum* AC 1979	3.3±1.3 efg	4.8±0.4 h	1.64±0.56 de	nd	nd	nd	0.50	MS	34.0± 4.3 bc Ψ	5.1±0.0 hi
3	CGN20503	*C. annuum* Bisbas	11.8±2.1 l	9.8±0.29 i	0.85±0.15 bcd	0.7±0.2 abc	0.4±0.1 abc	0.52±0.17 bc	0.67	MR	43.8± 3.4 b-f Ψ	4.1±0.2 d
4	CGN21534	*C. annuum* de Arbol	2.0±0.8 cde	0.2±0.4 a-d	0.19±0.38 abc	0.3±0.2 a	0.2±0.1 ab	0.88±0.25 ef	0.75	MR	46.5±14.0 c-g Φ	5.2±0.1 hij
5	CGN22830	*C. annuum* Serrano	1.0±0.8 abc	0.3±0.4 a-d	0.25±0.43 abc	nd	nd	nd	1.00	R	36.3± 1.3 bc ξ	5.0±0.1 gh
6	CGN23765	*C. annuum* CM 331	0.3±0.5 a	0.0±0.0 a	0.00±0.00 a	0.2±0.2 a	0.1±0.1 a	0.50±0.50 b	0.92	R	78.5± 6.6 jkl ξ	5.0±0.1 gh
7	PRI2007008	*C. annuum* PBC 535	7.0±2.2 ij	21.6±1.4 l	3.40±1.41 g	12.8±4.1 i	9.6±0.8 j	0.81±0.25 cd	0.17	S	135.3± 6.2 n Ψ	4.7±0.1 f
8	CGN16842	*C. annuum* L California Wonder	4.0±0.8 fgh	2.5±0.4 fg	0.65±0.17 abc	2.7±0.8 ef	2.2±0.4 fg	0.82±0.14 cd	0.50	MS	49.0±11.0 c-g Φ	7.4±0.1 q
9	CGN16873	*C. annuum* Kalifornische F.	12.3±1.0 l	0.8±0.5 de	0.06±0.04 ab	-	-	-	0.67	MR	27.8± 2.9 b Φ	5.7±0.1 kl
10	CGN16911	*C. annuum* Mild California	1.0±0.8 abc	0.2±0.2 a-d	0.08±0.14 abc	0.6±0.3 ab	0.6±0.4 bc	0.80±0.45 cd	0.75	MR	61.0±19.6 f-i Φ	7.3±0.1 pq
11	CGN19189	*C. annuum* California W. 300	1.5±0.6 bcd	0.2±0.4 a-d	0.25±0.35 abc	0.6±0.5 ab	0.1±0.2 a	0.07±0.15 a	0.92	R	63.0±10.1 g-j Φ	7.6±0.0 r
12	CGN20796	*C. annuum* Wunder v Kal.	1.0±0.8 abc	0.3±0.4 a-d	0.33±0.58 abc	2.2±0.5 ef	1.6±0.3 ef	0.73±0.18 cd	0.67	MR	40.5± 7.6 bcd Φ	7.7±0.1 r
13	CGN22120	*C. annuum* Gold California W.	2.5±1.3 de	0.3±0.2 a-d	0.12±0.10 abc				0.83	R	8.0± 0.8 a Φ	7.8±0.1 r
14	CGN22163	*C. annuum* Riesen v. Kal.	7.0±0.8 ij	3.0±0.4 fg	0.44±0.12 abc	0.8±0.2 a-d	0.6±0.1 bcd	0.77±0.14 cd	0.58	MR	62.3± 7.2 gj Φ	7.1±0.1 p
15	CGN16874	C. annuum Yolo Wonder 31-22	1.0±0.8 abc	1.1±0.2 e	0.96±0.32 cd	nd	nd	nd	0.67	MR	11.3± 1.3 a Φ	6.8±0.1 o
16	CGN21543	*C. annuum* Yolo Wonder Imp.B	7.0±0.8 ij	2.3±0.5 f	0.32±0.08 abc	nd	nd	nd	0.67	MR	59.3± 5.0 e-i Φ	6.5±0.0 n
17	CGN22852	*C. annuum* Yolo Wonder A	8.3±0.5 jk	0.1±0.3 abc	0.02±0.03 ab	1.8±0.8 de	1.2±0.6 de	0.68±0.12 cd	0.58	MR	71.0±18.6 h-k Φ	6.8±0.1 o
18	CGN22853	*C. annuum* Yolo Wonder B	2.3±0.5 de	0.1±0.2 ab	0.03±0.06 ab	1.5±0.4 b-e	0.8±0.4 cd	0.56±0.23 cd	0.58	MR	95.8± 2.9 m Φ	6.7±0.1 o
19	CGN23098	*C. annuum* Yolo Wonder L	1.5±0.6 bcd	0.3±0.4 a-d	0.25±0.35 abc	2.3±0.6 ef	1.7±0.4 ef	0.75±0.08 cd	0.58	MR	76.3± 8.3 i-l Φ	5.6±0.1 k
20	CGN23099	*C. annuum* Yolo Y	0.8±0.5 ab	0.1±0.3 abc	0.17±0.29 abc	1.6±0.5 cde	1.2±0.4 de	0.71±0.04 cd	0.67	MR	73.5± 3.1 i-l Φ	6.4±0.1 n
21	CGN23249	*C. annuum* Yolo Wonder	4.8±0.5 gh	0.7±0.5 c-e	0.15±0.10 abc	1.1±0.7 bcd	0.9±0.4 cd	0.83±0.17 de	0.50	MS	86.0±25.1 klm Φ	6.1±0.1 m
22	CGN17042	*C. baccatum* No. 1553	4.8±1.5 gh	13.1±1.3 j	3.07±1.38 fg	50.4±5.0 l	37.7±3.3 m	0.75±0.05 cd	0.17	S	123.5± 3.4 n Ψ	5.3±0.1 j
23	CGN23206	*C. baccatum* RU 72-51	10.0±1.8 kl	6.0±0.1 h	0.62±0.14 abc	28.0±2.8 k	18.9±2.9 l	0.68±0.15 cd	0.33	S	278.0± 7.6 p ξ	5.3±0.0 ij
24	CGN16994	*C. chinense* RU 72-194	5.5±1.3 hi	3.3±0.8 g	0.62±0.13 abc	3.4±0.3 fg	3.3±0.3 h	0.97±0.04 f	0.50	MS	42.5± 3.4 b-e ξ	4.8±0.1 fg
25	CGN16995	*C. chinense* RU 72-241	7.3±1.7 ij	20.9±1.1 l	3.02±0.80 fg	3.1±0.5 fg	2.2±0.4 fg	0.73±0.11 cd	0.33	S	37.3± 0.5 bcd ξ	4.4±0.1 e
26	CGN17219	*C. chinense* No.4661 Selection	17.3±1.7 m	11.6±0.7 j	0.68±0.05 abc	15.9±4.4 j	14.6±2.7 k	0.95±0.21 f	0.25	S	163.5±15.4 o ξ	4.1±0.1 d
27	CGN21469	*C. chinense* AC 2212	6.8±1.3 ij	0.2±0.2 a-d	0.03±0.04 ab	nd	nd	nd	0.83	R	35.8± 3.3 bc Φ	3.5±0.0 b
28	CGN21557	*C. chinense* No.4661	21.0±2.2 n	48.4±5.2 n	2.34±0.49 ef	nd	nd	nd	0.00	S	394.5±30.5 q ξ	4.5±0.1 e
29	CGN22829	*C. chinense* Miscucho Colorado	20.0±0.8 mn	11.4±1.3 i	0.57±0.08 abc	nd	nd	nd	0.50	MS	132.5± 6.6 n ξ	3.7±0.2 c
30	PRI1996108	*C. chinense* PI 281428	4.0±0.8 fgh	17.4±2.3 k	4.52±1.19 h	10.6±2.5 i	6.7±2.2 i	0.67±0.33 cd	0.17	S	89.3± 9.8 m ξ	3.4±0.1 ab
31	PRI1996112	*C. chinense* PI 315023	36.5±2.4 o	73.6±5.4 o	2.02±0.15 e	5.0±3.1 g	2.9±1.0 gh	0.72±0.34 cd	0.17	S	506.8±13.6 r ξ	3.2±0.1 a
32	CGN21546	*C. frutescens* L. Tabasco	18.3±2.4 mn	30.8±2.4 m	1.70±0.25 de	7.0±1.0 h	5.4±0.7 i	0.77±0.07 cd	0.08	S	54.5± 6.8 d-h Ψ	3.8±0.0 c

WD = whitefly density (whiteflies.leaf^{-1}); Ovi = Oviposition (eggs.cm^{-2}); OR = oviposition rate (eggs.whitefly^{-1}); ND = nymphal density (nymphs.cm^{-2}); NS = nymphal survival (surviving nymphs per hatching eggs). Means followed by standard deviation and different letters within columns are different by Duncan's multiple range test in 0.05 *P* significance. *p-value* of variance analysis for all parameters is less than 0.01. In the list of trichomes, the means are also followed by the type of trichome (ξ = nonglandular trichome, Φ = glandular trichome, and Ψ = both nonglandular and glandular trichomes); (S = susceptible if R index is less than or equal to 0.2, MS = moderately susceptible if R index is in between 0.21-0.5, MR = moderately resistant if RI is in between 0.51 – 0.8, and R = resistant if R index is larger than 0.8).

Table 3. Pearson's correlation coefficient between parameters used for whitefly resistance screenings including adult survival (AS), oviposition rate (OR), whitefly density (WD), oviposition (Ovi), nymphal density (ND), and nymphal survival (NS).

Screening methods		Clip-on Cage Test	Detached Branch Test		Screenhouse Test			
	Resistance Parameter	OR	WD	Ovi	OR	Ovi	ND	NS
Clip-on Cage Test	AS	.676**	.174	.298	.328	.608*	.630*	.655**
	OR	1	.510*	.631**	.468*	.804**	.816**	.609*
Detached Branch Test	WD		1	.753**	.299	.493*	.462*	.212
	Ovi			1	.786**	.674**	.608**	.169
	OR				1	.654**	.560*	.089
Screenhouse Test	Ovi					1	.988**	.396
	ND						1	.564*

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

Table 4. Pearson's correlation coefficient between parameters observed in detached branch test with pepper leaf characteristics. WD = whitefly density; Ovi = Oviposition; OR = oviposition rate

Resistance Parameters	WD	Ovi	OR
Glandular trichome density	-.406*	-.606**	-.525**
Nonglandular trichome density	.713**	.752**	.423*
Cuticle thickness	-.600**	-.663**	-.507**

** correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed)

Discussion

Different test show highly similar results for whitefly resistance in pepper

Breeders are always in search of simple evaluation methods that can be carried out efficiently and at the same time deliver meaningful results. To avoid contamination of other plant material, small-scale experiments at laboratory scale are preferred. To this purpose, we tested three different screening methods for evaluating whitefly resistance in pepper: methods involving intact plants (free-choice, no-choice) and detached branch (free-choice) assays. The results from the different tests were well correlated (Table 3), indicating that meaningful results are obtained. This is especially true for parameters related to oviposition. The strong correlation of the ORs measured with different methods shows that

oviposition is a good and reliable parameter. Furthermore, the high correlation between parameters in detached-branch test and screenhouse test show that the detached-branch test can be used as a quick and efficient method to evaluate whitefly preference either in small or in large-scale evaluations. Similar observations were made by Maharijaya *et al.* (2010) for thrips resistance in pepper.

Evaluating pepper germplasm for whitefly resistance

Large differences in whitefly resistance and/or preference levels were observed between accessions of pepper. In the no-choice test, the low AS and/or OR on *C. annuum* California Wonder 300, *C. chinense* No. 1720, *C. annuum* CM331 and *C. annuum* Bisbas show that these accessions have mechanisms to defend themselves against whiteflies. The plants might produce toxic compounds to oppose whiteflies and/or have physical barriers that prevent feeding (van Emden 2002). Also there are differences in egg-laying preference (Table 1). Oviposition is an element of the whitefly life cycle that is very difficult to control with biological control and/or any kind of pesticides, so decreasing whitefly oviposition would be helpful in whitefly management.

In the choice situation, several accessions of *C. annuum* and *C. chinense* AC 2212 were not preferred, whereas several others supported large populations of whiteflies. Whiteflies prefer the most suitable host and leave the non-preferred plants. Large differences were seen between the most preferred and the least preferred accessions in the choice tests. There are several factors that might contribute to whitefly preference. Host choice can be affected by leaf architecture and colour (Sippell *et al.* 1987), leaf pubescence (McAuslane 1996), cuticle thickness (Channarayappa *et al.* 1992b) and metabolites that play a role as repellent or attractant (Chermenskaya *et al.* 2009). Whiteflies choose the most suitable host for oviposition not only because they can feed on it, but also because the offspring should be able to survive (Nomikou *et al.* 2003). Oviposition preference and host plant selection by the female whitefly has a profound effect on the fitness of its offspring (van Lenteren and Noldus 1990). This phenomenon was seen in this screening test, where adult WD correlated with OR and OR correlated with nymphal density.

For several accessions, different resistance levels were recorded in the no-choice and free-choice tests. There were accessions that were susceptible in the no-choice test, but not preferred in a free-choice test. The difference could be due to the plants producing repellents or plants that have physical barriers resulting in avoidance by the whitefly. Also, it may be due to the attractiveness of other accessions in choice situations even though *B. tabaci* could live on the nonpreferred accession.

Trichome density and cuticle thickness as factors affecting whitefly resistance

The high positive correlations of non-glandular trichome density with WD and OR suggest that trichome density is associated with whitefly preference. These correlations are similar to what was found in tomato (Toscano *et al.* 2002), cotton (Flint and Parks 1990) and soybean (McAuslane 1996). Whiteflies prefer to lay eggs on hairy leaves rather than on leaves without trichomes. Non-glandular trichomes provide a more suitable microclimate for oviposition (Butter and Vir 1989). Furthermore, in tomato, glandular trichome exudates deter natural enemies of the whitefly, while the whitefly body is (partly) protected from the exudates by wax (Walling 2008).

Both types of trichomes were found in pepper. The glandular trichomes are much smaller than the non-glandular ones, and they were found on the majority of the sweet pepper accessions that were classified as resistant or moderately resistant. In cases where no glandular trichomes were present, such as in most *C. chinense* species, the accessions were susceptible to whiteflies. These observations suggest an important role for glandular trichomes in whitefly resistance. Trichome exudates can also be toxic and/or repellent to the whitefly itself (Mutschler *et al.* 1996). Secondary metabolites in pepper leaves, such as the ones produced in trichomes, may play an important role as oviposition deterrent, as was shown for leaf miners (*Liriomyza trifolii*) (Kashiwagi *et al.* 2007). However, glandular trichomes alone do not necessarily determine resistance or susceptibility. Accessions, such as *C. annuum* PBC 535, *C. baccatum* No. 1553 and *C. frutescens* L. Tabasco, have glandular trichomes but were susceptible. Non-glandular trichomes were found on most susceptible plants with the

exception of *C. annuum* CM331, which has only non-glandular trichomes and was still highly resistant. These differences show the diversity of whitefly resistance mechanisms among pepper accessions.

Cuticle thickness was negatively correlated with WD and OR, which suggests that it is an important factor for whitefly non-preference in pepper, especially in sweet peppers. Thick cuticles prevent the stylet from being inserted into the epidermis and reaching the phloem bundle (Janssen *et al.* 1989). Such resistance mechanisms have been reported in vegetables such as tomato, cotton and cassava to different kinds of phloem-feeding/piercing insects (Eigenbrode and Espelie 1995, Bellotti and Arias 2001, Jindal *et al.* 2008).

In conclusion, we have identified pepper accessions that differ in whitefly resistance and preference. Whitefly resistance and preference seem to be present in the germplasm evaluated, and this offers opportunities for doing genetic studies and breeding whitefly-resistant pepper varieties. We have identified *C. annuum* accessions CM331, Seranno and California Wonder 300 as the most promising. OR seem to be a good parameter for determining resistance.

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Chapter 4

Resistance to *Bemisia tabaci* in Tomato Wild Relatives

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Abstract

Bemisia tabaci is one of the most threatening pests in agriculture, particularly in Solanaceous crops such as tomato and pepper that are cultivated the open field in Indonesia. Pesticide application is often not effective and can be hazardous to humans and environment. The exploitation of plant natural defenses that are present in wild relatives of tomato, may offer a solution. To evaluate resistance parameters and to identify plant material with high levels of resistance, we screened a number of accessions of tomato wild relatives using three methods; a free-choice test in a screenhouse in Indonesia, a no-choice test with clip-on cages in a greenhouse and a leaf disc test in a climate-room in the Netherlands. Antibiosis resulting in low adult survival was the major component for resistance in tomato. However, other resistance component(s) may play a role as well. In some accessions there was a change in the resistance level over time. Several resistance parameters used in the different tests were well correlated. The best resistance source was an accession of *Solanum galapagense*, which had not been identified as being resistant in the past. This is of particular interest as this species is closely related to the cultivated tomato, which may facilitate introgression of the resistance component(s). Whitefly non-preference and resistance were associated with the presence of type IV trichomes. Other mechanisms might be involved since some accessions without type IV trichomes showed low nymphal density. The leaf disc test is a good *in vitro* alternative for the clip-on cage whitefly resistance screening, as shown by the high correlation between the results obtained with this test and the clip-on cage test. This offers breeders the possibility to carry out tests more efficiently.

Keywords: *Solanum*, whitefly resistance, trichome, antibiosis, antixenosis

Introduction

Bemisia tabaci (Gennadius) is one of the most important pests in agricultural crops worldwide. This whitefly is responsible for large reductions in crop yield and quality. Consequently, high costs are made for controlling it (Morales 2007). *Bemisia tabaci* causes direct damage by feeding on the phloem sap and it produces honeydew on which sooty molds can grow (Byrne and Miller 1990). This may result in physiological disorders of the plant, such as leaf wilting and irregular ripening of the fruit (Schuster *et al.* 1990b; McCollum *et al.* 2004). However, the main problem caused by *B. tabaci* is the damage done by the plant viruses they transmit (Morales and Jones 2004).

Tomato cultivation and production, particularly in tropical countries is highly dependent on pesticides. However, pesticides can be hazardous for the environment, growers and consumers. The exploitation of whitefly resistance originating from wild relatives of cultivated tomato is anticipated to be a more sustainable way of controlling whiteflies (Broekgaarden *et al.* 2011). Different levels of whitefly resistance have been reported for wild relatives of tomato including *S. pennellii, S. habrochaites, S. habrochaites* f. *glabratum, S. pimpinellifolium, S. chilense* (Maliepaard *et al.* 1995; Fancelli and Vendramim 2002; Toscano *et al.* 2002; Muigai *et al.* 2002, 2003; Maruthi *et al.* 2003; Baldin *et al.* 2005).

Whiteflies prefer hairy leaves (Toscano *et al.* 2002), but the presence and density of type IV and VI trichomes has a negative effect on whitefly adult survival and oviposition rate (Channarayappa *et al.* 1992a; Snyder *et al.* 1998). Exudates of these trichomes play a major role in whitefly resistance (Fancelli *et al.* 2005). Compounds implicated in whitefly resistance are acyl-sugars (Liedl *et al.* 1995; Mutschler *et al.* 1996), methyl-ketones and derivates of the sesquiterpene carboxylic acid (Frelichowski and Juvik 2005), which might act as repellants and/or natural pesticides (Bleeker *et al.* 2009).

Mapping studies have identified Quantitative Trait Loci (QTLs) for reduced oviposition of *Trialeurodes vaporariorum* on chromosomes 1 and 12 in *S. habrochaites* f. *glabratum* (Maliepaard *et al.* 1995). Five QTLs for acyl sugars

that confer whitefly resistance in *S. pennellii* LA176 were identified on chromosomes 2, 3, 4 and 11 (Mutschler *et al.* 1996). *Solanum habrochaites* LA1777 was also the source for QTLs showing a reduced egg deposition; these were located on the chromosomes 9, 10 and 12 (Momotaz *et al.* 2010). However, the combined effects of these QTLs explained only part of the variation present for whitefly resistance (Maliepaard *et al.* 1995; Lawson *et al.* 1997; Momotaz *et al.* 2010). From these results it was concluded that many genes might be involved in the whitefly resistance. When many genes/regions are involved the introduction of the resistance trait from the wild relatives into commercial cultivars is often difficult. Therefore, the identification of genes with a major effect on resistance is of utmost importance. Preferably these resistance components should be present in close relatives of the cultivated tomato as introgression of the resistance is easier in these cases (Hogenboom 1972).

Two types of assessments are used to evaluate whitefly resistance: free-choice tests and no-choice tests (Romanow *et al.* 1991; Erb *et al.* 1994). In a free-choice test, whiteflies are given the choice between two or more different hosts of which it is able to choose the most preferred host(s). In a no-choice test, only one host is accessible for the whitefly and whiteflies that cannot feed on it will be hampered in their growth or die. Therefore, both antibiosis and antixenosis, which may result from repellence or attraction of whiteflies, is assessed in free-choice tests, whereas no-choice tests much more assess antibiosis (Baldin and Beneduzzi 2010). Reliable parameters for whitefly resistance assessments are very important. Parameters used to describe resistances are density and/or survival of a particular developmental stage of whitefly including adults, eggs or nymphs (Maliepaard *et al.* 1995; Fancelli and Vendramim 2002; Maruthi *et al.* 2003). Those parameters might measure similar or different resistance factors. Furthermore, relationships between resistance parameters and other supposedly related parameters like honeydew production, sooty-mold growth and plant damage have not been evaluated yet. Also, the development of whitefly resistance during the growth of the tomato plant has not been analyzed. Therefore, the objectives of the present study were to evaluate methods and resistance parameters used for whitefly

resistance screening and to identify plant material that has high levels of resistance, preferably based on different mechanisms.

Materials and Methods

Plant and whitefly material

Forty-six accessions of tomato and related wild species were obtained from the Centre for Genetic Resources (CGN) and the collection of Plant Research International (PRI) – The Netherlands, the Asian Vegetable Research and Development Center (AVRDC) – Taiwan and PT East West Seed Indonesia (EWSI) – Indonesia. If clear differences were seen between individuals of one accession, they were considered as different accessions and they were given an individual number. This made the total number of evaluated accessions 52. Twenty six accessions (Table 1) were screened in 2008 under free-choice conditions in a screen house at EWSI, Purwakarta, West Java, Indonesia. Nine accessions (both resistant and susceptible) together with 26 until then unscreened accessions (Table 2) were evaluated under no-choice conditions using clip-on cages at Wageningen UR Plant Breeding, the Netherlands in 2009.

Non-viruliferous silverleaf whiteflies (*B. tabaci*, Mediterranean-Middle East-Asia Minor I), from the collection of the Laboratory of Entomology, Wageningen University – the Netherlands or the Plant-Pathology Department of Bogor Agricultural University – Indonesia, were used for screening.

Free-choice test

Twenty-six tomato accessions were evaluated using a free-choice test in an insect proof screenhouse. The experiment was conducted from September until December 2008 at EWSI. The screenhouse protected the plants from outside insects, heavy rainfall as well as strong sunlight. Seeds were sown in insect-free boxes and moved to clean cages after one week. One month later, three plants which had four or more shoots were selected from each accession. Four shoots of each selected plant were grafted onto two week old eggplants to avoid root diseases such as *Fusarium* wilt and nematodes. Two weeks after grafting, the plants were transplanted into a four liter-black bucket containing a rice husk and peat moss soil mixture. Four grafted plants originating from one original plant of

each accession were placed in a square together on a table one meter above ground level. There were two lines on each table with 35 cm between lines, 20 cm between plants within the line and 100 cm between tables. The plants were supported by a bamboo stick. Branches and flowers were pruned in order to one main stem. Two amaranth plants were placed in between every accession (Figure 1). There were three replications (derived from three individual plants) for each accession in the screenhouse. Five weeks after grafting, virus-free *B. tabaci* were introduced by placing heavily infested eggplants in the middle of the plants of each accession. During one week, the eggplants were shaken twice a day and left without watering. In this way, the whitefly adults were forced to look for other plants because the eggplants desiccated and died after 6 days (Muigai *et al.* 2003). The whitefly population development was studied by counting the number of adult whiteflies, eggs and nymphs, and also registering the whitefly related parameters including honeydew production, sooty-mold growth and plant damages at three different time points. The first evaluation was carried out on day 8 and 9 after infestation, the second evaluation was on day 22 and 23 and the third evaluation was on day 36 and 37. The number of adult whiteflies was determined by counting directly on the abaxial of lateral leaflets on the 3rd or 4th and 7th or 8th leaves from the apex; this direct counting is more reliable (less variance) than beating of the plant and counting in a tray (Gusmao *et al.* 2005). Egg and nymph numbers were determined on the same leaflets as where the adults were counted. The leaflets were cut from the plant to facilitate egg and nymph counting under a stereo microscope (10x). Also the leaf area was measured. Honeydew production, sooty-mold growth and plant damages were visually scored on a scale of 0 to 4. Scores used for honeydew production were (0) no honeydew, (1) one to five honeydew droplets on one leaf, (2) honeydew present on two or more leaves, (3) severe honeydew (more than five honeydew droplets per leaflet) present on one or two leaves, (4) severe honeydew present on three or more leaves. Scores for sooty-mold growth were (0) no sooty mold, (1) some sooty mold present on one leaf, (2) sooty mold present on two or more leaves, (3) heavy sooty mold (thick and covering more than 10% of leaflet area) present on one or two leaves and the others show no or a bit sooty mold, (4) heavy sooty mold present on three or more

leaves. Scores for plant damages were (0) no necrosis or wilting, (1) light necrosis or wilting present of one leaf, (2) light necrosis or wilting present of two or more leaves, (3) heavy necrosis or wilting (more than 30% of leaf area) of two leaves but the plant is still growing, (4) heavy necrosis or wilting of three or more leaves and plant growth is inhibited or the plant is dead.

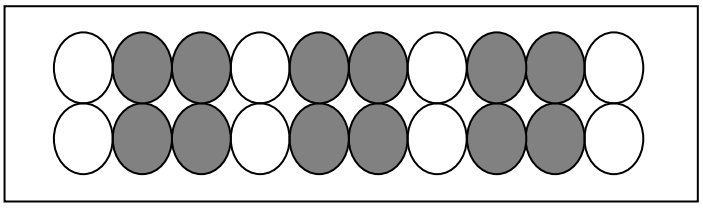

Figure 1. Setup in the screenhouse, four grafts of one individual plant of an accession are grown together (grey) and are separated from four plants of the next accession by two amaranth plants (white). Whitefly infested leaves were put in between the four grafts.

Data on adults, eggs, nymphs and leaf area in the free-choice test were used to determine adult-whitefly density (number of adult whiteflies.cm^{-2} of leaf), egg density (number of eggs.cm^{-2}), and nymphal density (number of nymphs.cm^{-2}). Log transformation was used to normalize adult-whitefly density data and transformation for egg and nymphal density.

No-choice tests

No-choice tests were carried out in April and May 2009 by using clip-on cages (Maliepaard *et al.* 1995) and leaf discs in Wageningen, The Netherlands. Seeds of each tomato accession were sown in peat-moss soil in a sowing box and after the third leaf stage, the plants were transplanted into 1.5 L pots containing peat-moss soil and placed on stainless steel tables in two randomized blocks (compartments) with one plant per accession per block. The temperature in the compartments was set to 20 ºC/15 ºC (day/night), a 16L:8D photoperiod was used and relative humidity was kept at 70%. One week before infestation, the temperature was raised gradually until it reached 27 ºC/18 ºC (day/night) two days before infestation. The plants were ready to be tested with clip-on cages and leaf discs.

Clip-on cage test.

Plants were infested with whiteflies six weeks after sowing. Five synchronized whitefly females (one to two days old) (*n*) were anesthetized with CO_2 and put into a clip-on cage (2 cm in diameter and 1 cm in height) and placed immediately on the abaxial of a leaflet of the 3rd or 4th leaf from the apex. Five clip-on cages were attached per plant. Four days after infestation (*d*) the clip-on cages were removed from the leaves and the dead and living whiteflies (*m*) were counted. The number of eggs (*e*) was counted under a stereo microscope (10x magnifications). The clip-on cages were reassembled at their original positions on each leaflet before new adult whiteflies started to emerge from the eggs. The emerging adults (a_i) were counted and removed from the cages every day (t_i) during a week. Pupal cases (*p*) were counted seven days after the first-emerging adult whiteflies. Adult survival (AS), oviposition rate (OR), pre-adult survival (PS) and development periods (DP) were calculated by using the equations of Maliepaard *et al.* (1995)as shown below.

$$AS = \left(\frac{m}{n}\right)^{1/d} \text{ day}^{-1} \qquad \text{(Formula 1)}$$

$$OR = \frac{2e}{d(m+n)} \quad \text{eggs.female}^{-1}\text{.day}^{-1}$$

(Formula 2)

$$PS = p/e \text{ whiteflies.egg}^{-1} \qquad \text{(Formula 3)}$$

$$DP = \frac{\sum(t_i.a_i)}{\sum a_i} \text{ days}$$

(Formula 4)

An Arcsin transformation was used to normalize data of adult survival and pre-adult survival data, and a square-root transformation for oviposition rate and development period.

Leaf disc test.

Nine accessions, selected on the basis of adult survival and oviposition rate in the clip-on cage test were used for the leaf disc test. One week after whitefly infestation in the clip-on cages test, four young leaflets at the 3rd, 4th or 5th node from the top were cut from each accession and put on a petri dish containing 1% agar and covered with paper which had four symmetrical holes (Figure 2). Each hole was two cm in diameter and therefore of the same size as the area under the

clip-on cage. Twenty synchronized whitefly females (one to two days old) were anesthetized with CO_2 and placed on the paper, the disc was closed with a cage (eight cm in diameter and six cm in height) containing nylon mesh (for air circulation). The cages were reversed and placed in the climate room at 27 ºC, a relative humidity of 70% and a photoperiod of 16L:8D. Alive and dead whiteflies as well as egg number were counted four days after infestation. The test was done in three replications. Adult survival and oviposition rate were calculated and normalized in the same way as in the clip-on cage test.

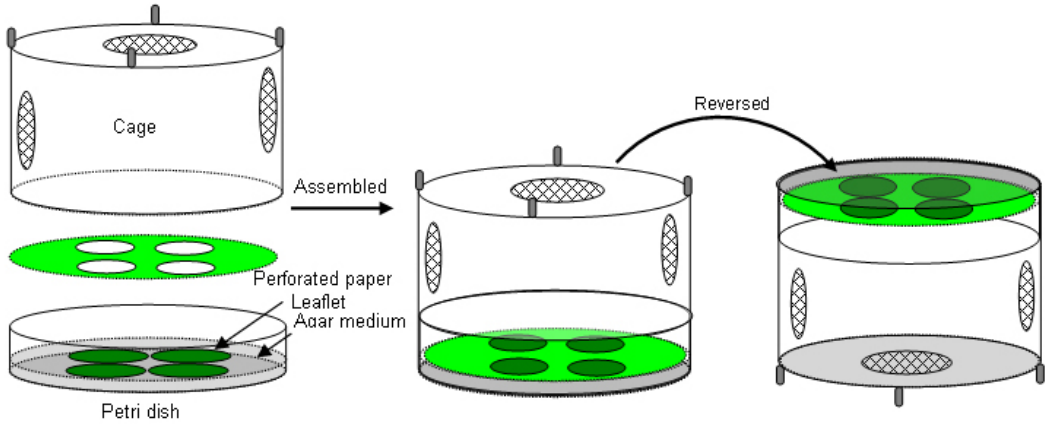

Figure 2. Experimental setup for the leaf disc test.

Observation of trichomes

Classification and identification of trichome types were made according to Luckwill (1943) based on the morphology and presence of glands. The leaflet opposite of the leaflet with the clip-on cage was taken from the plant. The trichomes were identified and counted in an area of 11.11 mm^2 (1/3 cm x 1/3 cm) on the right and left side of main vein at the leaflet base using a stereo dissecting microscope (40 to 100x magnifications).

Statistical analysis

Pearson's r correlation coefficients were calculated between whitefly parameters in both no-choice and free-choice tests and between whitefly resistance parameters and trichome density in the no-choice test. Spearman's rho correlation was used to calculate the correlation between honeydew production,

sooty-mold growth and plant damage in the free-choice test. Data were also subjected to two-way repeated measurement analysis of variance for free-choice test and analysis of variances for no-choice tests. Afterward, mean differentiation by Duncan's Multiple Range Test (DMRT) for accession and Least Significant Difference (LSD) for observation time of each accession. Statistical analysis was conducted with the software package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL).

Results

Whitefly resistance in free-choice test

In the free-choice test, several parameters were measured including adult-whitefly, egg and nymphal density at three successive time points as well as honeydew production, sooty-mold growth and plant damage. Table 3 shows the Pearson's r correlations between the different parameters. The data underlying these can be found in the Supplementary tables 4.1 to 4.4. Significant correlations were named slight if r = 0.20 – 0.40 moderate from 0.41 to 0.60, high from 0.61 – 0.80 and very high from 0.81 – 1.00. Adult-whitefly density was moderately correlated with egg and nymphal density, whereas, egg and nymphal density were very highly correlated with each other. Honeydew production was highly correlated with nymphal density, but only slightly with adult-whitefly and moderately with egg density. Sooty mold growth was highly correlated with plant damage, but both sooty mold growth and plant damage slightly or moderately correlated with the other parameters.

Table 1 shows for all accessions the development of adult and egg density over time in the free-choice test. The results of nymphal density, honeydew production, sooty-mold growth and plant damage are presented in the Supplementary tables 4.1 to 4.4. Adult-whitefly and egg density on most tomato accessions changed over time. For instance, the number of adults decreased sharply over time for *S. arcanum* CGN14355 and *S. lycopersicum* EWSI24294, whereas, the number of adults increased on most *S. habrochaites* and *S. pimpinellifolium* accessions. Furthermore, egg density also decreased on *S. cheesmaniae* CGN17086, *S. neorickii* CGN15816 and CGN15815, whereas egg density for all *S. habrochaites* accessions developed the other way around. The

changes mostly occurred between the first and second observation time and less between the second and third. The change can also be seen from the correlation between the three time points. The correlation of adult-whitefly density between first and second observation time was 0.454 (N = 75), between first and third observation time 0.413 (N = 75) and the correlation between second and third observation time was 0.931 (N = 75). The correlations between observations for the other parameters are presented in Supplementary Table 4.5.

Table 1. Means of adult whitefly density (whitefly.cm^{-2}) and egg density (egg.cm^{-2}) in the resistance screening of tomato accessions under free-choice condition; the mean followed by different letters in the parenthesis within columns are different according to Duncan's multiple range test and different letters in the brackets within lines are different according to Fisher's student test in 0.05 *p*-significance.

No	Accession Name	Observation time*					
		Adult whitefly density			Egg density		
		1	2	3	1	2	3
1	*S. galapagense* PRI95004/PY-8027	0.0 (a) [a]	0.1 (a) [a]	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]	0.5 (a) [a]
2	*S. galapagense* PRI95004/PY-8030	0.5 (e) [b]	0.5 (cde) [b]	0.3 (def) [a]	4.4 (d) [a]	8.1 (d) [ab]	8.4 (cd)[b]
3	*S. cheesmaniae* CGN15916	2.1 (jk) [a]	2.4 (k) [a]	1.4 (o) [a]	69.3 (m) [b]	52.1 (k) [a]	65.2 (i) [ab]
4	*S. cheesmaniae* CGN24039	0.8 (g) [a]	1.2 (ij) [a]	0.9 (mn) [a]	37.8 (j) [a]	79.7 (l) [b]	55.7 (i) [ab]
5	*S. cheesmaniae* CGN17086	2.2 (k) [b]	1.2 (j) [a]	0.9 (mn) [a]	60.4 (lm)[c]	14.5 (fg) [b]	9.1 (d) [a]
6	*S. arcanum* CGN14355	1.8 (j) [b]	0.3 (b) [a]	0.2 (cd) [a]	45.5 (jk) [a]	62.1 (k) [a]	37.7 (gh)[a]
7	*S. arcanum* CGN15877	0.9 (g) [b]	0.6 (ef) [b]	0.5 (ghi) [a]	9.5 (ef) [a]	12.8 (ef)[ab]	13.1 (e) [b]
8	*S. glandulosum* CGN15803	1.3 (hi) [c]	0.9 (gh) [b]	0.7 (jkl) [a]	26.3 (i) [a]	23.8 (i) [a]	21.6 (f) [a]
9	*S. glandulosum* CGN14357	0.5 (e) [a]	0.1 (a) [b]	0.1 (b) [b]	10.2 (ef) [a]	20.3 (hi) [b]	22.1 (f) [b]
10	*S. glandulosum* CGN14358	1.0 (g) [b]	0.5 (cde) [a]	0.5 (hij) [a]	5.4 (d) [a]	10.9 (e) [a]	9.0 (d) [a]
11	*S. habrochaites* f. *glabratum* CGN24035	0.4 (cd)[a]	0.9 (ghi) [b]	0.8 (lm) [b]	5.1 (d) [a]	6.7 (d) [ab]	10.4 (de)[b]
12	*S. habrochaites* f. *glabratum* PRI921237	0.2 (b) [a]	0.2 (a) [a]	0.2 (c) [a]	1.3 (b) [a]	2.9 (b) [a]	30.4 (g) [b]
13	*S. habrochaites* CGN15391	1.4 (i) [a]	3.5 (l) [c]	2.3 (p) [b]	18.8 (h) [a]	62.2 (k) [b]	62.2 (i) [b]
14	*S. habrochaites* LA1777	0.4 (de)[a]	0.4 (bcd) [a]	0.4 (fgh) [a]	3.0 (c) [a]	34.8 (j) [b]	41.6 (h) [c]
15	*S. habrochaites* LA1033	0.1 (b) [a]	0.8 (fg) [b]	0.8 (klm) [b]	2.3 (c) [a]	58.5 (k) [b]	54.0 (i) [b]
16	*S. lycopersicoides* CGN23973	0.3 (c) [a]	1.2 (j) [b]	1.1 (no) [b]	28.1 (i) [a]	35.8 (j) [a]	37.7 (gh)[a]
17	*S. lycopersicum* PRI91117	1.5 (i) [b]	0.9 (ghij) [a]	0.7 (jkl) [a]	11.3 (f) [a]	39.6 (j) [b]	42.4 (h) [b]
18	*S. lycopersicum* EWSI24294	2.1 (jk) [b]	0.6 (ef) [a]	0.6 (ijk) [a]	2.6 (c) [a]	40.2 (j) [b]	36.7 (gh)[b]
19	*S. lycopersicum* EWSI49444	0.5 (de)[a]	0.8 (gh) [b]	0.8 (lm) [b]	4.4 (d) [a]	4.7 (c) [a]	6.6 (bc)[a]
20	*S. neorickii* CGN15816	0.3 (c) [a]	0.5 (cde) [c]	0.4 (efg) [b]	10.2 (ef) [b]	4.6 (c) [a]	5.4 (b) [a]
21	*S. neorickii* CGN15815	0.7 (f) [b]	0.4 (bc) [a]	0.3 (de) [a]	14.6 (g) [b]	4.9 (c) [a]	6.2 (bc)[a]
22	*S. pennellii* CGN23952	3.3 (l)					
23	*S. peruvianum* CGN17052	1.2 (h) [a]	0.6 (de) [a]	0.5 (ghij) [a]	4.3 (d) [a]	16.8 (gh) [b]	19.1 (f) [b]
24	*S. peruvianum* CGN17047	2.0 (jk) [b]	0.9 (ghi) [a]	0.8 (klm) [a]	55.0 (kl) [b]	33.0 (j) [a]	42.2 (h) [ab]
25	*S. pimpinellifolium* CGN14401	0.5 (e) [a]	1.2 (j) [b]	1.2 (o) [b]	8.1 (e) [a]	37.8 (j) [b]	35.8 (gh)[b]
26	*S. pimpinellifolium* PRI91059	0.7 (f) [a]	1.1 (hij) [b]	0.8 (lm) [ab]	10.9 (f) [a]	12.4 (ef) [a]	13.6 (e) [a]

Observation time: 1) 8 and 9 days after infestation; 2) 22 and 23 days after infestation; and 3) 36 and 37 days after infestation.

Table 2. Means of whitefly resistance parameters and type-trichome density in clip-on cage test. Mean followed by different letters within columns are different by Duncan's multiple range test in 0.05 *p*-significance.

No	Accession Name	Whitefly Resistance Parameters				Trichome Density (trichomes.mm^{-2})	
		Adult survival (whitefly alive.day^{-1})	Oviposition rate (egg.whitefly^{-1}.day^{-1})	Preadult survival (whiteflies.egg^{-1})	Development period (days)	Type IV	Type V
1	*S. galapagense* PRI95004/PY-8027	0.0 a	0.1 a	0.0 a	No data	30	0
2	*S. galapagense* PRI95004/PY-8028	0.98 efg	6.9 hij	0.9 hi	22.7 abc	0[1)]	40
3	*S. galapagense* PRI95004/PY-8029	0.97 efg	9.7 klm	0.9 ghi	23.5 hijk	0[1)]	19
4	*S. galapagense* PRI95004/PY-8030	0.96 ef	5.7 gh	0.8 fghi	23.1 def	0[2)]	20
5	*S. galapagense* PRI95004/PY-8031	0.97 ef	5.6 gh	0.8 fghi	23.7 jkl	0[1)]	27
6	*S. cheesmaniae* LA1448	1.0 g	10.7 lm	0.9 hi	23.6 ijkl	0	17
7	*S. arcanum* CGN15531	0.98 efg	9.5 klm	0.9 ghi	23.6 ijkl	0	35
8	*S. arcanum* CGN14356	0.99 fg	9.4 klm	0.9 ghi	22.8 bcd	0	34
9	*S. arcanum* CGN15801	0.97 efg	3.8 ef	0.8 ghi	23.7 jkl	0	2
10	*S. arcanum* CGN15392	0.99 fg	5.2 fgh	0.3 b	24.5 m	0	45
11	*S. arcanum* CGN15799	0.97 efg	4.0 fg	0.9 ghi	23.9 l	0	25
12	*S. glandulosum* CGN14357	0.99 fg	10.1 klm	0.9 ghi	24.0 l	0	56
13	*S. habrochaites* f. *glabratum* CGN15792	0.79 bc	0.8 abc	0.5 cde	23.2 efgh	7	7
14	*S. habrochaites* f. *glabratum* CGN15879	0.72 b	1.8 cd	0.7 cde	23.3 efghi	3	13
15	*S. habrochaites* f. *glabratum* PI134417	0.72 b	0.3 ab	0.4 cd	25.9 n	29	0
16	*S. habrochaites* f. *glabratum* PI134418	0.0 a	0.2 a	0.0 a	No data	36	0
17	*S. habrochaites* f. *glabratum* PRI921237	0.82 c	1.9 cd	0.6 cd	23.3 fghij	21	10
18	*S. habrochaites* LA1718	0.78 bc	0.3 ab	0.0 a	No data	5	11
19	*S. habrochaites* LA4137	0.98 efg	4.3 ef	0.6 cdef	22.6 abc	8	0
20	*S. habrochaites* LA1777	0.89 d	1.2 bc	0.4 bc	22.6 ab	14	0
21	*S. lycopersicum* Moneymaker	1.0 g	6.3 hij	0.9 fghi	23.8 kl	0	30
22	*S. lycopersicum* PRI91117	1.0 g	5.9 ghi	0.6 cde	23.6 ijkl	0	24
23	*S. minutum* CGN15816	0.97 efg	5.1 fgh	0.9 hi	23.1 def	0	43
24	*S. neorickii* LA2072	0.98 efg	5.2 fgh	0.7 defg	22.5 a	0	31
25	*S. neorickii* LA2133	0.83 c	2.4 de	0.7 cdef	23.0 cde	25	0
26	*S. peruvianum* CGN17052	0.94 e	3.6 ef	0.9 ghi	23.6 ijkl	0	16
27	*S. peruvianum* CGN17046	1.0 g	10.7 lm	1.0 i	23.4 fghij	0	17
28	*S. peruvianum* PI126928/PY-8037	0.99 fg	8.1 jk	0.9 ghi	23.1 defg	0	44
29	*S. peruvianum* PI126928/PY-8038	0.99 fg	11.4 m	0.9 hi	23.5 ghijk	0	51
30	*S. pimpinellifolium* PRI91059	0.98 efg	10.9 lm	0.9 hi	22.9 bcde	0	28
31	*S. pimpinellifolium* LA1261	0.98 efg	8.6 jkl	0.9 ghi	22.8 abcd	0	32
32	*S. pimpinellifolium* LA1584/PY-8040	0.71 b	0.6 ab	0.7 efgh	23.3 efgh	21	2
33	*S. pimpinellifolium* LA1584/PY-8039	0.97 efg	6.7 hij	0.9 ghi	22.8 bcd	0[2]	26
34	*S. pimpinellifolium* CGN15912	0.97 efg	7.6 hij	0.6 cd	22.8 bcd	0	21
35	*S. pimpinellifolium* CGN15808	0.99 fg	7.8 ijk	0.9 hi	23.6 hijk	0	20

[1)] type IV trichomes were not found on the leaf lamina, but a few were present on the stem and leaf petioles;
[2)] a few type IV trichomes were found on the leaf lamina.

Solanum galapagense PRI95004/PY-8027 showed the lowest adult density at all time points. *Solanum habrochaites* f. *glabratum* PRI921237 behaved similar, whereas other accessions such as *S. habrochaites* LA1033 showed only a low adult density at the first observation point but not at the other two. For *S. glandulosum* CGN14357 this was the opposite.

The lowest egg density at all time-points was also found on *S. galapagense* (PRI95004/PY-8027). Some accessions were less preferred at a particular developmental stage. For instance, on all *S. habrochaites f. glabratum* accessions there were fewer eggs at the first and second observation point compared to the third. On *S. habrochaites* LA1777, LA1033 and *S. lycopersicum* EWSI24294 the number of eggs was low at the first observation point, compared to observations 2 and 3. For *S. lycopersicum* EWSI49444, *S. neorickii* CGN15816 and *S. neorickii* CGN15815 it was the opposite; they had fewer eggs during the second and third observation.

Table 3. Pearson's correlation between parameters used in the whitefly resistance screening of tomato accessions in the free-choice experiment.

Parameters	ED	ND	HD	SM	DM
Whitefly density (WD)	0.48** (228)	0.43** (225)	0.38** (225)	0.35** (228)	0.24** (228)
Egg density (ED)		**0.86** (225)**	0.49** (225)	0.20** (228)	0.35** (228)
Nymphal density (ND)			**0.62** (225)**	0.26** (225)	0.47** (225)
Honeydew (HD)				0.30** (234)	0.50** (234)
Sooty mold (SM)					**0.61** (234)**
Plant damages (DM)					

** correlation is significant at the 0.01 level (2-tailed); * correlation is significant at the 0.05 level (2-tailed).

Whitefly resistance in no-choice tests

Two types of no-choice tests were used; clip-on cage and leaf disc tests. The results of the clip-on cage test are shown in Table 2. Pearson's correlation coefficient between adult survival (AS) and oviposition rate (OR) was 0.726 (N = 315), between AS and pre-adult survival (PS) was 0.591 (N = 315) and between OR and PS was 0.623 (N = 288). The developmental period (DP) did not correlate with AS, OR or PS (-0.12 to -0.07; N = 288). Adult survival, OR, PS and DP were not significantly different between the two blocks used and among replications.

However, PS was different between the two blocks (*p-value* less than 0.05). There were significant differences among tomato accessions for all parameters (*p-values* less than 0.01). Adult survival ranged from 0.0 to 1.0 whitefly alive.day^{-1} (Table 2). *Solanum pimpinellifolium* LA1584/PY-8040 and four *S. habrochaites* accessions were slightly less resistant than the two most resistant accessions (*S. galapagense* PRI95004/PY-8027 and *S. habrochaites f. glabratum* PI134418). Oviposition rate ranged from 0.1 to 11.4 eggs.female^{-1}.day^{-1}. Six accessions (*S. galapagense* PRI95004/PY-8027, *S. habrochaites f. glabratum* PI134418, PI134417, CGN15792, *S. habrochaites* LA1718 and *S. pimpinellifolium* LA1584/PY-8040), with a low AS, were also having the lowest OR (Table 2). Three accessions (*S. galapagense* PRI195004/PY-8027, *S. habrochaites f. glabratum* PI134418 and *S. habrochaites* LA1718) which were low in AS and OR, were also low in PS. The development period ranged from 22.5 to 25.9 days. The shortest DP was found on *S. neorickii* LA2072, *S. pimpinellifolium* LA1261, *S. galapagense* PRI95004/PY-8028, *S. habrochaites* LA4137 and LA1777.

In the leaf disc test we compared 9 accessions to the clip-on cage test. The resistance levels observed for the resistant (*S. galapagense* PRI95004/PY-8027, *S. habrochaites f. glabratum* PI134417 and PI134418, and *S. pimpinellifolium* LA1584/PY-8040), moderately resistant (*S. habrochaites f. glabratum* CGN15879 and *S. habrochaites* LA1718) and susceptible accessions (*S. galapagense* PRI95004/PY-8028, *S. peruvianum* PI126928/PY-8038 and *S. lycopersicum* MM) were similar in the leaf disc and clip-on cage tests (Supplementary table 4.6). This was also clear from the high correlation between the leaf disc and clip-on cage tests (R = 0.88 for AS and R = 0.93 for OR).

Correlation between no-choice and free-choice tests

Nine accessions were tested in both free-choice and clip-on cage tests. Pearson correlation between parameters in free-choice and no-choice tests can be seen in Table 4. Adult survival correlated with adult-whitefly, egg and nymphal density at all time-points. On the other hand, OR and PS highly correlated with egg and nymphal density at the first observation time only.

Table 4. Pearson correlation between parameters in free-choice and no-choice tests (N = 9)

Parameters	Observation time	Clip-on cage test			
		AS	OR	PS	DP
Adult whitefly	1	0.79*	0.55	0.54	0.66
	2	0.59	0.45	0.47	0.44
	3	0.70*	0.46	0.52	0.63
Egg density	1	0.87**	0.87**	0.79*	0.66
	2	0.74*	0.42	0.33	0.65
	3	0.64	0.22	0.18	0.74*
Nymphal density	1	0.76*	0.81**	0.80**	0.59
	2	0.81**	0.54	0.48	0.68*
	3	0.86**	0.48	0.46	0.84**

** correlation is significant at the 0.01 level (2-tailed); * correlation is significant at the 0.05 level (2-tailed).

Trichome diversity and its relationship to whitefly resistance parameters

Of the seven types of trichomes (I-VII) type IV and/or V trichomes were predominantly present on the abaxial leaf surface. Type I, III and VII were mostly absent. Type VI was present on the abaxial side of the leaves of all accessions, but in low numbers. It was frequently found on stem and leaf petioles. Trichome type I and/or III were found on the stem of the plant, leaf petiole and on the veins and only rarely on the adaxial leaf surface. The number of type IV and V trichomes was different among the tomato accessions (Table 2). The occurrence of type IV trichomes ranged from 0.0 to 36.2 trichomes.cm^{-2}. All accessions which were resistant as shown by low AS, OR and PS had type IV trichomes causing a high correlation between type IV trichomes and whitefly resistance parameters (Table 5). Most susceptible accessions had many type V trichomes and no type IV trichomes, which also show from the correlation between susceptibility and resistance with presence of trichomes V and IV respectively (Table 5). However, three accessions, *S. arcanum* CGN 14355 and *S. glandulosum* CGN14358, which were evaluated in free-choice test, and *S. arcanum* CGN15392 which was only evaluated in the clip-on cage test, did combine the absence of type IV trichomes with whitefly resistance.

Discussion

Parameters for whitefly resistance assessments

Whitefly developmental stages as parameter for resistance

In the initial stage of a whitefly infestation the adults have to choose a host plant for feeding and/or oviposition. Selection of the host plant may depend on several factors such as leaf architecture and color (Sippell *et al.* 1987), leaf pubescence and trichome type and density (McAuslane 1996; Snyder *et al.* 1998; Toscano *et al.* 2002), cuticle thickness (Channarayappa *et al.* 1992b) and compounds that play a role in repelling or attracting whiteflies (Chermenskaya *et al.* 2009). Subsequent stages of the whitefly development depend on the initial selection or survival of the adults. Therefore the different stages in the whitefly development may be correlated in a free-choice test and this is also what we observe (Table 3). However, some resistance parameters are much more strongly correlated then others. Although very significant, there was a correlation of only 0.48 between whitefly density and egg density on a host plants whereas egg density and nymph density are highly correlated (0.86). Lower correlation between adult density and egg or nymphal densities shows that resistance factors in adult density and egg/nymphal density may be different.

Table 5. Pearson's correlation between parameters in no-choice test with different types of trichomes (N = 140 for adult survival, oviposition rate and pre-adult survival; and N = 128 for developmental period).

Parameters	Trichome density		
	Type IV	Type V	Type VI
Adult survival	-0.82**	0.67**	-0.42**
Oviposition rate	-0.79**	0.75**	-0.30**
Pre-adult survival	-0.64**	0.60**	-0.24**
Developmental periods	0.07	0.03	0.06

**. correlation is significant at the 0.01 level (2-tailed); *. correlation is significant at the 0.05 level (2-tailed).

The high correlation between egg and nymphal densities suggest that no resistance factor(s) are present that effect egg hatching. All nymphal stages including instar 1 to instar 4 were observed, so oviposition (egg density) was apparently affected by an antibiotic and/or preference factor(s) which were recognized by the adult female. This hypothesis was already proposed by van Lenteren and Noldus (1990) and Nomikou *et al.* (2003) who suggest that

oviposition preference and host plant selection by the female whitefly has a profound effect on the fitness of its offspring.

Adult and egg density in the free-choice test, especially in the beginning of infestation, may be influenced by preference factors. However, the high correlation between whitefly density in the free-choice test and adult survival in no-choice test (Table 4), which much more assesses antibiosis than antixenosis, points at antibiotic factors as the main cause for the differences. Egg and nymphal densities in the free-choice test also highly correlated with oviposition rate and pre-adult survival in the no-choice test (Table 4). However, the correlation is only there for egg and nymphal density at the first observation time. This observation time was relatively similar with that for oviposition rate and pre-adult survival in clip-on cage test; plants were about 6 weeks old. The poorer correlations for the other time points may therefore be caused by the different development changes of the host plant, which may affect resistance. Resistance of some accessions increased whereas for others it decreased (Table 1).

Adult survival in the no-choice test was highly correlated with other parameters determined in the same test and also with all parameters in the choice test (Table 4). This strongly suggests that factor(s) affecting adult survival are the major factor in tomato defense. Antibiotic agents, such as acyl-sugar and methyl ketones, have been identified affecting insect growth in some tomato wild relatives (Lin *et al.* 1987; Liedl *et al.* 1995).

The developmental period was not correlated to the other parameters. This indicates that it is regulated by other mechanisms and in our studies it is not playing a major role in whitefly resistance. Also others have shown that the developmental period was not simply linked to adult survival, oviposition rate and pre-adult survival (Romanow *et al.* 1991; Bas *et al.* 1992; van Giessen *et al.* 1995).

Correlation between honeydew production, sooty mold growth, plant damage and whitefly resistance

Whiteflies produce honeydew (Blua and Toscano 1994) which contains several sugars and amino acids (Byrne and Miller 1990), which are good substrates for sooty-mold growth (McCollum *et al.* 2004). Whitefly infestation

and sooty-mold growth were found to result in physiological disorder and plant damage (Morales 2007). Our results show that only nymphal density correlates with honeydew production (Table 3), although both adult whiteflies and nymphs produce honeydew (Blua and Toscano 1994). The high correlation is most likely due to the fact that honeydew production of nymphs is much more regular than that of adults. Plant damage did slightly to moderately correlate with resistance parameters as well as honeydew production, and it highly correlated with sooty-mold growth (Table 3). Sooty mold growth contributes in several ways to the plant damage as it inhibits light transmission into leaf tissue which result in reducing photosynthesis and physiological disorders (Filho and Paiva 2006; Morales 2007).

Leaf disc test for whitefly resistance assessment

Adult survival and oviposition rate in the leaf disc test is highly correlated with adult survival and oviposition rate in the clip-on cage test. Therefore, leaf disc test may be a good alternative for whitefly resistance assessment using the clip-on cages. As an *in vitro* test, the leaf disc test has some advantages. It allows conducting the test in a more controlled environment, less space is needed and it is safer especially when there are viruses involved in the experiment. It is also possible to carry out the test in a free-choice situation. However, some improvements such as the addition of appropriate nutrition and antifungal agents are needed when one would like to assess the whole whitefly life cycle. The fact that we find a high correlation between the clip-on cage (*in-vivo* test) and the leaf disc tests (*in-vitro* test) suggests that detaching or wounding tomato leaves does not or slightly effects the resistance. Similar results were reported for an *in vitro* test used to screen for thrips resistance in pepper (Maharijaya *et al.* 2011).

Whitefly resistance and preference in accessions of tomato wild relatives

Level of whitefly resistance and preference in tomato accessions

The results show accession dependent responses to the whiteflies in the no-choice and choice test. Some accessions were fully resistant, whereas others were completely susceptible (Tables 2 and 3). One of the most striking examples was

accession PRI95004. This *S. galapagense* (syn: *Lycopersicum cheesmanii* f. *minor*) accession is derived from the genetically heterogeneous *S. galapagenense* accession. Both morphological characters and trichome types varied between individuals of this accession. In total five different homogenous groups were found, of which PY-8027 and PY-8030 are shown (Table 1 and 2). *Solanum galapagense* PY-8027, was highly resistant with high density of type IV trichomes. The four others were susceptible and were lacking the high density of type IV trichomes, like PY-8030. The resistant selection PY-8027 gave no adult survival and almost no oviposition in the no-choice test and was not preferred in the free-choice tests. The accession has never before been reported to be resistant to *B. tabaci*. *Solanum galapagense* is genetically close to commercial tomato (Perralta *et al.* 2008) which may make it easier to use in commercial breeding programs. After testing with *Keiferia lycopersicella* (Walsingham) less damage and lower numbers of larvae were found on another *S. galapagense* accession (Schuster 1977).

The level of whitefly resistance in the *S. habrochaites* accessions was variable. This species has been exploited as resistance source to several pests (Lin *et al.* 1987; Eigenbrode and Trumble 1993; Momotaz *et al.* 2010). In our no-choice test *S. habrochaites* LA 1718 showed some level of resistance (Table 2), due to a low oviposition rate. In the choice assay (Table 1) LA1777 and LA1033 showed resistance only in the beginning of whitefly infestation and they became more susceptible over time. Previous research showed that LA 1777 was less preferred in a choice test (Muigai *et al.* 2003) and less virus incidence was detected after infestation by viruliferous whitefly (Maruthi *et al.* 2003). In our evaluation most accessions of *S. habrochaites* f. *glabratum* were not preferred by whitefly with PI134418 being the most resistant accession. Our results confirm earlier results (Toscano *et al.* 2002; Fancelli and Vendramim 2002; Muigai *et al.* 2003; Baldin *et al.* 2005).

Solanum pimpinellifolium LA1584 also showed heterogeneity within the accession. Resistance observed was due to a low adult survival and oviposition rate. This accession was also reported as resistant due to low nymphal survival (Fancelli and Vendramim 2002), but it was preferred in a free-choice test (Baldin

et al. 2005). Some other accessions from *S. arcanum, S. glandulosum, S. lycopersicum* and *S. neorickii* showed partial resistance for adult density, egg density or pre-adult survival. From those accessions, only *S. arcanum* was reported to be partially resistant to whitefly (Channarayappa *et al.* 1992a; Muigai *et al.* 2003).

Whitefly resistance changes over time

The number of whiteflies that can be sustained by an accession depends on the suitability of the host as food resources (Hirano *et al.* 1995), resistance levels (antibiosis) and microclimatic factors (Horowitz 1986). Resistance of most accessions changed between the first and second observation (Table 1). On the other hand, only minor changes occurred between the second and the third observation time for most accessions. These results show that successful whitefly colonization on a new host is largely dependent on host suitability at the time the first infestation takes place. During that period, interactions between the host plant and phloem-feeding insects occur that may change host plant suitability (Broekgaarden *et al.* 2010). The interaction can increase the resistance in the host plant (induction) or decrease it (suppression) (Broekgaarden *et al.* 2007). Bas *et al.* (1992) also observed resistance differences between younger and older plants. Effects of tomato age and infestation time were also reported in the resistance of tomato plants against potato moth (*Phthorimala operculella*) (Gurr and McGrath 2001).

Influence of trichome types on whitefly preference and resistance

Trichomes have been considered as the most important pest resistance factor. Seven types of trichomes are known in tomato of which type I, IV, VI and VII are glandular trichomes, and type II, III and V are non-glandular trichomes (Gurr and McGrath 2001; Simmons and Gurr 2005).

The presence, density and distribution of the trichome types depend on the tomato genotype, organs/tissue, age and environmental conditions (Wilkens *et al.* 1996; Gurr and McGrath 2001; Kang *et al.* 2010). *Solanum galapagense* has no type II and III, few type I, VI and VII, very few type V, but very abundant type IV trichomes (Simmons and Gurr 2005; Simmons *et al.* 2005). In our results type IV

trichomes are present on the most resistant *S. galapagense* accession. Also *S. habrochaites* and *S. habrochaites* f. *glabratum* had high densities of type IV and VI trichomes (Eigenbrode and Trumble 1993; Simmons and Gurr 2005).

From our results it is clear that the most resistant and not preferred tomato accessions had a high density of glandular type IV trichomes. Other researchers also reported that the presence of this trichome type highly correlated with resistance to whiteflies and other pests (Dimock and Kennedy 1983; Channarayappa *et al.* 1992a; Snyder *et al.* 1998; Muigai *et al.* 2003). Although glandular trichomes seem to play an important role in whitefly resistance, it is actually the compounds within the trichomes that are critical. For instance, *S. habrochaites* LA 1777 and LA 1033, have a similar density of type IV trichomes, but they differ in resistance to *Helicoverpa zea* and *Spodoptera exigua* and in the constitution of trichome exudates (Frelichowski and Juvik 2001). Examples of such exudates are methylketones such as 2-tridecanone and 2-undecanone which are present at high concentrations in type IV and VI of trichomes and are believed to have an insecticidal effect on several arthropods (Lin *et al.* 1987; Kashyap *et al.* 1991; McDowell *et al.* 2011). Glandular trichomes can also produce zingiberene and sesquiterpene compounds which play role as repellence (Maluf *et al.* 2001; Bleeker *et al.* 2009; Kang *et al.* 2010). Different compounds were identified in *S. pennellii* and *S. pimpinellifolium* type IV trichomes. Here, the type IV trichomes contain a high amount of acyl-sugars which make the trichomes sticky (Liedl *et al.* 1995; Mutschler *et al.* 1996; Fancelli *et al.* 2005; Rodriguez-Lopez *et al.* 2011). Importance of the trichome content is also shown by the fact that the metabolite content of different types of trichomes within an accession/species is more similar than the same type of trichome from different accessions/species of tomato (McDowell *et al.* 2011).

In contrast to glandular trichomes, non-glandular trichomes, especially type V, are not involved in pest resistance. Whiteflies prefer hairy leaf (Toscano *et al.* 2002). Non-glandular trichomes provide also a more suitable microclimate for oviposition and protects the eggs and larvae from their enemies (Butter and Vir 1989). A glandular trichome-based resistance mechanism is not the only mechanism in tomato to get whitefly resistance. Whitefly resistance was also

found in accessions without glandular trichomes such as in *S. arcanum* CGN14355 and CGN15392, and *S. glandulosum* CGN14358. Other mechanisms such as leaf-surface hardness and cuticle thickness or mesophylic-leaf compounds may play a role in the whitefly resistance mechanism as well. Thick cuticles cannot be pierced by the whitefly's stylet (Janssen *et al.* 1989).

In conclusion, correlations of parameters within and between free-choice and no-choice tests show that antibiosis is the major factor for whitefly resistance in tomato accessions. Leaf disc tests are an alternative *in vitro* method that can be used for whitefly resistance screening. Whitefly resistance level of tomato accessions varied and can change over time. *Solanum galapagense* PRI95004/PY-8027, which is closely related to commercial tomato, is highly resistant to whitefly over time. Some other accessions from *S. habrochaites f. glabratum, S. pimpinellifolium, S. arcanum and S. glandulosum* showed partial resistance. These accessions are potential sources for resistance factor(s), which may be exploited in breeding programs in tomato aimed at whitefly resistance.

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Chapter 5

Identification and QTL Mapping of Whitefly Resistance Components in *Solanum galapagense*

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Abstract

Solanum galapagense is closely related to the cultivated tomato and shows a very good resistance towards whitefly. A segregating population resulting from a cross between the cultivated tomato and a whitefly resistant *S. galapagense* was created and used for mapping whitefly resistance and related traits, which made it possible to unravel the genetic basis of the resistance. Quantitative trait loci (QTLs) for adult survival co-localized with type IV trichome characteristics (presence, density, gland longevity and gland size). A major QTL (*Wf-1*) was found for adult survival and trichome characters on Chromosome 2. This QTL explained 54.1% of the variation in adult survival and 81.5% of the occurrence of type IV trichomes. A minor QTL (*Wf-2*) for adult survival and trichome characters was identified on Chromosome 9. The major QTL was confirmed in F3 populations. Sixteen metabolites segregating in the F2 mapping population were associated with *Wf-1* and/or *Wf-2*. The *Wf-1* locus was associated with acyl sugar production. These results show that whitefly resistance in *S. galapagense* seems to inherit relatively easy compared to whitefly resistance from other sources and offers great prospects for resistance breeding as well as elucidating the underlying molecular mechanism(s) of the resistance.

Keywords: *Bemisia tabaci*, *Solanum*, metabolomics, SNP markers, genetic map.

Introduction

Whiteflies (*Bemisia tabaci* Genn.) cause serious problems in the cultivation of tomatoes and other vegetable crops mainly because they can be the vector of a large number of harmful viruses (Morales and Jones 2004). Feeding of whiteflies also inhibits plant growth (Schuster *et al.* 1990a) and the honeydew produced by the whiteflies can promote sooty mold growth, which leads to physiological disorders (McCollum *et al.* 2004). Natural enemies and/or pesticides are not effective enough in open field cultivation to prevent the unpredictable outbreaks of whiteflies (Hirano *et al.* 1995). Moreover, pesticides are known to be harmful for growers and can result in pesticide-resistant whiteflies (Erdogan *et al.* 2008). A plant variety that is naturally resistant to whiteflies may be a good alternative to control *B. tabaci* and the viruses it distributes (Broekgaarden *et al.* 2011).

Whitefly resistance has been found in wild relatives of tomato such as *S. pennellii*, *S. habrochaites*, *S. chilense*, *S. pimpinellifolium* and *S. galapagense* (Baldin *et al.* 2005; Fancelli and Vendramim 2002; Firdaus *et al.* 2012; Muigai *et al.* 2003; Toscano *et al.* 2002). The resistance parameters used in these studies were density and/or survival of whitefly adults, eggs and nymphs (Firdaus *et al.* 2012; Maliepaard *et al.* 1995; Muigai *et al.* 2003). These parameters showed that the resistance mechanisms were based on antixenosis and/or antibiosis (Baldwin *et al.* 2005; Channarayappa *et al.* 1992a; Toscano *et al.* 2002). Mortality may be caused by physical barriers preventing the whiteflies to feed on the phloem sap (Toscano *et al.* 2002) or by toxic compounds (Kehr 2006).

Whitefly resistance in wild relatives of cultivated tomato and potato is suggested to be associated with glandular trichomes (Erb *et al.* 1994; Oriani *et al.* 2011; Muigai *et al.* 2003; Rodriguez-Lopez *et al.* 2011). Of the seven trichome types found in tomato and its wild relatives, four types are glandular (Luckwill 1943). The presence of type IV and VI trichomes is highly correlated with whitefly resistance (Channarayappa *et al.* 1992a; Dimock and Kennedy 1983; Firdaus *et al.* 2012; Muigai *et al.* 2003). Glandular trichomes might play a role as physical barrier and/or source of compounds deterrent and/or toxic to whiteflies (Dimock and Kennedy 1983; Toscano *et al.* 2002). However, not all tomato

accessions with type IV trichomes are resistant (Frelichowski and Juvik 2001; Muigai *et al.*2003), suggesting that the content of the trichomes also plays an important role. Acyl sugars are the major exudates of type IV trichomes in *S. pennellii* and *S. pimpinellifolium* (Blauth *et al.* 1998; Fancelli *et al.* 2005; Mutschler *et al.* 1996; Rodriguez-Lopez *et al.* 2011). Methyl-ketones and derivates of sesquiterpenes carboxylic acids are major exudates of type IV and VI trichomes in *S. habrochaites spp* (Chatzivasileiadis and Sabelis 1997; Eigenbrode *et al.* 1994; Farrar and Kennedy 1991; Frelichowski and Juvik 2001; Kennedy *et al.* 1991).

Genetic factors underlying whitefly resistance were identified by Quantative Trait Loci (QTL) mapping studies (Blauth *et al.* 1998; Maliepaard *et al.* 1995; Momotaz *et al.* 2010; Mutschler *et al.* 1996). In *S. habrochaites* LA1777, the QTLs for oviposition rate on chromosomes 9, 10 and 11 co-localized with QTLs for type IV trichomes (Momotaz *et al.* 2010). However, in *S. habrochaites f. glabratum* CGN1.1561 the QTLs for egg deposition and the QTLs for presence of glandular trichomes did not co-localize, which may point at another mechanism (Maliepaard *et al.* 1995). In *S. pennellii*, twelve QTLs were detected for presence and density of type IV trichome and production of acyl sugar (Blauth *et al.* 1998; Mutschler *et al.* 1996). However, backcross plants containing the five QTLs for acyl sugar production did not produce elevated levels of acyl sugars, suggesting that additional QTLs are needed (Lawson *et al.* 1997). These studies showed that whitefly resistance can be based on several mechanisms involving many genes. It is likely that the genetics underlying the difference in resistance is easier to unravel when more closely related species like *S. pimpinellifolium* and *S. galapagense* are used as the source of the resistance.

For successful introgression breeding a comprehensive knowledge of the genetic basis of the different whitefly resistance factors is needed and preferably a closely related wild relative to be able to minimize linkage drag as much as possible. In this study we used a *S. galapagense* accession with a very high level of resistance to study the genetics of the resistance and to identify components involved in the resistance.

Materials and methods

Plant and whitefly materials

An F2 population of 230 individuals was obtained after self-pollination and seed collection of an F1 plant originating from the cross between *S. lycopersicum* cv. Moneymaker^{tmvR} PRI91117 and *S. galapagense* PRI95004 (Firdaus *et al.* 2012). The parents were obtained from Wageningen UR Plant Breeding Wageningen, the Netherlands. Seeds were sown in peat-moss soil in a sowing box and seedlings transplanted after the third leaf stage into 1.5 L pots containing peat-moss soil and maintained in an insect-proof greenhouse with a 16h light and an 8h dark photoperiod (16L:8D), 20 ºC/16 ºC (day/night) and 70% relative humidity from October 2009 to March 2010 in Wageningen, the Netherlands. Plants were pruned to maintain a manageable size. Cuttings were made from each genotype for whitefly screenings in Wageningen, the Netherlands and Purwakarta, Indonesia. Non-viruliferous silverleaf whiteflies (*B. tabaci*, group Mediterranean-Middle East-Asia Minor I), from the collection of the Laboratory of Entomology, Wageningen University, the Netherlands or local haplotype of *B. tabaci* of the Plant-Pathology Department of Bogor Agricultural University, Indonesia, were used for screening. Sequence analysis was also done for whitefly identification (Firdaus et al 2012).

Whitefly resistance tests

Free-choice test. A free-choice test was carried out in Purwakarta, Indonesia from November 2009 to February 2010. In the Netherlands three cuttings were made from the two parents, four F1 plants and 120 F2 plants, and shipped to Indonesia in October 2009. The plantlets were grafted onto three week old eggplant (*Solanum melongena* cv. EG203) rootstocks to prevent nematode problems. Two cuttings per genotype were randomly arranged on tables one meter above the ground level in an insect-proof screen house. In a screen house the plants are protected from unwanted insects, heavy rainfall and intense sunshine. There were two rows on each table with 35 cm between rows and 20 cm between plants. Amaranthus plants were put in between genotypes as border plants. Branches and flowers of the tomato plants were pruned regularly to get one main stem and to avoid fruit setting. Six weeks after grafting, the plants were infested

with virus-free *B. tabaci* by placing heavily infested eggplants between the plants. The eggplants were shaken twice a day and left without watering, therefore the eggplants dried out and died after a few days forcing the whiteflies to move on (Muigai *et al.* 2003). Eight days after infestation, the number of adult whiteflies on the backside of lateral leaflets on the 3rd or 4th and 7th or 8th leaf from the top were counted (Gusmao *et al.* 2005). One day later egg, nymph numbers and leaf area were determined under a stereo microscope (10x) on the same, now detached leaflets. Data on adults, eggs, nymphs and leaf area in choice test were used to calculate adult-whitefly density (number of adult whiteflies.cm^{-2}), egg density (number of eggs.cm^{-2}) and nymph density (number of nymphs.cm^{-2}). Log transformation was used to normalize the data.

No-choice tests. Clip-on cages were used for these tests according to Maliepaard *et al.* (1995). In Indonesia, the test was carried out on five weeks old plants (one week before the free choice test). Synchronized whiteflies (one to two days old) were anesthetized by putting them at 4 ^{o}C for about 10 minutes and females were selected. Five whitefly females (*n*) were collected with an aspirator and transferred to a clip-on cage (two cm in diameter and one cm in height), three cages were attached to the underside of a leaflet of the 3rd or 4th leaf from the top. Four days after infestation (*d*) the clip-on cages were removed from the leaves and the death and living whiteflies (*m*) were counted. The number of eggs (*e*) was counted under a stereo microscope. In Wageningen, one cutting was made from each of the 189 F2 individuals and four cuttings from each parent. Lateral branches and flowers were regularly removed. One week before infestation, the temperature was raised gradually until it reached 27 ºC/18 ºC (day/night) two days before infestation. Whitefly infestation was done six weeks after the cuttings were made. Synchronized whiteflies (one to two days old) were anesthetized with CO_2 for female selection. Female selection and infestation were done in a similar way as in Indonesia. The same parameters as in Indonesia were measured and additionally pupal cases (*p*) were counted 8 days after the first adult appeared, which was around 17-21 days after infestation. Adult survival (AS), oviposition rate (OR) and pre-adult survival (PS) were calculated by using the equations as shown below (Maliepaard *et al.* 1995).

$AS = \left(\frac{m}{n}\right)^{1/d}$ survival.day^{-1} (Formula 1) $OR = \frac{2e}{d(m+n)}$ eggs.female^{-1}.day^{-1} (Formula 2)

$PS = p/e$ whiteflies.egg^{-1} (Formula 3)

An ArcSin transformation was used to normalize adult survival and pre-adult survival values, and a square-root transformation was used for oviposition rate.

Trichome type identification and counting. Different types of trichomes were identified based on Luckwill (1943). Trichomes were counted on the abaxial side of the lateral leaflets which were used for the clip-on cage test one and two days after infestation (young leaves) of the no-choice tests had started. The leaflets were cut from the plant and three circles were made by using a perforator (1.1 mm in diameter) on the right and/or left sides of the main vein at the beginning of the leaflet. The trichome types on these circles were identified and counted by using a stereo dissecting microscope (40 to 100 times magnification). R45 was calculated by dividing type IV density by the sum of the density of type IV and V. Gland longevity and size of the type IV trichomes were measured in the test done in the Netherlands. Gland longevity is given by the percentage of type IV trichomes still present three weeks after infestation (old leaves). The scores were: (0) value between 0 and 0.49, (1) value between 0.5 and 0.99, and (2) value of 1 (no type V trichomes). For the gland size, the scores were made based on the proportion between small (15 to 25 μm) and big (45 to 55 μm) gland size of type IV trichomes. The score for gland size was (0) when the minority of the glands were big and (1) when the majority of the glands were big.

Correlation between resistance parameters and trichomes. Correlations between resistance parameters and density of type IV, V and VI trichomes were calculated with the Pearson's correlation method, whereas the correlation between presence of type I and III trichomes and scores for ratio, longevity and size of type IV trichomes were calculated with the Spearman's correlation method (Steel and Torrie, 1980). The resistance parameters and traits of the tests in Indonesia and the Netherlands were compared with paired samples t-test analyses. The analyses

were done by using SPSS 19.0.0.1 package (SPSS® Inc. an IBM® Company). Heritability of the resistance parameters and traits were calculated based on Burton (1952): $h^2 = (VF_2-(VP_1+VP_2+VF_1)/3)/VF_2$, where h^2 = broad sense heritability, VF_2 = variance of F_2, VP_1 = variance of parent 1, VP_2 variance of parent 2 and VF_1 = variance of F_1.

Genomic DNA extraction and genotyping

Approximately two cm^2 fresh young leaves were collected and grinded using the Retsch Mixer Mill MM301® according to the manufacturer's manual. Afterwards, the genomic DNA was extracted according with the maxiprep method as described in the KingFisher® 96 manual (Thermo Labsystems). The DNA was quantified and qualified using the NanoDrop 1000 V.3.7 (Thermo Fisher Scientific Inc) and gel electrophoresis. Fifty ng per µl DNA solution were prepared for genotyping. A Single Nucleotide Polymorphism (SNP) Infinium array (made for other purposes at Wageningen UR Plant Breeding) contained 5528 SNPs, of which 1654 were polymorphic between our *S. lycopersicum* and *S. galapagense* parents. Genotyping was carried out by Service XS, Leiden, the Netherlands.

Genetic linkage analysis and QTL mapping

A genetic linkage map of SNP markers data was calculated using JoinMap® 4.0 (Van Ooijen 2011). SNP markers that were monomorphic or difficult to score were removed. Markers that showed an identical segregating pattern were considered as one marker. Finally, of the 1654 SNP markers polymorphic between the parents, 589 markers were used to construct a genetic map. The genetic map was constructed based on recombination frequency with a minimum LOD-score 2.0 and maximum likelihood was used as mapping algorithm. In the regression mapping, linkages with recombination frequency less than 0.4 and LOD score higher than 1.0 were used. MapQTL® 6 (Van Ooijen 2009) was used to determine significant associations between markers and phenotypic traits. The genetic linkage and QTL maps were drawn using MapChart 2.2 (Voorrips 2002).

Gas chromatography-mass spectroscopy (GC-MS) and liquid chromatography-mass spectroscopy (LC-MS)

Two cuttings per F2 genotype were used to analyse the chemical content by GC-MS. After six weeks, the 3rd and 4th leaf from the top (the same leaf stages as used for clip-on cages test) were harvested and immediately frozen in liquid nitrogen and kept at -80 ^{o}C until they were prepared for metabolite analyses. Frozen leaves were grinded to a fine powder and 400 mg of the powder was dissolved in anhydrous dichloromethane (>99.8%, Sigma-Aldrich) and 0.75 µg.ml^{-1} Heptadecanoic acid methyl ester (Methylheptadecanoate) was added as an internal standard. The solution was homogenized by vortexing and centrifuged at 1500 rpm for 10 minutes. The supernatant was dried on a bed of 2 cm disodium sulphate (Na_2SO_4) using an 8 mm diameter glass funnel, with a one cm plug of salinized glass wool fibres (O_2Si) at the bottom and 2 cm disodium sulphate (Na_2SO_4) in the upper part. The extract was injected on 7683 series B injector (Agilent Technologies) into a 7890 A GC (Agilent) coupled to a 5975 C quadrupole mass selective detector (Agilent Technologies). Chromatography was performed on an ZB-5MS column (Phenomenex, 30 meter, 0.25 mm inner diameter, 0.25 µm film thickness) with a 5 meter retention gap. The temperature of the injector was set to 250 ^{o}C for GC and 260 ^{o}C for MS. The temperature of the column was programmed at 45 ^{o}C for 1 min, and raised gradually by 10 ^{o}C min^{-1} up to 300 ^{o}C and kept at 300 ^{o}C for 7 minutes. Helium was used as carrier gas and the column flow was 1 ml.min^{-1}. The column effluent was ionised by electron impact at 70eV. Mass spectra were obtained from 35 – 400 m/z.

The ten most resistant and ten most susceptible genotypes were selected for LC-MS analysis. Five hundred mg of frozen-leaves powder were extracted with 1.5 ml Methanol-Formic acid solution (99% Methanol absolute - Sigma-Aldrich and 0.133% (95%-Formic acid - Sigma-Aldrich). The extracts were homogenized by shaking for about 1 minute and were sonicated for 10 minutes and then centrifuged at 2500 rpm for 10 minutes. The supernatant was filtered by using RC4® minisart 0.45 µm filter. Afterwards, 30 µL of filtrate was put into a vial with glass insert and closed with the cap.

An untargeted metabolomics approach was applied to process the raw GC-MS and LC-MS data (Hall and Hardy 2012, Tikunov et al 2005). MetAlign software (Lommen 2009, metalign.nl) was used to extract and align all mass signals (s/n≥ 3). Absent values were randomized between 0.1 and 3 times the noise. 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 using MSClust software (Tikunov *et al.* 2005, 2011). Metabolites were putatively identified by matching the mass spectra of obtained metabolites to authentic reference standards and the NIST08 (National Institute of Standards and Technology, Gaithersburgh, MD, USA), Wiley, and Wageningen Natural compounds spectral libraries and by comparison with retention indices of the literature calculated using a series of alkanes and fitted with a third order polynomial function (Strehmel *et al.* 2008). To identify resistance related metabolites, the mass abundances of metabolites obtained from GC-MS were subjected to QTL mapping. Metabolites which had QTLs at the same position as QTLs for AS were considered as potentially related to whitefly resistance. The effect of parent alleles of the QTLs on the abundance of related metabolites in the F2 population was also compared by using univariate analysis followed by least significant difference (LSD) test of the IBM® SPSS® 19 package (Steel and Torrie, 1980). The abundance of acyl sugars obtained from LC-MS was compared between resistant and susceptible bulks, using the student t-test.

Confirmation of the QTLs in F3 populations

Nine F2 plants were selected of which we knew one or more QTLs would segregate after selfing. Forty F3 seeds of each selected F2 plant were sown in peat-moss soil in sowing boxes. Genomic DNA was extracted and a number of markers were determined using KASPar system (KBiosciences, UK). Markers were chosen in the identified QTL regions (Table 1). Based on the marker data, ninety six F3 plants were selected and phenotyped for whitefly resistance level in a no-choice experiment. The effects of the QTLs on Chromosome 2 and Chromosome 9 were calculated.

Table 1. SNP markers used in confirmation of QTLs in the F3 population.

No	Marker position*)	Chromo-some	Sequence	Allele	
				S. galapa-gense	*S. lycoper-sicum*
1	47987080	02	ATCATTTTTTAGGAC[**G/A**]GATTTATATTCTTGT	G	A
2	48838396	02	AAACTTGCAGGTACT[**G/A**]CGACCTCCTATGATC	G	A
3	49271932	02	GATTCTTCCACGCCT[**A/C**]GCTCTTCTTCTGCAG	A	C
4	49456276	02	GGAAAATAGTTTGTG[**T/C**]ATTAAAAGAGCAGAA	T	C
5	49486956	02	GTTGCCTAGTTCAAC[**G/A**]TTTGTTTACGCAACA	A	G
6	14931849	09	TCAGATGGTGATTCC[**T/C**]CACCTTACAGAAAAT	T	C
7	23385770	09	GCAGCCGTTGCAGTC[**T/C**]CAATTTCGCCCACAA	T	C

*) Marker position was according to version 2.30 of the tomato sequence

Results

Whitefly resistance and trichome properties

The average values for adult survival (AS), oviposition rate (OR), pre-adult survival (PS) and trichomes of the parents, F1 and F2 populations are shown in Table 2. *Solanum lycopersicum*, the susceptible parent, did not have type IV trichomes, whereas, *S. galapagense*, the resistant parent, did not have type V trichomes. The presence of type IV trichomes was dominant in the Netherlands, but under Indonesian conditions both types of trichomes were present on the F1 leaves (Table 2). Also in the F2 population there were differences in trichome distribution between the Netherlands and Indonesia. Of the F2 population 100 genotypes were analyzed in both countries. In the Netherlands both types of trichomes were simultaneously present in 15% of the population and in Indonesia this was 49%. In the Netherlands more genotypes (63%) had only type IV trichomes compared to Indonesia (32% = 38 genotypes). The proportion of genotypes without type IV trichomes did not much differ much between the two countries (22% in the Netherlands and 19% in Indonesia). The average AS and OR of the F2 population in Indonesia was significantly lower than in the Netherlands and the trichome type IV and VI concentrations in the Netherlands were significantly higher than in Indonesia. The AS screening in the Netherlands showed that there were 75 resistant genotypes and 92 susceptible genotypes (Figure 1). This figure also shows that all 75 resistant genotypes had type IV and no type V trichomes (R45 = 1), 30 other genotypes that also had type IV

trichomes and no type V trichomes were susceptible. All together 124 genotypes had type IV trichome and 43 genotypes did not have it.

In the Netherlands there was a high correlation between AS and OR and a lower correlation between AS and PS (Table 3). The AS of the tests in Indonesia and the Netherlands were significantly correlated. The correlation between AS in the Netherlands and the parameters of the free-choice test in Indonesia were significant but the correlation was lower than with the parameters of the no-choice test in Indonesia. Presence of type I trichomes and properties of type IV trichomes such as density, ratio, gland longevity and size negatively correlated with AS and OR and to a lesser extent also with PS (Table 3). Type IV and type V trichomes can both be present but there is a high positive correlation of 0.85 between the presence of type IV and the absence of type V trichomes. High correlations were also found among trichome type I, III, IV and V; no correlation was found between type VI and the other trichome types (Table 4).

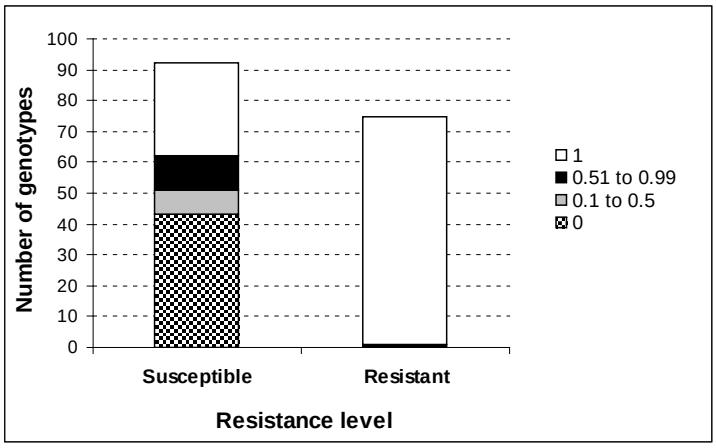

Figure 1. Genotype distribution of F2 population based on resistance levels (S = susceptible, AS = 0.6 to 1.0; and R = resistant; AS = 0.0 to 0.59) and the ratio of type IV and type V trichomes (1 = all type IV; 0.51 to 0.99 = type IV was dominant; 0.1 to 0.5 = type V was dominant and 0 = all type V)

Linkage map of the SNP markers

Fourteen genetic linkage groups were constructed based on 589 segregating markers (loci) using a population of 182 F2 plants (Figure 2). Two chromosomes (Chromosomes 1 and 3) were represented by two linkage groups (due to recombination hot spots). In total, the genetic map spans 1259 cM and the order of the SNP markers in genetic linkage map was identical with that of the physical map (not shown). Figure 2 also shows the distribution of the markers on the physical map.

Table 2. Whitefly resistance related characteristics of the plant material used in the Netherlands and Indonesia.

Country	Adult survival	Oviposition rate	Pre-adult survival	Trichome Presence		Trichome Density			Gland characters of type IV trichomes	
				Type I	Type III	Type IV	Type V	Type VI	Longevity	Size
Netherlands										
P1 (*S. lycopersicum*)	1 ± 0	4.63 ± 0.12	0.30 ± 0.06	0	1	0 ± 0	283.8 ± 71.3	7.5 ± 2.4	nd	nd
P2 (*S. galapagense*)	0 ± 0	0 ± 0	0 ± 0	1	0	326.3 ± 21.9	0 ± 0	15.0 ± 1.0	2	1
F1	0.45 ± 0.20	2.15 ± 0.60	0.60 ± 0.1	1 ± 0	0.1 ± 0.1	281.8 ± 28.6	0 ± 0	7.7 ± 1.4	1	1
F2	0.52 ± 0.03	2.86 ± 0.21	0.35 ±0.02	0.79 ±0.03	0.25 ± 0.03	173.8 ± 9.4	92.9 ±10.6	9.2 ± 0.4	1.5 ±0.1	0.46 ± 0.04
Heritability	0.98	0.72	0.72	1	0.78	0.83	0.74	0.61	1	1
Indonesia										
P1 (*S. lycopersicum*)	1 ± 0.	1.55 ± 0.15	nd	0 ± 0	1 ± 0	0 ± 0	279.0 ± 3.6	14.1 ± 0.5	nd	nd
P2 (*S. galapagense*)	0 ± 0	0 ± 0	nd	1 ± 0	0 ± 0	208.7 ± 9.6	0 ± 0	3.1 ± 0.2	nd	nd
F1	nd	nd	nd	1 ± 0	0 ± 0	146.4 ± 14.5	62.0 ± 31.3	11.5 ± 1.3	nd	nd
F2	0.28 ± 0.04	0.80 ± 0.13	nd	0.77 ± 0.04	0.35 ± 0.04	100.3 ± 7.6	87.6 ± 8.7	6.9 ± 0.5	nd	nd
Heritability	0.87	0.99	nd	1.00	1.00	0.89	0.72	0.98	nd	nd
p-value	<0.001	<0.001	nd	0.657	0.01	<0.001	0.97	<0.001	nd	nd

nd = not determined

Mean (followed by standard error of mean) and heritability of resistance parameters and trichome properties of parents, F1 and F2 population in the tests in Indonesia and the Netherlands and significance of the difference (*p-value*) between both countries. Presence of type I and III was scored: 0 for absence and 1 for presence. Gland longevity was scored 0 to 2 based on division of type IV density in old leaflet (value two or three days after infestation compared to value three weeks after infestation); score 0 for 0.0 to 0.49,score 1 for 0.5 to 0.99 and score 2 for 1 or the absent of type V trichomes in the old leaflet. Gland sizes was scored as 0 if most glands were small (15 to 25 μm) and 1 if most glands were large (45 to 55 μm).

Table 3. Correlation between whitefly resistance parameters and trichome properties of experiments carried out in the Netherlands and Indonesia. Adult survival in the Netherlands (ASNL) was used as a reference point.

Test location	Method	Parameters	ASNL	Presence of trichome type		Density of trichome type			Type 4 trichome properties		
				I	III	IV	V	VI	R45‡	longevity	Size
The Netherlands	Clip-on cage test	Adult survival		-.55**(169)	.56**(168)	-.63**(168)	.62**(169)	-.15(169)	-.74**(168)	-.61**(125)	-.58**(132)
		Oviposition rate	.80**(169)	-.45**(168)	.49**(167)	-.57**(167)	.54**(168)	-.14(168)	-.68**(167)	-.64**(124)	-.52**(131)
		Pre-adult survival	.39**(111)	-.41**(110)	.42**(109)	-.35**(109)	.30**(110)	-.09(110)	-.44**(109)	-.17(67)	-.09(73)
Indonesia	Clip-on cage test	Adult survival	.57**(61)	-.26*(74)	.36**(73)	-.69**(74)	.54**(74)	.04(74)	-.65**(74)	nd	nd
		Oviposition rate	.45**(61)	-.18(74)	.31**(73)	-.49**(74)	.59**(74)	-.01(74)	-.59**(74)	nd	nd
	Free-choice test	Whitefly density	.25*(93)	-.14(117)	.23*(113)	-.157(117)	.15(115)	-.05(112)	-.35**(115)	nd	nd
		Egg density	.39**(97)	-.32**(123)	.28**(120)	-.449**(119)	.41**(121)	-.07(119)	-.58**(121)	nd	nd
		Nymphal density	.22*(80)	-.30**(101)	.29**(99)	-.38**(101)	.25*(100)	-.13(98)	-.45**(100)	nd	nd

Adult survival in the Netherlands (ASNL) was used as a reference point.
Number of plants in the analysis are between brackets; *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed); ‡ = ratio between type IV and V densities. n.d. = not determined.

Table 4. Correlation between trichome types based on test in the Netherlands

Trichome type	Type III	Type IV	Type V	Type VI
Type I	-.86**(175)	1**(176)	-.71**(176)	.11(176)
Type III		-.86**(175)	.75**(176)	-.07(175)
Type IV			-.85**(176)	.11(176)
Type V				-.02(176)

Number of plants are between brackets; *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

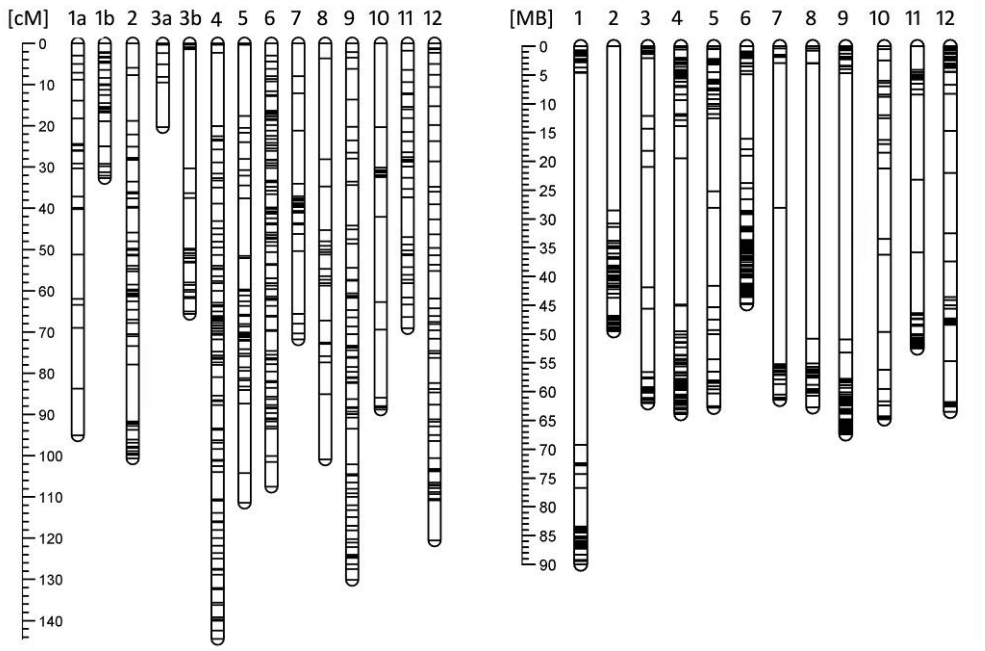

Figure 2. Genetic linkage map based on a F2 population of a *S. lycopersicum* x *S. galapagense* cross and calculated by JoinMap 4.1 package (van Ooijen, 2011) (left). In total 589 SNP markers were used, the marker positions are also given on the physical map of *S. lycopersicum* version 2.30 (Right).

QTL mapping

QTLs for AS were identified on Chromosome 2 and 9 (Figure 3). A major QTL (LOD = 28.1) was found on Chromosome 2 and named *Wf-1*. *Wf-1* explained 54.1% of genetic variation (Table 5). Another QTL (LOD = 5.8) was located on Chromosome 9 and named *Wf-2*. The *Wf-2* QTL explained 14.8% of the genetic variation. The presence of the *Wf-1* allele of the resistant parent (*Wf-1gal*) in homozygote state reduces adult survival in the F2 lines to that of the resistant parent (Table 6). In the F2 population *Wf-2* does not have an effect on AS and ratio of type IV/V trichome (R45) when *Wf-1gal* is present in homozygote state (Table 6). With the data obtained in Indonesia, only the major QTL on Chromosome 2 could be detected. The QTLs for trichome type I, III, IV and V co-localized with the QTLs for AS and OR (Table 5). The QTLs for type IV trichome density and R45 are also shown in Figure 3. The major QTL on Chromosome 2 was also associated with gland longevity and size of type IV trichomes. Additional QTLs for gland longevity and for gland size were found on Chromosome 5 and Chromosome 7 (Table 5). The trichome QTLs were also detected with the data collected in Indonesia, but with lower LOD scores.

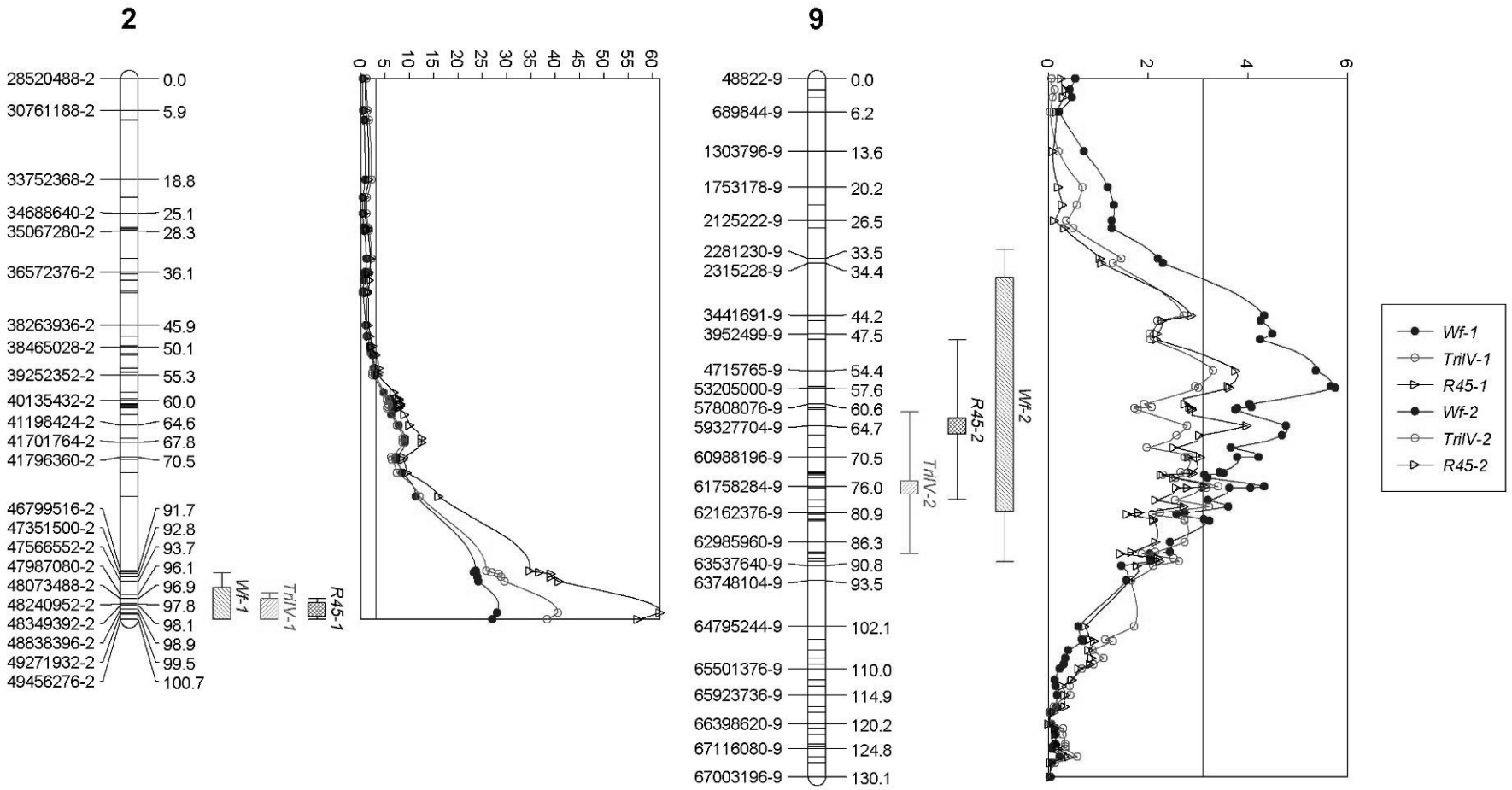

Figure 3. The whitefly resistance loci on Chromosome 2 and Chromosome 9. Adult survival (*Wf*-1, *Wf*-2), type IV trichome density (*TriIV*) and type IV divided by the sum of type IV and Type V (*R45*) are associated with both loci. The numbers left of the schematic chromosomes show the physical position of the SNP markers and the right numbers show the genetic distance in centiMorgan (cM). The LOD values are at the top of the graph.

Table 5. Results of QTL mapping for whitefly resistance and presence of different trichome types in the mapping population derived from a cross between *Solanum lycopersicum* x *S. galapagense*.

No	Traits	QTL Location	The Netherlands					Indonesia				
			Position (cM)	LOD score	% Expl.	Additive	Dominance	Position (cM)	LOD score	% Expl.	Additive	Dominance
1	Adult survival (survival.day^{-1})	Ch# 2 (*Wf-1*)	99.5	28.1	54.1	-0.41	0	98.1	15.6	65.7	-0.34	-0.27
		Ch# 9 (*Wf-2*)	57.6	5.75	14.8	-0.21	-0.07					
2	Oviposition rate (eggs.female^{-1}.day^{-1})	Ch# 2 (*Wf-1*)	98.9	19.5	41.7	-2.39	-0.13	77.9	5.96	33.6	-0.83	-0.75
		Ch# 9 (*Wf-2*)	76	4.26	11.1	-1.26	-0.28					
3	Pre-adult survival (whiteflies.egg^{-1})	Ch# 2 (*Wf-1*)	96.1	3.38	13.3	-0.14	0.01	n.d				
4	Type I presence	Ch# 2 (*Wf-1*)	98.9	31.2	56.4	0.34	0.32	93.7	6.1	21.2	0.22	0.19
		Ch# 9 (*Wf-2*)	70.5	4.91	12.2	0.19	0.11	76	3.46	12.7	0.18	0.20
5	Type III presence	Ch# 2 (*Wf-1*)	97.8	33.5	59	-1.02	-1.09	92.8	5.8	21.1	-0.31	-0.05
		Ch# 9 (*Wf-2*)	44.2	3.84	9.7	-0.39	-0.53	57.6	3.25	12.4	-0.25	0.01
6	Type IV presence	Ch# 2 (*Wf-1*)	98.9	31.2	56.4	0.34	0.32	98.9	10.6	35.1	0.29	0.21
		Ch# 9 (*Wf-2*)	70.5	4.91	12.2	0.19	0.11					
7	Type IV density	Ch# 2 (*Wf-1*)	98.9	40.9	66.3	120.01	89.11	98.9	16.8	49	80.52	14.07
		Ch# 9 (*Wf-2*)	76	3.4	8.7	51.49	3.66					
8	Type IV gland longevity	Ch# 2 (*Wf-1*)	96.1	14.5	38.9	0.77	0.25	n.d				
		Ch# 5	62.6	3.13	10.1	-0.203	0.394					
9	Type IV gland size	Ch#2 (*Wf-1*)	91.7	6.2	19.1	0.36	0.09	n.d				
		Ch# 7	37.9	3.47	11.2	0.012	0.333					
10	Type V density	Ch# 2 (*Wf-1*)	99.5	57.5	78.4	-140.85	-123.96	98.9	13.3	41.2	-80.30	-38.02
		Ch# 9 (*Wf-2*)						57.6	3.78	14.4	-0.25	0.04
11	R45	Ch# 2 (*Wf-1*)	99.5	63.1	81.5	46.15	38.82	99.5	16.8	49.3	39.08	12.81
		Ch# 9 (*Wf-2*)	64.7	3.98	10.1	16.17	16.89					
12	Type VI density	No QTL										

Notes: % Expl. = percent of explained phenotypic variation, *n.d.* = not determined

Table 6. Mean of adult survival of parents and F2 population grouped based on alleles of *Wf-1* (in chromosome 2) and *Wf-2* (Chromosome 9).

Adult survival	**Chromosome 9**		
	Wf-2lyc\ Wf-2lyc	***Wf-2lyc\ Wf-2gal***	***Wf-2gal\ Wf-2gal***
Chromosome 2			
Wf-1lyc\ Wf-1lyc	0.91 e (22)	0.87 de (16)	0.89 e (13)
Wf-1lyc\ Wf-1gal	0.66 cd (16)	0.55 c (40)	0.30 b (21)
Wf-1gal\ Wf-1gal	0.0 a (3)	0.07 ab (19)	0.08 ab (16)
S. lycopersicum	0.99 e (3)		
S. galapagense			0.0 a (3)
Ratio of type IV trichomes (R45)	**Chromosome 9**		
	Wf-2lyc\ Wf-2lyc	***Wf-2lyc\ Wf-2gal***	***Wf-2gal\ Wf-2gal***
Chromosome 2			
Wf-1lyc\ Wf-1lyc	0.04 a (22)	0.06 a (17)	0.14 a (13)
Wf-1lyc\ Wf-1gal	0.86 b (16)	0.92 b (43)	1.0 b (22)
Wf-1gal\ Wf-1gal	1.0 b (3)	1.0 b (19)	1.0 b (18)
S. lycopersicum	0.0 a (3)		
S. galapagense			1.0 b (3)

Wf-1lyc and *Wf-2lyc* are alleles of *S. lycopersicum*; *Wf-1gal* and *Wf-2gal* are alleles of *S. galapagense*. Different letters after the mean show significant different based in Duncan's multiple range test in level significance of 0.05. Number of plants is indicated in parentheses.

GC-MS and LC-MS Analysis

In the GC-MS analysis of DCM extracts of the F2 population a total of 96 metabolites were detected. The segregation of the abundance of these 96 metabolites was followed in the F2 population. A total of 16 of the 96 metabolites were clearly associated with *Wf-1* and/or *Wf-2* (Table 7). Nine metabolites had a QTL at *Wf-1* or *Wf-2*, three metabolites had QTLs at *Wf-1* and *Wf-2* of which one had a third QTL at Chromosome 3. The remaining four metabolites had a QTL at *Wf-1* or *Wf-2* in combination with another one on Chromosome 6 or 7. Table 7 also shows the effect of the alleles of *S. galapagense* or *S. lycopersicum* on the relative abundance of the metabolites. Seven metabolites were more abundant when the *S. galapagense* allele was homozygously present and seven metabolites were more abundant when the *S. lycopersicum* allele was homozygously present, two metabolites had the highest abundance in the heterozygous state. Metabolites that were more abundant in *S. galapagense* were associated with *Wf-1*, while those being more abundant in *S. lycopersicum* were not associated with *Wf-1* (Table 7). Eleven out of the sixteen metabolites could be putatively identified. Of the 644 putative metabolites in the LC-MS analysis, twenty-eight acyl sugars were identified and nine of the acyl sugars (all acyl sucroses) were found at higher

relative concentrations (*p-value* < 0.001) in the 10 most resistant F2-plants than those in the 10 most susceptible F2-plants (Figure 4).

Table 7. Metabolites co-localizing with QTLs for adult survival on chromosome 2 and/or 9.

No	Metabolites Identity	QTL description						Alleles		
		Chromosome	Position	LOD	% Explained	Additive	Dominance	*ll*	*gl*	*gg*
1	Unknown (Met 1202)	2	99.5	7.18	18.5	0.4	-0.3	2.1a	2.2a	2.9b
2	Unknown (Met1376)	2	99.5	7.98	20.3	3.0	-2.7	15.1a	15.4a	21.0b
3	Phytol (Met2279)	2	96.1	4.61	12.3	0.3	-0.3	1.9a	1.9a	2.6b
4	Isovaleric anhydride (only spectrum, no RI, Met1319)	2	99.5	17.39	39	8.8	-6.3	13.1a	15.7a	30.8b
		9	54.4	3.46	9.4	4.4	-0.5	14.1a	18.0b	22.0c
5	Unknown (Met2620)	2	92.8	4.47	11.9	-0.1	0.1	1.30b	1.32b	1.18a
		9	45.1	3.12	8.5	0.0	0.1	1.29ab	1.33b	1.22a
6	Heptacosane (Met3988)	2	96.9	6.79	17.5	27.5	-19.1	109.47a	115.46a	157.04b
		9	73.8	4.65	12.4	23.6	-7.9	109.78a	115.17a	144.20b
		3	0.3	5.08	13.4	29.4	-10.1			
7	Dodecanoic acid (Met1160)	2	99.5	11.87	28.6	15.7	-11.7	11.5a	15.5a	43.0b
		6	49.1	3.79	10.2	7.1	-10.9			
8	Dodecanoic acid chloride (Met1332)	2	99.5	17.54	39.3	4.3	-3.2	5.2a	6.2a	13.7b
		6	49.1	3.8	10.2	1.6	-2.6			
9	Tetramethyl-2-hexadecene isomer (Met1411)	9	73.5	3.36	9.1	-2.1	-0.3	16.4b	14.2a	13.2a
10	Neophytadiene isomer I (Met1487)	9	73.5	4.02	10.8	-1967.9	-231.7	17051.4b	15043.9a	13756.2a
11	Tetramethyl-2-hexadecene isomer (Met1593)	9	73.5	4.16	11.2	-26.6	-6.1	202.6b	173.4a	160.5a
12	Neophytadiene isomer II (Met1637)	9	73.5	3.89	10.5	-102.0	-14.6	882.1b	774.6a	711.4a
13	Neophytadiene isomer III (Met1825)	9	73.5	4.19	11.2	-74.4	-7.1	633.5b	555.2a	507.5a
14	Alkane (Met4051)	9	50.6	3.19	8.7	1.5	10.1	26.8a	36.2b	28.9a
15	Unknown (Met1741)	9	73.5	4.28	11.5	-28.5	-12.5	199.3b	167.5a	156.9a
		7	21.2	3.2	8.7	10.2	-33.8			
16	Unknown (Met1953)	9	73.5	3.27	8.9	-10.9	-7.3	93.24b	81.04a	77.38a
		7	16.1	3.33	9.0	4.7	-17.6			

*) likely chlorophyll breakdown product; The average of metabolite abundance is followed by letters of least significance difference (LSD) test between group based on alelles of resistant parent (*g*) and susceptible parent (*l*). Different letters indicated significantly different of mean at p-value less than 0.05.

Confirmation and reduction of QTLs in F3 population

The confirmation experiment gave somewhat different results. The effect of *Wf-1* on AS and R45 was confirmed in F3 populations (Table 8). As in the F2

population, *Wf-1gal* had a strong effect on adult survival and R45. The *Wf-2* allele in this confirmation did not affect adult survival (Table 8).

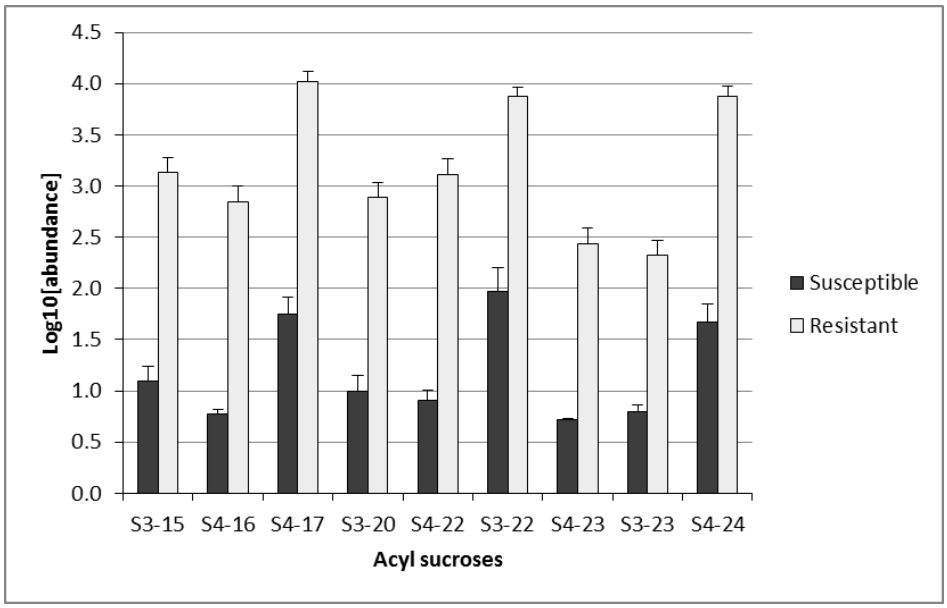

Figure 4. Abundance of acyl sugars that were significantly different (*p-value* < 0.01) between resistant and susceptible bulks. The S shows sucrose, number after S shows number of acyl groups and number after hyphenation shows total carbon atoms of all the acyl moieties

Table 8. Adult survival and percentage of type IV trichomes of parents and F3 populations grouped based on alleles of *Wf-1* (in chromosome 2) and *Wf-2* (Chromosome 9).

Adult survival	**Chromosome 9**		
	Wf-2lyc\ Wf-2lyc	***Wf-2lyc\Wf-2gal***	***Wf-2 gal\Wf-2gal***
Chromosome 2			
Wf-1lyc\ Wf-1lyc	0.99 c (5)	0.97 c (5)	0.98 c (18)
Wf-1lyc\ Wf-1gal	0.59 b (15)	0.68 b (10)	0.61 b (9)
Wf-1gal\ Wf-1gal	0.17 a (15)	0.49 b (8)	0.02 a (9)
S. lycopersicum	0.99 c (6)		
S. galapagense			0.0 a (6)
Ratio of type IV trichomes (R45)	**Chromosome 9**		
	Wf-2lyc\ Wf-2lyc	***Wf-2lyc\ Wf-2gal***	***Wf-2gal\ Wf-2gal***
Chromosome 2			
Wf-1lyc\ Wf-1lyc	0.0 a (5)	0.0 a (5)	0.0 a (18)
Wf-1lyc\ Wf-1gal	0.69 c (15)	0.36 b (10)	0.42 b (9)
Wf-1gal\ Wf-1gal	1.0 d (15)	0.99 d (8)	1.0 d (9)
S. lycopersicum	0.0 a (6)		
S. galapagense			1.0 d (6)

Wf-1lyc and *Wf-2lyc* are alleles of *S. lycopersicum*; *Wf-1gal* and *wf-2gal* are alleles of *S. galapagense*. Mean values are presented. Different letters after the mean show significant different based in Duncan's multiple range test in level significance of 0.05. Number of plants is indicated in parentheses.

Discussion

A major QTL confers high levels of whitefly resistance

Solanum galapagense PRI95004 is very resistant to whiteflies (Firdaus *et al.* 2012). The QTL mapping revealed two QTLs for adult survival, one major QTL on Chromosome 2 (*Wf-1*) and one minor QTL in Chromosome 9 (*Wf-2*). The F2 plants with *Wf-1gal* allele of *S. galapagense* in homozygous condition had an equally low adult survival as the resistant *S. galapagense* parent. During the screening in Indonesia, only the *Wf-1* locus was detected. This is probably due to the more precise screening in the Netherlands. Alternatively, the climatical conditions in the Netherlands and Indonesia may have caused the difference. Also in the number and ratio of the different trichomes, differences were seen in plants grown in the Netherlands and Indonesia. In the F2 population the locus *Wf-2* played only a role in plants heterozygous for *Wf*-1, plants with the allele *Wf-2gal* homozygous were clearly more resistant. In the F3 populations *Wf-1* was confirmed but, although it is not significantly different, it seems that *Wf-2* is also needed to get complete resistance (Table 8). Whether this was due to the small sample of the F3 populations or the presence of two clearly deviating plants is not known but this will be further investigated in future studies. The results show that a homozygous introgression with *Wf-1gal* will lead to resistant plants with low AS, OR and PS.

So far, there was no QTL detected for adult survival in tomato. However, QTLs for oviposition rate were found in *S. habrochaites* f. *glabratum* (Maliepaard *et al.* 1995) and *S. habrochaites* f. *typicum* (Momotaz *et al.* 2010). In *S. habrochaites* f. *glabratum*, two QTLs for oviposition rate of the greenhouse whitefly (*Trialeurodes vaporariorum*) were found on chromosomes 1 (*tv-1*) and 12 (*tv-2*). Besides a different location of the QTLs, the effect of the QTLs was also less obvious and probably more loci are required to get low levels of oviposition of the greenhouse whitefly in *S. habrochaites* f. *glabratum*. In *S. habrochaites* f. *typicum* (Momotaz *et al.* 2010) QTLs for oviposition rate were found on chromosomes 9, 10 and 11. The QTL on Chromosome 9 was not mapped in the same region as our *Wf-2*.

The fact that no QTLs were found in the free choice test might be caused by the unreliable resistance screening due to a low level of infestation pressure during the test. Furthermore, in a free choice test, whitefly infestation is not only the result of antibiosis and/or physical barriers, but also repellents can play a role (van Emden 2002; Bleeker *et al.* 2009).

Wf-1 and *Wf-2* co-localize with QTLs for trichomes

The QTL for the absence/presence of type I, III, IV and V trichomes and type IV properties such as gland longevity and size co-localized with the resistance QTLs. This is in agreement with previous reports where whitefly resistance was shown to be dependent on the presence of type I and IV glandular trichomes. QTLs for trichomes were identified in *S. habrochaites* (Maliepaard *et al.* 1995; Momotaz *et al.* 2010). In the Maliepaard study the QTLs for the presence of type IV and type VI trichomes were located on different chromosome as the OR QTLs showing that the lower OR of the greenhouse whitefly in *S. habrochaites* was not related to the presence of type IV or type VI trichomes. In another study (Momotaz *et al.* 2010) the QTLs for oviposition rate of *B. tabaci* and the type IV trichome density did co-localize. Although the resistance was closely related with the density of type IV trichomes, the differences in number, position and effect of the QTLs described in Momotaz *et al.* (2010) show that the resistance of *S. habrochaites* is quite different from that found in *S. galapagense*. The presence of glands distinguishes type I/III and type IV/V. The relationship among these trichome types has also been reported by others based on their inheritance and metabolomic profiles (Blauth *et al.* 1998; McDowell *et al.* 2011).

In earlier studies (Maliepaard *et al.* 1995; Momotaz *et al.* 2010), QTLs were described for type IV density on Chromosome 9, but these QTL were positioned 56.5 -59.0 Mb apart from the *Wf-2* QTL. This shows that we found a previously not identified QTL.

Whitefly resistance of *S. galapagense* and trichomes

The resistance factors do not only prevent adult survival but also reduce or prevent oviposition. Whereas, the low correlation between AS/OR and PS points to different resistance mechanisms *Solanum galapagense* showed a very low AS. The high negative correlation between type I or IV trichomes and AS show that

these trichomes play an important role in adult survival probably by the production of sticky and/or toxic exudates. Trapped whiteflies were often seen on this kind of trichomes (also described by Toscano *et al.* 2002). This supports the hypothesis that this resistance mechanism acts before feeding, which may prevent virus transmission. Negative correlations between type IV trichomes and insect survival were also reported in *S. pennellii*, *S. habrochaites* and *S. pimpinellifolium* (Channarayappa *et al.* 1992a; Dimock and Kennedy 1983; Muigai *et al.* 2003).

No resistant genotype was found without type IV trichomes (Figure 1) and the resistance was highly correlated with type IV trichome density and R45 (Table 3). However, also a number of susceptible genotypes had type IV trichomes and no type V trichome (Figure 1), showing that the resistance was not only determined by merely the presence of type IV trichomes but also by additional characters such as size, longevity and specific exudates. Similar results were also reported in accessions of *S. habrochaites* (Frelichowski and Juvik 2001; Momotaz *et al.* 2010). The very low correlation between type VI trichome and resistance showed that type VI trichomes do not play a role in our mapping population. This was different in a cross of an accession of *S. habrochaites* in which the resistance was associated with type VI trichomes (Chatzivasileiadis and Sabelis 1997; Lin *et al.* 1987).

Co-localization of QTLs for metabolites and whitefly resistance

All eight metabolites with a QTL on chromosome 2 in our study have a higher concentration when the *S. galapagense* allele is present. In other studies whitefly resistance is believed to be associated with acyl sugar production (in *S. pennellii*). Isovaleric acid and dodecanoic acid have a QTL on Chromosome 2 and might be involved in acyl sugar biosynthesis (Blauth *et al.* 1999; Walters and Steffens 1990). Unfortunately we did not map the loci for acyl sugars in our population. In an interspecific cross of *S. pennellii* and *S. lycopersicum*, five QTLs related to acyl sugars were mapped on Chromosome 2, 3, 4 and 11 (Mutschler *et al.* 1996). Two of them were located on Chromosome 2 but 5 cM and 67 cM away from *Wf-1*. Although the position of one of the QTLs was close to *Wf-1*, the effect was different; *Wf-1* has a strong effect on whitefly resistance, whereas the acyl sugar QTL had only a small additive effect on acyl sugar accumulation in *S. pennellii*

(Lawson *et al.* 1997; Mutschler *et al.* 1996). In another study with an intraspecific cross of two *S. pennellii* accessions, at least 12 QTLs were found for type IV trichome density and acyl sugar concentration on Chromosomes 2, 3, 4, 5, 6, 7, 9, 10 and 11 (Blauth *et al.* 1998). These QTLs included the five QTLs from the study of Mutschler *et al.* (1996). These studies showed that acyl sugar production is controlled by many genes in *S. pennellii*. We identified only two QTLs regulating the resistance and resistance related metabolites. This simple genetic model might be due to the fact that only a small number of acyl sugars is involved in the resistance originating from the *S. galapagense* accession, or that there is a regulator gene which is able to activate many genes for acyl sugar production in *S. lycopersicum*. The involvement of acyl sugars in the resistance is supported by the high production of acyl sucroses in the ten resistant F2 plants.

The metabolites with a QTL on Chromosome 9 and not on chromosome 2 had lower relative concentrations in the presence of the *S. galapagense* allele. These compounds were neophytadiene isomer I, II and III, and tetramethyl-2-hexadecene isomers and seem to enhance whitefly susceptibility in the cultivated tomato.

Inheritance of whitefly resistance and trichomes

Overall the results from the screenings in Indonesia were not as clear as those in the Netherlands, especially in the free-choice test different responses to whitefly infestation were seen. This might be due to the different whitefly biotypes that were used in the Netherlands and Indonesia. In pepper accessions that were tested in both countries with the same whitefly biotype, the whitefly resistance values were highly correlated (r = 0.61 to 0.82) (Firdaus *et al.* 2011). The difference might be caused by differences in the environment that also resulted in differences in the ratio of trichomes and mean AS in the same mapping population in the two countries. For F1 plants, R45 was different in the Netherlands and Indonesia. The presence of type IV trichomes was dominant in the Netherlands and intermediate in Indonesia. An interdependent presence of type IV and V trichomes was also found in mapping populations of *S. lycopersicum* x *S. habrochaites* (Maliepaard *et al.* 1995; Momotaz *et al.* 2010; Snyder and Carter 1984) and an intraspecific cross of *S. pennellii* (Blauth *et al.* 1998). The presence and density of type IV trichomes

are known to be influenced by light intensity and leaf age (Wilkens *et al.* 1996), but other factors like humidity and/or temperature may also play a role in the formation of the different trichomes. Our results show that the presence of type IV trichomes was controlled dominantly by one gene, but there might be interaction of genes for the ratio of type IV/V trichomes (R45). Lenke and Mutschler (1984) also found that the inheritance of type IV trichomes in an interspecific cross of *S. lycopersicum* x *S. pennellii* was not complex and segregation patterns indicated that only two unlinked genes were involved. The same trichome inheritance was also observed in a study with *S. habrochaites* f. *glabratum* (Freitas *et al.* 2002).

Metabolites related to whitefly resistances

Mono- and sesquiterpenes, methyl ketones and acyl sugars are secondary metabolites that are known to be associated with whitefly resistance in tomato (Chatzivasileiadis and Sabelis 1997; Eigenbrode *et al.* 1994; Fancelli *et al.* 2005; Farrar and Kennedy 1991; Frelichowski and Juvik 2001; Lin *et al.* 1987; Mutschler *et al.* 1996; Oriani and Vendramim 2010; Rodriguez-Lopez *et al.* 2011). Monoterpenes such as p-cymene are abundantly present in *S. pennellii* LA716 (Bleeker *et al.* 2009). Monoterpenes also play a role as repellent of *B. tabaci* (Bleeker *et al.* 2009) and western flower thrips (*Frankliniella occidentalis*) (Janmaat *et al.* 2002). In our study we did not find QTLs for monoterpenes located on Chromosome 2 and/or Chromosome 9. Phytol, a diterpenoid compound, is one of the putative whitefly resistance related metabolites. The role of phytol in resistance might be that phytol is a precursor of tocopherols (Valentin *et al.* 2006) that play a role as antiherbivory agent (Neupane and Norris 1991). Neophytadiene isomers I, II and III, and tetramethyl-2-hexadecene isomers were measured and QTLs on Chromosome 9 were found. These compounds are metabolites resulting from the degradation of phytol (Didyk *et al.* 1978). However, the observed presence of neophytadienes may be an artefact of the analysis (Grossi *et al.* 1996). Heptacosane is a metabolite that can be highly produced in tomato (Srinivasan *et al.* 2006) and is the main constituent of the leaf cuticle (Reina-Pinto and Yephremov 2009). So far, this compound was reported in studies on the oviposition rate in *Helicoverpa armigera* (Srinivasan *et al.* 2006) and as attractant to parasitoids (Paul *et al.* 2008).

One of the other identified metabolites was dodecanoic acid, a free fatty acid compound, that is one of intermediate metabolites of methyl ketones. In *S. habrochaites* f. *glabratum* a fatty acid can be hydrolysed and decarboxylated into a methyl ketone (Fridman *et al.* 2005). However, in our population we did not find any difference in the concentration of methyl ketones between susceptible and resistant genotypes. Therefore, it is not likely that methyl ketones play a role in the whitefly resistance coming from *S. galapagenese*. Dodecanoic acid might also be one of intermediate metabolites of acyl sugars. Fatty acid compounds are very abundant in glandular trichomes of *S. pennellii* LA716 and the main constituent in acyl sugar biosynthesis (Blauth *et al.* 1999; Mutschler *et al.* 1996; Walters and Steffens 1990). We showed that some acyl sugars in the resistant bulk of F2 plants were present in much higher concentrations (Figure 4) indicating that the production of acyl sugars was the main resistance factor in *S. galapagense*.

Prospects for resistance breeding in cultivated tomato based on the *Wf-1* locus.

The resistant parent (*S. galapagense*) and cultivated tomato (*S. lycopersicum*) are close relatives that are grouped in the same clade of the phylogenetic tree (Rodriguez *et al.* 2009). The use of a more closely related species in introgression breeding makes breeding more straight forward because the differences between the parents are less complex and therefore makes the genetics behind the resistance less complex. The difference between almost complete resistant plants and almost complete susceptible plants could be traced back to two loci: *Wf-1* and *Wf-2*. Since the resistance, trichome properties and some metabolites all map in these regions it is likely that a major regulatory gene is involved that affects all traits mentioned.

The *Wf-1* locus is located at the bottom of Chromosome 2. This region is on the genetic linkage map 7.8 cM and 2.5 Mb on the physical map. In tomato, the gene density in euchromatin region is about 6.7 kb/gene (Wang *et al.* 2006). Therefore, this region would cover approximately 370 genes and this was confirmed with the known annotated tomato sequence where approximately 360 genes are predicted in this region (http://solgenomics.net). In future studies we will carry out recombinant screens to further delineate the chromosomal fragment harboring *Wf-*

1gal. Better characterisation of the resistance will make a very focussed breeding possible resulting in commercial varieties with very high levels of whitefly resistance based on the *S. galapagense* source.

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Chapter 6

General Discussion

The direct and indirect damage of whiteflies in tomato and pepper growing areas can seriously reduce yield. No whitefly resistant cultivars are on the market yet although resistance has been identified in wild relatives of tomato and pepper (Fancelli and Vendramim 2002; Muigai *et al.* 2002; Muigai *et al.* 2003; Toscano *et al.* 2002; Maruthi *et al.* 2003; Baldin *et al.* 2005; Laska *et al.* 1986). No resistant varieties have been developed because the breeding for whitefly resistance in tomato and pepper has been hampered due to the complexity of the resistance. To unravel this complexity we evaluated whitefly life history parameters such as adult survival and oviposition rate, studied the occurrence and properties of trichomes as well as secondary metabolite profiles. We started off with evaluating germplasm form pepper and tomato for whitefly resistance. Based on the results from these evaluations it was decided to focus further research on tomato.

For more detailed genetic studies the best and most promising accession of a closely related wild tomato from the Galapagos Islands, *S. galapagense* PRI95004 was chosen and crossed with *S. lycopersicum*. Choosing a taxonomically very close relative of tomato is expected to make the introduction of the resistance in modern varieties less complex and therefore more feasible. After selfing of the F1-hybrid an F2 mapping population of 189 individuals was obtained. The resistance level and resistance related traits were measured in the individual plants of the mapping population. Each plant was also genotyped with more than a thousand polymorphic single nucleotide polymorphism (SNP) markers. QTL mapping studies based on the phenotyping and genotyping data resulted in the identification of chromosomal regions harbouring loci related to whitefly resistance and resistance related traits. Additional to the screening and mapping studies the implications of the large diversity present in whitefly populations around the world is discussed.

Reliable assessment of whitefly resistance in tomato and pepper

There are several parameters that can be used to assess levels of whitefly resistance in plants. They all measure different life history parameters, such as adult survival, oviposition rate, pre-adult survival, developmental period in no-choice tests or density of adult whiteflies, eggs, nymphs and pupal cases in choice

tests (Toscano *et al.* 2002; Fancelli and Vendramim 2002; Muigai *et al.* 2003; Oriani and Vendramim 2010; Romanow *et al.* 1991; Erb *et al.* 1994). Differences between resistance parameters can be caused by different resistance mechanisms underlying the different traits, which in turn can depend on single or multiple genes and these genes affect often more than one resistance parameter. By studying individual resistance parameters in mapping populations based on crosses between the most resistant source and the cultured tomato we aimed at unravelling the genetic basis of the resistance.

For the resistance assessment, we did choice and no-choice tests which allowed us to evaluate resistance parameters based on the different stages of whitefly life history. The parameters were determined by using screen house and clip-on cage tests (*in vivo*) in combination with detached branch and detached leaf disc tests (*ex vivo*). Our results showed that the parameters used in the *in*- and *ex vivo* tests are highly correlated, showing that *ex vivo* tests can be used as a simple and efficient method to evaluate whitefly resistance.

In the no-choice tests, adult survival and oviposition rate are highly correlated. The correlation with pre-adult survival is lower indicating that different mechanisms might play a role. In no-choice situations the prevention of feeding by plant characteristics may result in the death of most of the whiteflies. Such plant characteristics can be physical barriers such as the sticky glandular trichomes (Toscano *et al* 2002), cuticle thickness (Channarayappa *et al.* 1992b) or the production of toxic compounds such as terpenoids (Maluf *et al.* 2001; Freitas *et al.* 2002), methyl ketones (Dimock and Kennedy 1983; Chatzivasileiadis and Sabelis 1997) and acyl sugars (Goffreda *et al.* 1989; Liedl *et al.* 1995; Rodriguez-Lopez *et al.* 2011). In a no-choice test all these plant traits make the overall living conditions for the whiteflies poor, resulting in lower adult survival and oviposition rate. Resistance factors which specifically affect one parameter also seem to be present, as was shown by the poor correlation between adult survival/oviposition rate and pre adult survival. Similar observations have also been made in other studies (Romanow *et al.* 1991; Bas *et al.* 1992; van Giessen *et al.* 1995).

In choice tests, there is a low correlation between egg and whitefly density and a higher correlation between egg and nymph density. This suggests that plant

characteristics prevent whiteflies (although they are present) to lay eggs, but no mechanisms to prevent the development of the eggs into nymphs. Whitefly preference is the first step in a whitefly infestation, preference factors can be leaf characters such as color, shape, pubescence or trichome density, cuticle thickness, plant age and plant architecture (Bellotti and Arias 2001; Sippell *et al.* 1987; McAuslane 1996; Snyder *et al.* 1998; Toscano *et al.* 2002; Channarayappa *et al.* 1992b; Walker and Perring 1994). Headspace compounds of the leaf play an important role as whitefly attractant or repellent (Chermenskaya *et al.* 2009; Bleeker *et al.* 2009). Preference might also be due to "recognizing" the suitability of the host plant for nymph survival (Nomikou *et al.* 2003; van Lenteren and Noldus 1990). This hypothesis is supported by our results which showed a high correlation between egg density and nymph density. Whitefly feeding and egg laying apparently can be on different positions of the plants or on different plants (van Lenteren and Noldus 1990).

The high correlation between adult survival in no-choice tests and whitefly and egg density in choice test points at antibiotic factors as the main causes for non-preference in choice situations. A resistance mechanism that inhibits whitefly feeding will also reduce virus transmission.

Whitefly resistance in pepper and tomato

Whitefly resistance in pepper was related to cuticle thickness and glandular trichome density. A thick cuticle was found in some commercial cultivars of peppers such as *C. annuum* California Wonder and *C. annuum* Yolo Wonder which show higher levels of resistance. Glandular trichomes are believed to play an important role in resistance to whiteflies in tomato but glandular trichomes in tomato are quite different from those in pepper. In pepper they are much smaller than the size of any of the trichome types in tomato. The glandular trichomes in pepper were mostly present on the glabrous leaves with a thick cuticle, which was not preferred by whiteflies. Whitefly resistance in pepper is supposedly much more caused by cuticle thickness than by the presence of glandular trichomes. The resistant accession *C. annuum* CM33, which has a thin cuticle and hairy leaves without glandular trichomes was an exception. This accession probably has a different resistance mechanism.

The types of trichomes play an important role in whitefly resistant tomatoes. Glandular trichomes, especially type I, IV and VI, were abundant on resistant accessions of species such as *S. habrochaites* f. *glabratum, S. habrochaites* f. *typicum, S. pimpinellifolium* and *S. pennellii* (Erb *et al.* 1994; Mutschler *et al.* 1996; Fancelli and Vendramim 2002; Muigai *et al.* 2003; Oriani and Vendramim 2010; Rodriguez-Lopez *et al.* 2011; Chatzivasileiadis and Sabelis 1997; Antonious 2001; McDowell et al 2011; Chapter 4). In the resistance screening we found resistant accessions from *S. habrochaites* f. *glabratum, S. pimpinellifolium* and *S. galapagense*. *Solanum galapagense* was the most resistant accession with a high density of type IV trichomes. This accession had never before been reported as resistant to whiteflies. Other resistance mechanisms (non-glandular trichome-based resistance) were found in *S. arcanum* CGN 14355, *S. glandulosum* CGN14358 and *S. arcanum* CGN15392 which combined the absence of type IV trichomes with a reduction of pre-adult survival.

Glandular trichomes are required for a high level of whitefly resistance in tomato

High levels of whitefly resistance were found in accessions with an abundance of type IV trichomes and tomato accessions without type IV trichomes never had a high resistance level. In the F2 population between *S. galapagense* and cultivated tomato all resistant genotypes had a high density of type IV trichomes (Chapter 5). These results show the important role of the glandular trichome in obtaining high levels of resistance. Glandular trichomes might act as a physical barrier, interfering with whitefly landing, or with feeding and oviposition (Dimock and Kennedy 1983; Snyder and Carter 1984; Channarayappa *et al.* 1992a).

The presence of type IV trichome was positively correlated with the presence of type I trichomes and negatively correlated with type III and/or type V trichomes. The high correlation between type IV and type I makes it impossible to analyse the individual roles of these trichome types. The similarity of type I and IV trichomes also can be seen in their metabolite content (McDowell *et al.* 2011). Although type IV trichomes play an important role in whitefly resistance, the

presence and density of type IV trichomes cannot be used as the only indicator for whitefly resistance. In our mapping population 24.5% (41 genotypes) of the F2 population had a high density type IV trichomes but were not resistant. Similar observations were made for *S. habrochaites* f. *typicum* LA1033. This accession was reported susceptible to *Helicoverpa zea* and *Spodoptera exigua* although it had the same glandular trichomes as the resistant accession *S. habrochaites* f. *typicum* LA1777 (Frelichowski and Juvik 2001).

Quality and content of trichomes determine whitefly resistance in tomato

Trichome exudates are believed to be the main resistance factor. The difference in the level of resistance between *S. habrochaites* LA1777 and LA1033 is caused by different trichome exudates (Frelichowski and Juvik 2001). To see whether this is also the case in our mapping population we collected leaf material of individual plants and did a metabolomic analyses with GC-MS and looked for associations between individual metabolites and whitefly resistance. Another method to look for metabolomic compounds is LC-MS, we used this method to analyse the ten most resistant and most susceptible individuals of the F2 population. Especially acyl sugars were found to be different between the two groups. Acyl sugars were present in higher concentrations in the resistant plants. Acyl sugars are sticky exudates and known to contribute to whitefly resistance in *S. pennellii* and *S. pimpinellifolium* (Freitas *et al.* 2002; Resende *et al.* 2002; Fancelli and Vendramim 2002; Fancelli *et al.* 2005; Rodriguez-Lopez *et al.* 2011). Other trichome exudates such as terpenes and methyl ketones are also known to have a negative effect on whitefly infestation (Freitas *et al.* 2002; Bleeker *et al.* 2009; Chatzivasileiadis and Sabelis 1997; Antonious 2001; Fridman *et al.* 2005), but they were not found in high concentrations in our resistant plants and also no QTL co-localization of these metabolites and resistance was found in our F2-population.

Other characteristics of trichomes are size and longevity (of type IV trichomes). Size and longevity of glandular trichomes might contribute to the resistance. Such trichome characteristics might explain our finding that accessions of *S.*

habrochaites were resistant in the first week after infestation, but afterwards (three to five weeks) they became more susceptible. That plant age can affect the level of whitefly resistance was already known (Bas *et al.* 1992). The mapping study with our F2 population showed that trichome size and longevity are negatively correlated with whitefly adult survival.

Genetic background of whitefly resistance in *S. galapagense* PRI95004

An advantage of *S. galapagense* is that it is genetically very close to commercial tomato (Rodriguez *et al.* 2009) which is expected to make the introgression of the whitefly resistance trait through introgression breeding less complex. So far, QTLs for oviposition rate have been identified in *S. habrocahiates* and *S. pennellii* (Maliepaard *et al.* 1995; Momotaz *et al.* 2010; Mutschler *et al.* 1996; Lawson *et al.* 1997) which are not closely related to the cultivated tomato (Rodriguez *et al.* 2009).

A high density genetic map based on about 1000 SNP markers was made using the F2-population and QTL mapping revealed two chromosomal regions associated with resistance. The first and major QTL (*Wf-1*) is located at the bottom of Chromosome 2 and the second one (*Wf-2*) is located on Chromosome 9. The major QTL *Wf-1* can determine full resistance if present in homozygous state. The identified QTLs for many resistance related parameters such as presence and density of type I, III, IV and V trichomes, glandular head size and head longevity of type IV trichomes, co-localized with the QTLs for adult survival and oviposition rate. Also differences in the concentration of some metabolites found with GC-MS analysis were associated with the same resistance locus, the concentration of eight metabolites was associated with *Wf-1* and the concentration of eight other metabolites with *Wf-2* and not with *Wf-1*. The metabolite QTLs on Chromosome 2 are likely to be associated with whitefly resistance because the metabolites are precursors of acyl sugars, like isovaleric acid and dodecanoic acid (Blauth *et al.* 1999; Walters and Steffens 1990). Metabolites QTLs only associated with *Wf-2* might be involved in processes like chlorophyll breakdown. The production of these metabolites may enhance the susceptibility of cultivated tomato to whitefly.

The number, chromosome locations and/or resistance effects of QTLs for whitefly resistance earlier described in *S. habrochaites* f. *glabratum* (Maliepaard *et al.* 1995), *S. habrochaites* f. *typicum* (Momotaz *et al.* 2010) and for acyl sugar production in *S. pennellii* (Mutschler *et al.* 1996; Lawson *et al.* 1997) are different from the QTLs identified in our study with *S. galapagense*. The QTLs for whitefly resistance in *S. galapagense* are stronger and fewer in number than in the previous studies. The genetic background of whitefly resistance in *S. galapagense* is therefore likely to be different from the previously identified QTLs in other species. The co-localisation of all the resistance related QTLs suggests a major regulator gene that influences several different metabolomic pathways. The *Wf-1* region is about 7.8 cM on the genetic linkage map and 2.5 Mb on the physical map and contains about 360 genes. The candidate gene(s) for whitefly resistance may be one of the identified genes in *S. lycopersicum*, but it may also be that the gene conferring resistance has no homologue in the genome of cultivated tomato and therefore it should be identified through fine mapping and sequencing of the resistant accession. Fine mapping should be relatively easy since there is a high level of recombination in this region. Ultimately this will lead to cloning and further characterisation of the major gene (allele) from *S. galapagense* responsible for the high level of resistance to *B. tabaci*.

Bemisia tabaci diversity and the implication for whitefly resistance

Bemisia tabaci is known as a morphologically cryptic species complex (Frohlich *et al.* 1999; Perring 2001; De Barro 2012). Different species have been identified based on the sequence of a fragment of the mitochondrial cytochrome c oxidase subunit I (*mtCOI*). The grouping is based on a 3.5% threshold value of genetic divergence for the *mtCOI* sequence and so far thirty-six species have been recognized (Chapter 2). This number may increase with increasing number of *B. tabaci* samples from areas of the world which have not been sampled yet. Two of the 36 groups (Asia II 12 and Asia IV) were added because of our sampling in Indonesia and China and five groups of the 36 (Asia II 11, Japan 1, Japan 2, African, SubSahara Africa 5) were added as we reanalysed all recently added data (November, 30th 2010) in the database (www.ebi.ac.uk). That several of these presumed species are true species was shown by mating incompatibility (Brown *et*

al. 1995a, Frohlich *et al.* 1999; De Barro *et al.* 2005; Xu *et al.* 2010; Wang *et al.* 2011). However, it is still largely unclear if the 3.5% threshold used to delimit species is correct. It may in fact even be too stringent as reproductive incompatibilities were also shown between *B. tabaci* populations with less than 3.5% divergence (De Barro and Ahmed 2011; De Barro 2012). Therefore, it was suggested to use other genes as well for species delimitation (Boykin *et al.* 2012; De Barro 2012).

Haplotyping whitefly populations is interesting, especially to study differences in invasiveness, effectiveness of natural enemies and transmitted viruses (De Barro 2012). We found 387 haplotypes in the database and nineteen extra haplotypes through our sampling. Knowledge about whitefly haplotypes is of utmost importance in the management of this pest and its related diseases, especially to identify invasive and dangerous haplotypes.

The diversity of *B. tabaci* shows that this species, especially the universal groups, can adapt easily to a new location or habitat. *Bemisia tabaci* is reported to be able to easily develop resistance to pesticides (Dittrich *et al.* 1990; Cahill *et al.* 1995; Crowder *et al.* 2006; Erdogan *et al.* 2008). Moreover, *B. tabaci* is a generalist pest that attacks hundreds of host plants species. The diversity of *B. tabaci* is a challenging aspect in the development of resistant tomato cultivars. During the resistance screenings in our study we used the most invasive haplotype (B-Biotype/ Mediterranean-Middle East-Asia Minor 1) and an Indonesian haplotype. It will be interesting to see if the whole *Bemisia* complex is susceptible to the resistance we identified in *S. galapagense.* Nevertheless, a good agricultural practice should use resistant cultivars in combination with other management measures such as avoiding monocultures and natural enemies (Broekgaarden *et al.* 2011).

Concluding remarks

Whitefly resistance screening methods and parameters were evaluated in Indonesia and the Netherlands. Detached leaf assays on tomato and pepper are good alternative methods to complex *in vivo* tests. Detached leaf tests also allow conducting the test in more controlled environments.

We have identified sources of resistance towards whitefly in accessions of pepper and tomato. In tomato, the resistance is highly correlated with density and type of glandular trichomes, but other resistance mechanism might also play a role.

The most resistant tomato accession was *S. galapagense* PRI95004 which was completely resistant. In this accession, an antibiotic factor highly affects adult survival and oviposition rate in no-choice tests and whitefly and egg density in choice tests. The level of transmission of viruses was not studied, but based on results obtained in *S. pimpinellifolium* (Rodriguez-Lopez et al 2011) it is expected to be lower than on cultivated tomato. The introgression of the resistance factors into cultivated tomato might be easier and possibly with less linkage drag as *S. galapagense* is closely related to cultivated tomato.

The presence and a sufficient number of type I and/or IV trichomes is required for resistance in *S. galapagense* but the content of the trichome is also very important for resistance. One major QTL (*Wf-1*) on Chromosome 2 was associated with adult survival, oviposition rate, type I, III, IV and V trichome presence/density, size and longevity of glandular trichome heads and several metabolites. When this QTL is homozygously present the plants are fully resistant. A minor QTL (*Wf-2*) was identified on Chromosome 9. Our study on *B. tabaci* populations showed that *B. tabaci* is a diverse species complex containing at least 36 different species. It remains to be established if the identified source of resistance from *S. galapagense* will be effective against all cryptic *B. tabaci* species.

Phenotypic characteristics and the genetic basic of the whitefly resistance present in *S. galapagense* have been studied in this thesis. Further investigations are needed to identify the genes involved and elucidate the resistance mechanism. It is expected that all information together will allow the development of whitefly resistant tomato varieties via classical breeding.

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Summary

Whiteflies (*B. tabaci* Genn.) are a serious threat in tomato and pepper cultivation. The most prominent damage is related to their role as virus vector. Whiteflies also cause direct damages such as silvering of leaves, irregular ripening of fruits and nutrition deficiency. To control whiteflies pesticides and biological control have been used. However, pesticides are not only dangerous to humans and the environment, but also result in a quick development of whiteflies resistant to the insecticide. Biological control is difficult in open field cultivation, such as often is the case in Indonesia. Plant resistance may offer a more lasting and more effective control. In the modern germplasm of tomato and pepper no good resistance against whiteflies is available. Crossable wild relatives of tomato (and pepper) with high levels of resistance offer a solution. However, the introgression of resistance factors from wild relatives into cultivated tomato turned out to be difficult and no resistant cultivars are available yet. Few studies have been performed on whitefly resistance in pepper. In our study we covered different aspects of the whitefly-plant interaction, including the characterization of different whitefly populations, evaluation of whitefly resistance parameters, screening tomato and pepper germplasm and genetic studies based on the most resistant wild tomato accession. The objective of this research was to identify genetic variation in tomato and pepper accessions for the level of resistance to whitefly. As a follow up a more detailed genetic study involving the most resistant tomato source was executed. Ultimately this has to lead to the development of resistant commercial tomato cultivars.

This thesis does not only describe the current knowledge of whitefly resistance in tomato and pepper but does describe also the genetic diversity and taxonomical status of *B. tabaci* based on a fragment of the mitochondrial cytochrome oxidase 1 gene. In total, 387 unique haplotypes were identified from a total of 1089 sequences that were available on November, 30th 2010. An additional fifteen haplotypes were identified in the fifty whitefly populations we

collected in Indonesia, Thailand, India and China in 2008. Phylogeny reconstruction of the *B. tabaci* complex based on these sequences resulted in the identification of 36 groups with a genetic divergence of more than 3.5%, which are considered to be cryptic species. However, other researchers have also found mating incompatibility between groups that were less than 3.5% divergent from each other, showing the need of further investigations on species delimitation in the *B. tabaci* species complex.

In total forty-four accessions of peppers were evaluated for whitefly resistance in the Netherlands and Indonesia. Plants characteristics supposedly related to resistance were cuticle thickness and antibiotic factors in the leaves. Some accessions derived from *C. annum* California Wonder with a thick cuticle were resistant indicating that thick cuticles possibly cannot be punctured by whiteflies. However, *C. annuum* CM331 has a thin cuticle and was also resistant showing that other resistance mechanisms can be present as well.

In Chapter 4, a total of 53 tomato accessions representing 11 species was screened for whitefly resistance. The level of resistance was assessed by looking at different parameters for the whitefly life cycle such as adult survival, oviposition rate, number of nymphs and pupal cases. In the best source of resistance adult survival was severely reduced (up to 90% dead flies after one day). This resistance was highly correlated with the density of type I and IV glandular trichomes. These trichomes might be the most effective to protect the plant from whiteflies. Our results also showed that the amount of honeydew was correlated with nymph density. The highest level and most durable resistance was found in an accession of *Solanum galapagense*. Other accessions which showed resistance to whiteflies were accessions from *S. pimpinellifolium* and *S. habrochaites*. However, the level of resistance in *S. habrochaites* became less on older plants. Other resistances which are not based on glandular trichome might be present in accessions of *S. glandulosum* and *S. arcanum* which combined the absence of type IV trichomes with a reduction in pre-adult survival of the whitefly. Resistance parameters and assessment were evaluated which resulted in accurate evaluation on whitefly resistance in pepper and tomato accessions. *In*

vitro tests with detached leaves turned out to be a good alternative for whitefly resistance screenings.

A genetic study of resistance and resistance related traits was based on the resistance in *S. galapagense* PRI95004. This accession was chosen because it was the most resistant accession and a very close relative of cultivated tomato. The F2 mapping population showed a correlation between low adult survival and low oviposition rate. Type IV trichomes were essential for the resistance. However, genotypes with a high density of type IV trichomes are not necessarily resistant meaning that the contents of trichome glands also play an important role. The ratio between type IV and V trichomes, size and longevity of trichome glands and metabolite content all are important parameters. Type V trichomes on the leaf made the plant more susceptible. A SNP based, high density genetic linkage map was used for a QTL mapping study. Two QTLs for adult survival and oviposition rate were found. The first locus (*Wf-1*) is located on Chromosome 2 and the second one (*Wf-2*) is located on Chromosome 9. *Wf-1* has a very strong effect on the resistance and in homozygous condition results in a complete resistance. The QTLs were confirmed in an F3 mapping population. But the interaction between the QTLs in different experiments needs to be further investigated for a better understanding of the genetic control. Surprisingly, all traits that were related to resistance were located at the same positions as the QTLs for adult survival and oviposition rate. A number of metabolites that were identified through GC-MS analysis also mapped in these regions. Some of the metabolites that co-localized with *Wf-1* were related to acyl sugar biosynthesis. This was confirmed by the results of LC-MS analysis, showing that the concentration of nine acyl sugars in the ten most resistant F2 genotypes was much higher than that of the ten most susceptible genotypes. The metabolites co-localizing with *Wf-2* were related to other biological processes such as the suppression of chlorophyll breakdown.

Our results show that the genetic background of whitefly resistance in *S. galapagense* was controlled by two loci, and that the major locus is sufficient to confer full resistance, when present in homozygous state. This locus might be a regulator gene that controls multiple genes involved in resistance related traits. The position of *Wf-1* in a chromosomal region with a high level of recombination

will make it feasible to clone and further study the gene responsible for the very high level of resistance in *S. galapagense*. But even without cloning the prospects of breeding for cultivars with high levels of whitefly resistance are very promising.

Samenvatting

Wittevliegen (*B. tabaci* Genn.) zijn een serieuze bedreiging in tomaten- en peperteelten. De meeste schade wordt veroorzaakt doordat wittevliegen veel virussen verspreiden. Wittevliegen veroorzaken ook directe schade zoals het verkleuren van bladeren (*silver leaves*), het onregelmatig rijp worden van vruchten en een tekort aan nutriënten voor de plant. Om wittevliegen te bestrijden worden zowel pesticiden als biologische maatregelen gebruikt. Pesticiden zijn echter ook gevaarlijk voor mens en milieu en resulteren vaak in een snelle ontwikkeling van resistentie bij de wittevlieg. In Indonesië worden pepers en tomaten veelal geteeld in het open veld wat biologische bestrijding moeilijk maakt. Resistentie van de plant kan mogelijk een langduriger en effectievere controle geven. In moderne rassen van tomaat en peper zijn geen voorbeelden te vinden van rassen met een goede resistentie. Kruisbare wilde verwanten van tomaat (en peper) met hoge resistentieniveaus bestaan er wel en bieden een oplossing. Het inkruisen van resistentiefactoren uit deze wilde verwanten bleek echter moeilijk te zijn en heeft tot nu toe niet geresulteerd in resistente rassen. Er zijn maar enkele studies gedaan aan wittevliegresistentie in peper. In deze studie behandelen we verschillende aspecten van de wittevlieg-plant interacties zoals het karakteriseren van verschillende wittevlieg populaties, het evalueren van wittevlieg resistentie parameters in de tomaten en peper genenpool en genetische studies. Het doel van het onderzoek was om in tomaten en peper accessies genetische variatie voor (het niveau van) wittevliegresistentie te vinden. De meest resistente accessie van tomaat is vervolgens gebruikt voor kruisingen en genetische studies. Uiteindelijk moet dit leiden tot de ontwikkeling van resistente tomatenrassen.

Dit proefschrift beschrijft niet alleen de huidige kennis over wittevliegresistentie in tomaat en peper maar behandelt ook de genetische diversiteit en taxonomische status van de wittevlieg (*B. tabaci)* zelf. Dit werd gedaan met behulp van de sequentie van een fragment van het mitochondrieel cytochrome oxidase 1 gen. In totaal werden 387 unieke haplotypes geïdentificeerd

in een publiek toegankelijke database met 1089 sequenties. Aan deze set werden vijftien haplotypen toegevoegd die we zelf hadden gevonden in de door ons verzamelde vijftig wittevliegpopulaties. Deze populaties zijn verzameld in Indonesië, Thailand, India en China in 2008. Een fylogenetische studie van het *B. tabaci* complex gebaseerd op bovengenoemde sequenties resulteerde in 36 groepen met een genetische divergentie van meer dan 3.5%, wat als grens genomen wordt om te kunnen praten over verschillende soorten. Dat deze grens arbitrair is laten andere onderzoekers zien die kruisingsincompatibiliteit vonden tussen groepen met minder dan 3.5% divergentie. Meer onderzoek aan de indeling in het *B. tabaci* species complex is zeker nodig.

In totaal zijn 44 accessies van pepers geëvalueerd op wittevlieg resistentie in Nederland en Indonesië. Plant eigenschappen zoals de dikte van de waslaag op het blad en antibiotische factoren in de bladeren kunnen gerelateerd zijn aan hogere niveaus van resistentie. Sommige *C. annuum* California Wonder afgeleide rassen met een dikke waslaag waren resistent, dit suggereert dat een dikke waslaag mogelijk niet doorboord kan worden door wittevliegen. Maar dit is niet het enig mogelijk mechanisme, *C. annuum* CM331 heeft een dunne waslaag en was toch resistent.

In Hoofdstuk 4 wordt de screening op wittevliegresistentie besproken van in totaal 53 tomatenaccessies die elf soorten vertegenwoordigden. Het niveau van resistentie werd bepaald aan de hand van verschillende parameters zoals overleving van volwassen vliegen, eileg, het aantal nimfen en poppen. In de beste bron van resistentie was de overleving van volwassen wittevliegen sterk gereduceerd (tot 90% mortaliteit na 1 dag). Deze resistentie was sterk gecorreleerd met de dichtheid van type I en IV glandulaire trichomen. Deze trichomen blijken de meest effectieve manier zijn om de plant te beschermen tegen wittevliegen. Onze resultaten laten ook zien dat de hoeveelheid honingdauw gecorreleerd is met nymfdichtheid. Het hoogste resistentieniveau werd gevonden in een accessie van *Solanum galapagense*. Andere accessies met verhoogde resistentieniveaus waren *S. pimpinellifolium* en *S. habrochaites* accessies. Het niveau van resistentie in *S. habrochaites* daalde bij het ouder worden van de plant. Resistenties die niet gebaseerd waren op glandulaire trichomen zijn gevonden in

accessies van *S. glandulosum* en *S. arcanum*, hier waren geen type IV trichomen maar er was wel een lagere overleving van jongere wittevliegen. Het bepalen van resistentieparameters resulteerde in een betrouwbare evaluatie van wittevliegresistentie in tomaat en peper. *In vitro* testen met losse bladeren bleken een goed alternatief voor de resistentiebepalingen.

De genetische studie naar de achtergrond van de resistentie was gebaseerd op de resistentie in *S. galapagense* PRI95004. Deze accessie was gekozen omdat het de meest resistente accessie was en omdat het behoort tot een erg nauw verwante soort van de cultuurtomaat. In de F2 karteringspopulatie werd een correlatie gevonden tussen een lage overleving van volwassen vliegen en een lage eileg. Type IV trichomen waren essentieel voor de resistentie maar er werden ook niet resistente genotypen waargenomen met een hoge dichtheid van type IV trichomen. Dit geeft aan dat ook de inhoud van de glandulaire trichomen een belangrijke rol speelt. De verhouding tussen type IV en V trichomen, maar ook grootte en duurzaamheid van de klieren en aanwezigheid van bepaalde metabolieten zijn belangrijke parameters. Type V trichomen op het blad zijn gecorreleerd met vatbaarheid. Een genetische koppelingskaart met veel merkers (Single Nucleotide Polymorphisms, SNPs) maakte het mogelijk een karteringsstudie studie uit te voeren. Twee QTLs (Quantitative Trait Locus) voor overleving van volwassen wittevliegen en eileg werden geïdentificeerd. Het eerste QTL (*Wf-1*) was gelokaliseerd op Chromosoom 2 en het tweede QTL (*Wf-2*) op Chromosoom 9. *Wf-1* heeft een groot effect en maakt, indien homozygoot aanwezig, de plant volledig resistent. De QTLs zijn bevestigd in F3 karteringspopulaties. De interactie tussen de QTLs onder verschillende omstandigheden moet nog beter onderzocht worden. Alle resistentie gerelateerde eigenschappen werden op dezelfde, bovengenoemde posities op de chromosomen gekarteerd. Een aantal metabolieten die gevonden waren in GC-MS analyses karteerden ook in dezelfde gebieden. Sommige metabolieten die co-localiseerden met *Wf-1* waren gerelateerd aan acyl suiker biosynthese. Dit werd bevestigd door de LC-MS data, waar de concentraties van negen acyl suikers in de tien meest resistente F2 genotypen veel hoger waren dan in de 10 meest vatbare genotypen.

The metabolieten die co-lokaliseerden met *Wf-2* waren gerelateerd met biologische processen zoals de onderdrukking van chlorofyl afbraak.

Onze resultaten laten zien dat de genetische achtergrond van wittevliegresistentie in *S. galapagense* gecontroleerd wordt door twee loci, en dat het belangrijkste locus voldoende is om volledige resistentie te krijgen. Dit locus kan een regulerend gen zijn met een invloed op de expressie van meerdere genen. De positie van *Wf-1* in een chromosoomgebied met een hoge recombinatie frequentie maakt het mogelijk het resistentiegen te kloneren en verder te bestuderen. Maar zelfs zonder vervolgstudies is het nu mogelijk nieuwe tomatenrassen te selecteren met een hoog niveau van resistentie tegen wittevliegen.

Ringkasan

Kutu kebul (*Bemisia tabaci* Genn) merupakan ancaman serius bagi petani tomat dan cabai. Kerusakan paling tampak akibat hama ini terkait dengan perannya sebagai vektor berbagai macam virus tanaman. Kutu kebul juga menyebabkan kerusakan langsung seperti daun keperakan, pemasakan buah yang tidak merata dan kekurangan nutrisi. Pengendalian kutu kebul menggunakan pestisida dan agen hayati sudah dilakukan, tetapi pestisida tidak hanya berbahaya bagi manusia dan lingkungan, tetapi juga mempercepat munculnya strain kutu kebul yang tahan terhadap pestisida. Pengendalian menggunakan agen hayati juga sulit, khususnya pada sistem pertanian lahan terbuka seperti di Indonesia. Ketahanan tanaman diyakini merupakan pengendalian yang lebih tahan lama dan lebih ampuh. Pada plasma nutfah tomat modern tidak ada kultivar tahan terhadap kutu kebul. Oleh karena itu, kerabat liar tomato (dan cabai) yang tahan terhadap serangan kutu kebul bisa dijadikan jalan keluar. Akan tetapi, proses pemindahan faktor-faktor ketahanana dari kerabat liar ke tomat komersial mengalami kesulitan sehingga sampai saat ini belum ada kultivar tomat atau cabai komersial yang tahan. Begitu juga kajian dan penelitian ketahanan pada cabai masih sangat sedikit. Penelitian kami melingkupi aspek-aspek interaksi kutu kebul dan tanaman yang meliputi karakterisasi populasi kutu kebul, evaluasi parameter ketahanan terhadap kutu kebul, penapisan plasma nutfah tanaman tomat dan cabai serta kajian genetik aksesi tomat liar yang paling tahan terhadap kutu kebul. Tujuan penelitian ini untuk mengidentifikasi keberagaman genetik aksesi-aksesi tomat dan cabai dalam ketahanannya terhadap kutu kebul. Selanjutnya, kajian genetik secara rinci pada aksesi tomat yang paling tahan juga dilakukan. Ujungnya, penelitian ini menjadi awal pengembangan kultivar-kultivar tomat komersial tahan hama kutu kebul.

Thesis ini tidak hanya menggambarkan pengetahuan terkini mengenai ketahanan tomat dan cabai terhadap hama kutu kebul, tetapi juga mengkaji keragaman genetik dan status taksonomi hama kutu kebul berdasarkan gene

sitokrom oksidase I mitokondria (*mtCOI*). Sejumlah 387 haploid kutu kebul teridentifikasi dari 1089 sekuen yang ada di GeneBank pada 30 November 2010. Lima belas haploid kutu kebul juga berhasil diidentifikasi dari 50 populasi kutu kebul yang diambil dari Indonesia, Thailand, India dan China pada tahun 2008. Rekonstruksi filogeni kutu kebul berdasaran gen mtCOI berhasil mengidentifikasi 36 kelompok dengan perbedaan genetik lebih dari 3.5 % dan hasil ini mendukung hama ini sebagai kumpulan banyak spesies yang mirip. Tetapi, peneliti-peneliti lain juga menemukan ketidakmampuan kawin antara kelompok dengan perbedaan genetik lebih rendah dari 3.5 %. Oleh karena itu, penyelidikan lebih lanjut perlu dilakukan untuk menentukan batasan dan penamaan species-species hama ini.

Empat puluh empat aksesi cabai telah dievaluasi ketahanannya terhadap kutu kebul di Belanda dan Indonesia. Komponen tanamanan yang diduga terkait ketahanan cabai terhadap hama kutu kebul adalah ketebalan kutisel dan faktor antibiotik di daun tanaman. Beberapa aksesi turunan paprika *Capsicum annum* California Wonder memiliki kutisel yang tebal dan tahan terhadap kutu kebul. Tanaman ini menunjukkan kemungkinan kutisel yang tebal tidak dapat ditembus oleh belalai kutu kebul. Tetapi, *C. annuum* CM331 yang memiliki kutisel tipis tapi tahan menunjukkan kemungkinan adanya mekanisme ketahanan yang lain.

Pada Bab 4, Lima puluh tiga aksesi tomat yang terdiri dari 11 species telah ditapis untuk ketahanannya terhadap kutu kebul. Tingkat ketahanan diukur dengan mengamati parameter pada siklus hidup kutu kebul yang meliputi daya hidup kutu dewasa, rata-rata bertelur, jumlah nimfa dan bekas pupa. Pada aksesi paling tahan, daya hidup kutu kebul sangat rendah (sampai 90% kutu kebul dewasa mati setelah satu hari). Ketahanan ini sangat berhubungan dengan kerapatan trikom kelenjar tipe I dan IV. Trikom-trikom ini memberikan perlindungan yang paling efektif dari hama kutu kebul. Hasil kami juga menunjukkan bahwa embun madu berhubungan dengan kepadatan nimfa kutu kebul. Tingkat ketahanan paling tinggi dan lama ditemukan pada sebuah aksesi *Solanum galapagense*. Aksesi-aksesi lain yang menunjukkan tahan terhadap serangan kutu kebul adalah *S. pimpinellifolium* and *S. habrochaites*. Tetapi tingkat ketahanan pada *S. habrochaites* menurun pada tanaman yang lebih tua. Sistem ketahahan lain yang tidak berdasar pada trikom kelenjar mungkin ada pada aksesi *S. glandulosum* and *S. arcanum*. Kedua aksesi

ini tidak memiliki trikom tipe IV tapi mampu menghambat pertumbuhan nimfa kutu kebul. Parameter dan uji ketahanan yang telah dilakukan menunjukkan hasil pengukuran ketahanan yang akurat. Uji ketahanan secara *in vitro* menggunakan potongan daun merupakan alternatif yang baik dalam penapisan ketahanan terhadap kutu kebul.

Genetik ketahanan dan sifat-sifat terkait ketahanan diteliti berdasarkan ketahanan pada *S. galapagense* PRI95004. Aksesi ini dipilih karena paling tahan terhadap serangan kutu kebul dan berkerabat sangat dekat dengan kultivar tomat komersial. Pemetaan populasi F2 menunjukkan hubungan kuat antara daya hidup dan bertelur kutu kebul. Trikom tipe IV bukan penentu utama ketahanan, tetapi kandungan kelenjar trikom mengambil peranan penting terhadap ketahanan. Rasio antara trikom tipe IV dan V, ukuran dan panjang hidup kelenjar trikom dan kandungan metabolit merupakan parameter-parameter ketahanan yang sangat penting. Keberadaan trikom tipe V membuat tanaman menjadi lebih rentan. Menggunakan keragaman nukleotida tunggal (single nucleotide polymorphism, SNP), peta tautan genetik digunakan untuk pemetaan lokus sifat kuantitatif (QTL). Dua QTL telah ditemukan untuk daya hidup dan bertelur kutu kebul. Lokus pertama (*Wf-1*) terlatak di kromosom 2 dan lokus kedua (*Wf-2*) berada di kromosom 9. *Wf-1* berperan sangat kuat pada ketahanan tanaman. Pada keadaan homozigot, lokus ini menghasilkan ketahanan yang penuh seperti tetua yang tahan. QTL ini juga dikonfirmasi pada populasi F3. Tetapi interaksi antara kedua QTL perlu diinvestigasi lebih lanjut untuk mengetahui kendali genetiknya secara lebih baik. Menariknya, semua sifat yang terkait dengan ketahanan memiliki QTL yang sama dengan daya hidup dan bertelur kutu kebul. Sejumlah metabolit yang diidentifikasi menggunakan GC-MS juga terpetakan pada lokasi yang sama. Beberapa metabolit yang memiliki QTL pada *Wf-1* terkait dengan proses pembentukan asil-gula. Hasil ini dibuktikan oleh hasil analisis LC-MS yang menunjukkan bahwa konsentrasi sembilan jenis asil-gula pada sepuluh genotipe tahan jauh lebih tinggi daripada sepuluh genotipe rentan. QTL metabolit-metabolit yang berada di *Wf-2* terkait dengan proses biologis lainnya seperti penghambatan kerusakan klorofil.

Hasil kami menunjukkan bahwa latar belakang genetik ketahanan kutu kebul pada *S. galapagense* dikendalikan oleh dua lokus, dengan satu lokus utama yang mampu menhasilkan ketahanan penuh pada keadaan homozigot. Lokus ini mungkin gen pengatur yang mengendalikan beberapa gen yang terlibat pada sifat-sifat terkait ketahanan kutu kebul. *Wf-1* berada pada bagian kromosom yang memiliki tingkat rekombinasi yang tinggi dan ini memungkikan mengklon dan mengkaji gen yang bertanggung jawab terhadap ketahanan *S. galapagense*. Tetapi, walau pun tanpa pengklonan, QTL ini sangat menjanjikan untuk pemuliaan tomat tahan hama kutu kebul.

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Wageningen, 29 May 2012

Syarifin Firdaus

About the author

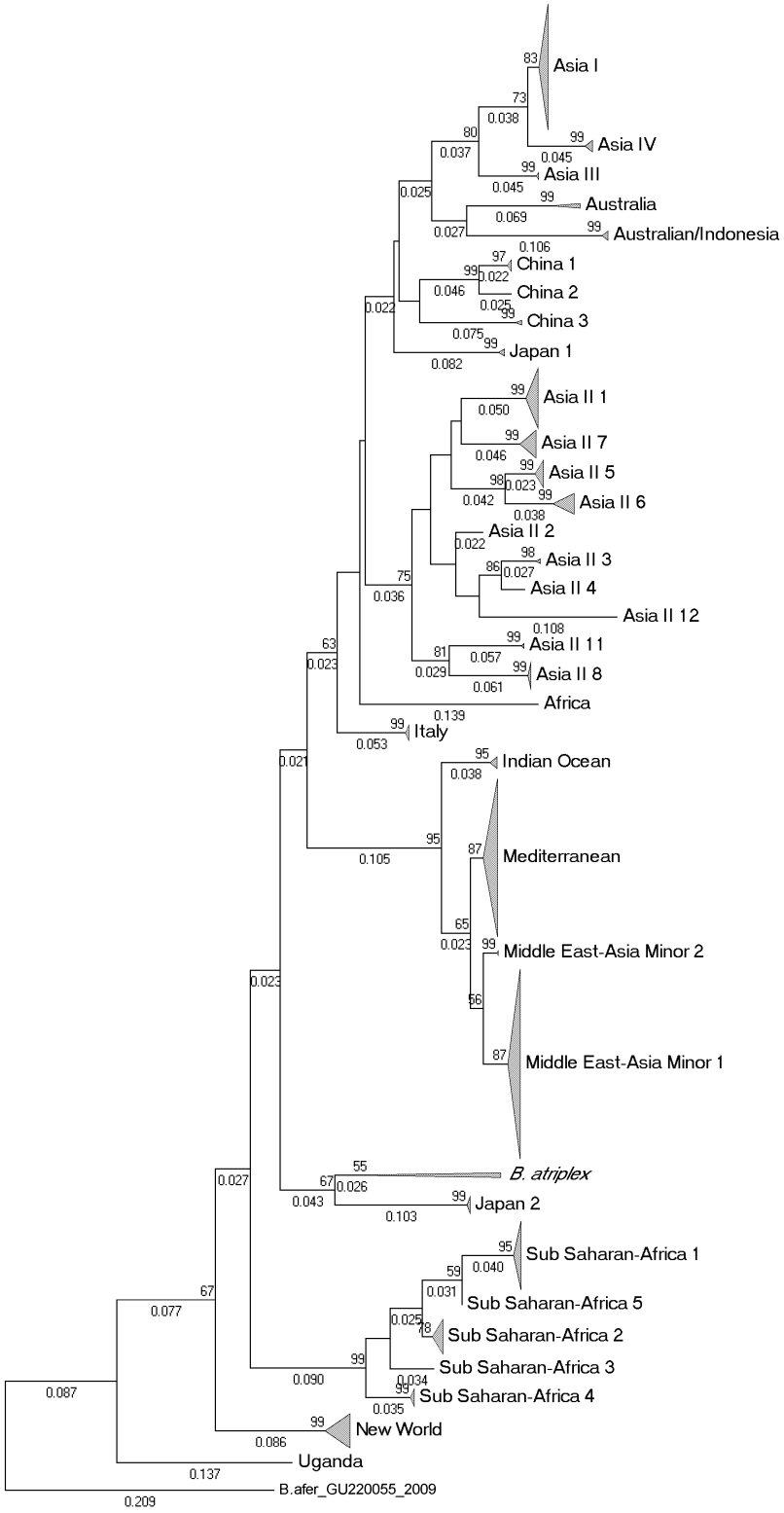
Syarifin Firdaus was born on September, 5th 1978 in Malang, East Java Indonesia. Natural science has inspired him to direct his study to take this division in his Senior High School. After completion the Senior High School, he took undergraduate program with specialization in genetics at Department of Biology, Faculty of Mathematics and Sciences, State University of Malang (UM) and finished in 2002. Curiosity in the genetics leaded him to collect financial support by working hard as a teacher, a consultant in education and a farmer. Those efforts allowed him to continue his study to master program at Department of Biology, Faculty of Mathematic and Science, Bogor Agricultural University (IPB) in 2003. In the master program, he was specialized in plant genetic of abiotic stress. After finishing the master program, he worked as a researcher at Research Center for Bioresources and Biotechnology (RCBIO), Bogor Agricultural University. In 2006, he had a great chance to compete with other candidates to study in Wageningen University and Research Center (WUR) through scientific program between Indonesia and the Netherlands (SPIN project) program which entitle Indonesian Solanaceae (INDOSOL) and funded by KNAW. In 2007, he was chosen as one of five PhD candidates and then started his PhD in Plant Breeding Department, WUR on January, 28th 2008. He can be reached by email syarifinfir@yahoo.com.

Publications

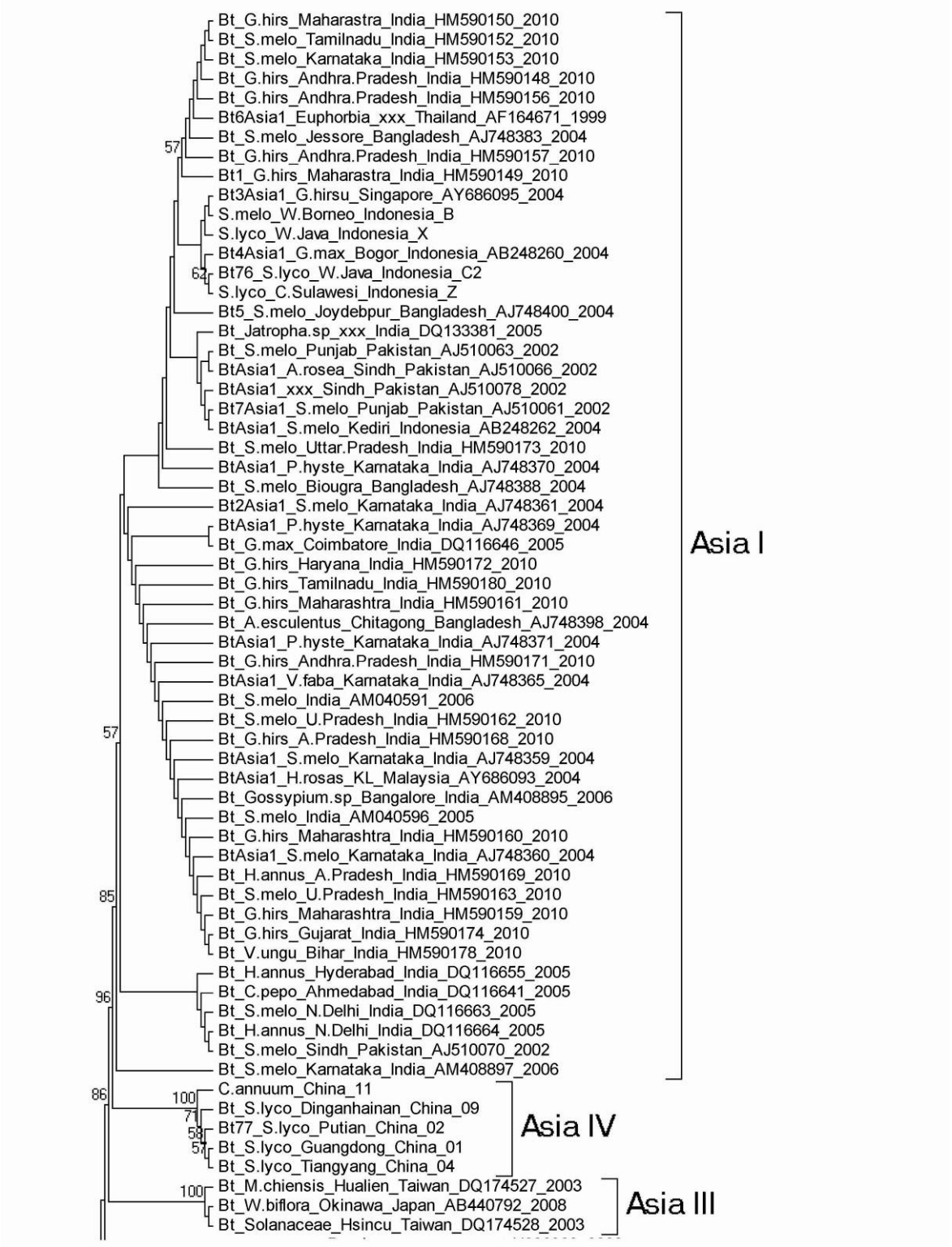
1. Suharsono, S, **S. Firdaus**, U.W. Suharsono (2008). Isolation and Cloning of cDNA Fragment of Gene Encoding for Multidrug Resistance Associated Protein from *M. affine*. Makara 12 (2), 102-107.
2. **Firdaus, S**, A.W. van Heusden, A. Harpenas, E. Darmo Jaya Supena, R. Visser and B. Vosman (2011) Identification of Silverleaf Whitefly Resistance in Pepper. Plant breeding 130, 708—714.
3. **Firdaus, S**, A.W. van Heusden, N. Hidayati, E. Darmo Jaya Supena, R. Visser and B. Vosman (2012) Resistance to *Bemisia tabaci* in Tomato Wild Relatives. Accepted for publication in Euphytica.
4. **Firdaus, S**, B. Vosman, N. Hidayati, E. Darmo Jaya Supena, R. Visser and A.W. van Heusden (2012) The *Bemisia tabaci* species complex: additions from different parts of the world. Accepted for publication in Insect Science.
5. **Firdaus, S**, A.W. van Heusden, R. Mumm, N. Hidayati, E. Darmo Jaya Supena, R. Visser and B. Vosman (2012) Identification and QTL Mapping of Whitefly Resistance Components in *S. galapagense*. Submitted for Publication in Theoretical Applied Genetics Journal.

Supplementary data, tables and figures

Supplementary Data 2.1 Sequences and related information that were used to reconstruct the phylogenetic tree of *B. tabaci* can be requested by email to syarifinfir@yahoo.com

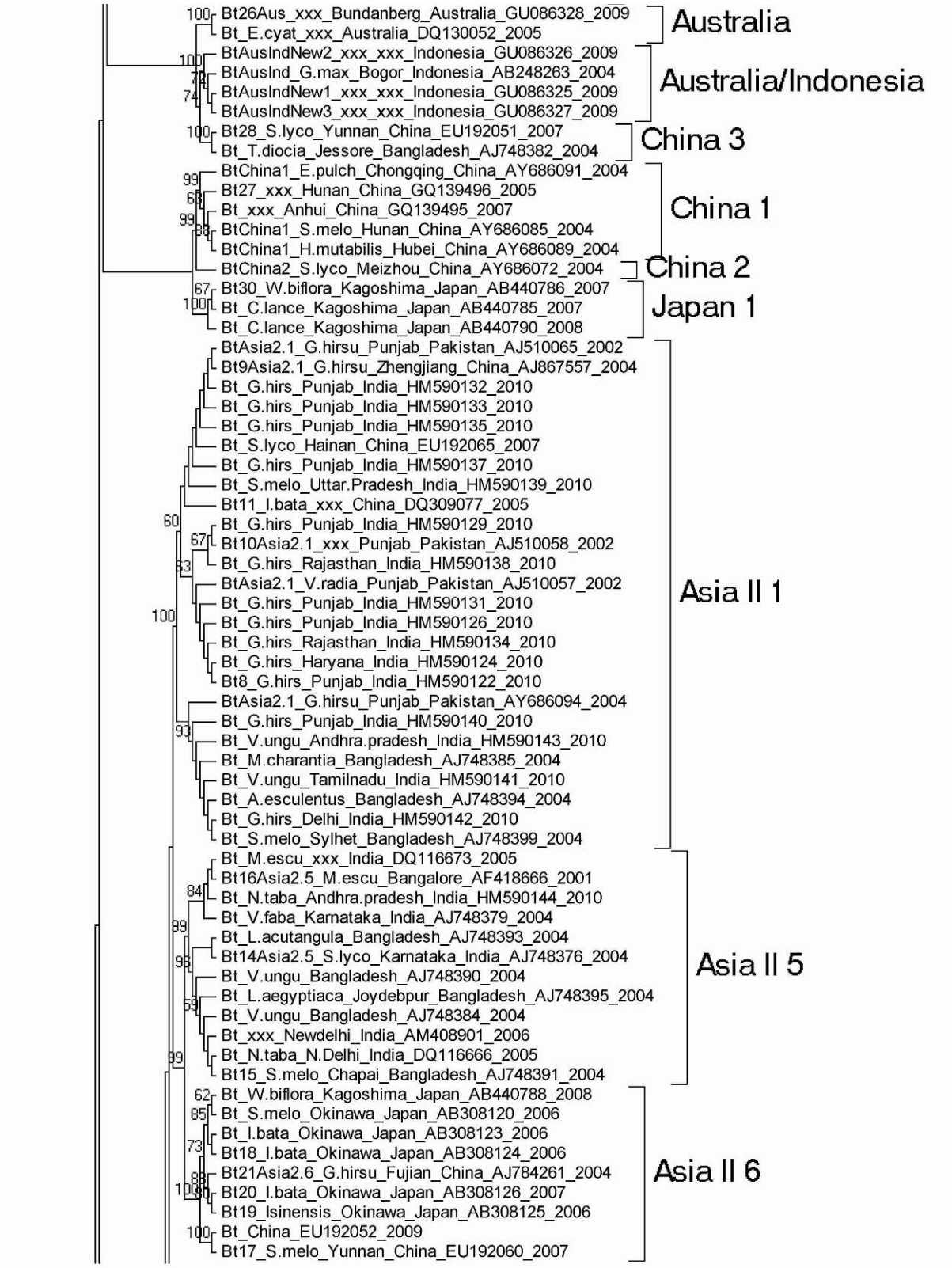


Supplementary figure 2.1 Phylogenetic tree of global *Bemisia tabaci* populations estimated by using Maximum Likelihood. Triangle Height represents number of haplotypes within the group and triangle width represent the highest genetic divergence among haplotypes within the group. Number above branches represents the percentage of 1000 bootstraps and number below branches represents genetic distance.



Supplementary figure 2.2 Phylogenetic tree of global *Bemisia tabaci* estimated by using maximum parsimony. Number above branches represents the percentage of 1000 bootstrap. Group were made based on genetic divergence less than 3.5%. Each taxon name contained Bt(group based on Dinsdale *et al.* 2010)(number for duplicated haplotype_Host plant_Location_Country_ Accession number in the database_Year of collection. xxx means no data is available. Aus/Ind is Australia/Indonesia, MEAM is Middle East-Asia Minor and SubSahAf is Sub Sahara-Africa.

Con't Supplementary figure 2.2



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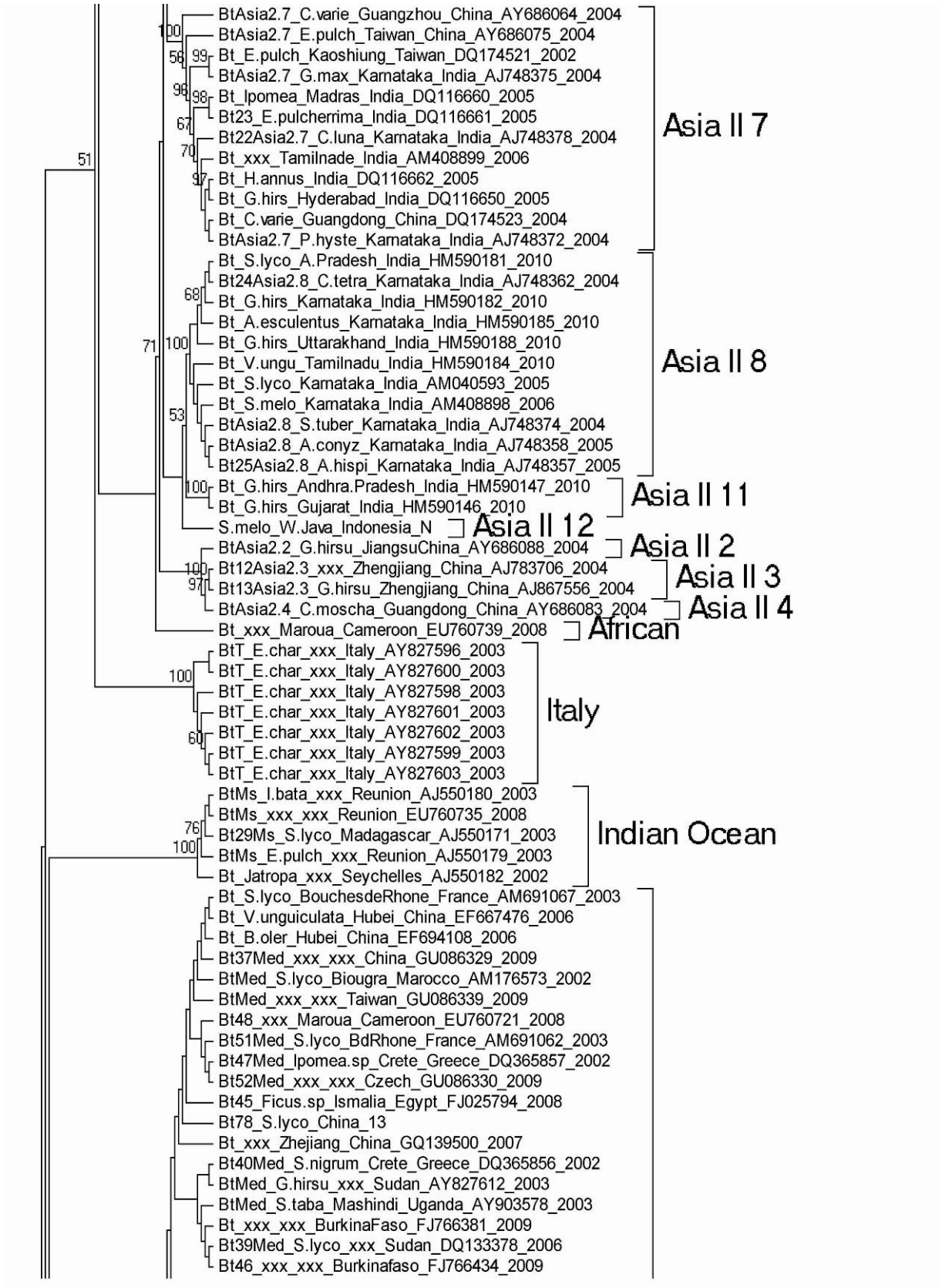

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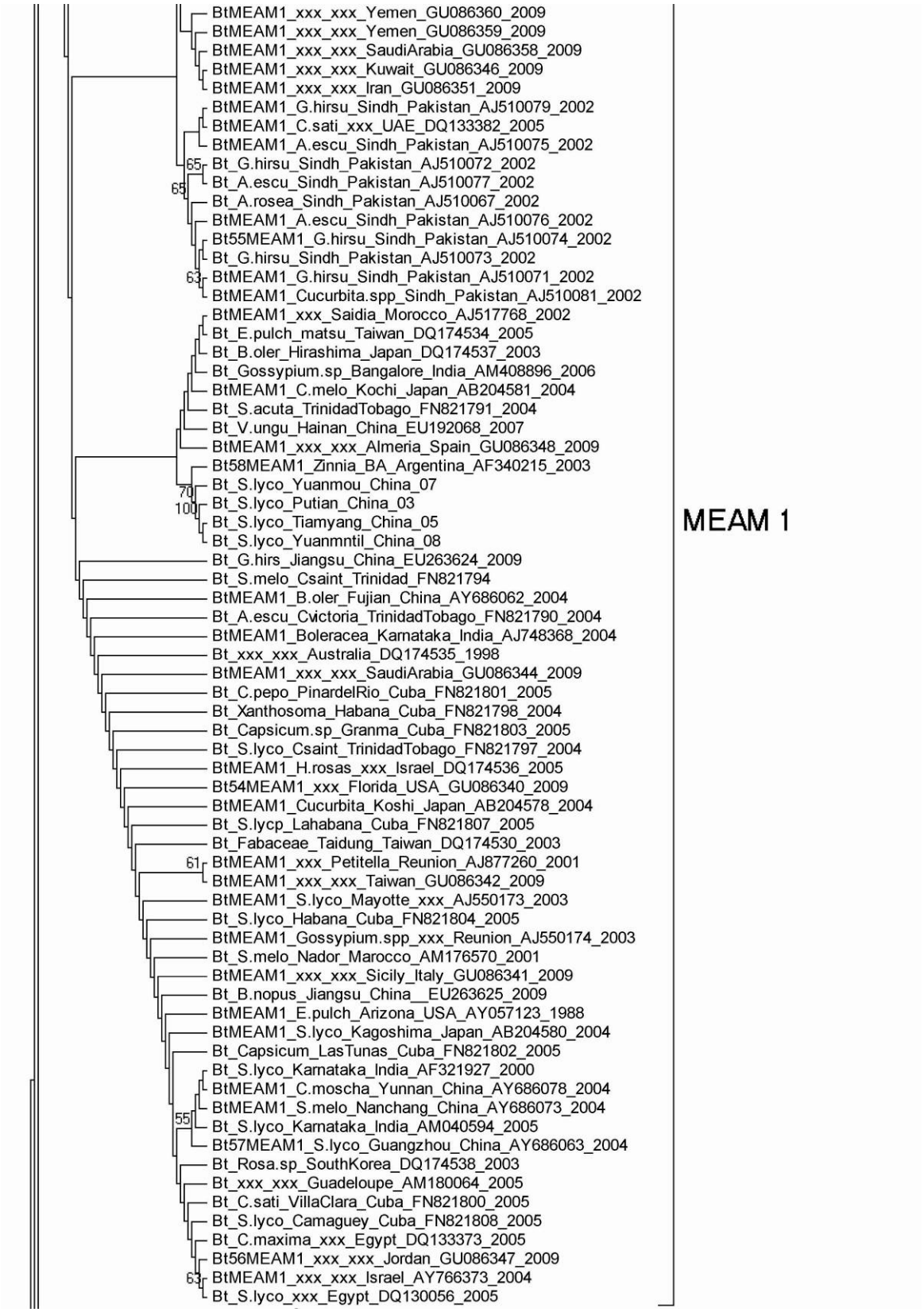

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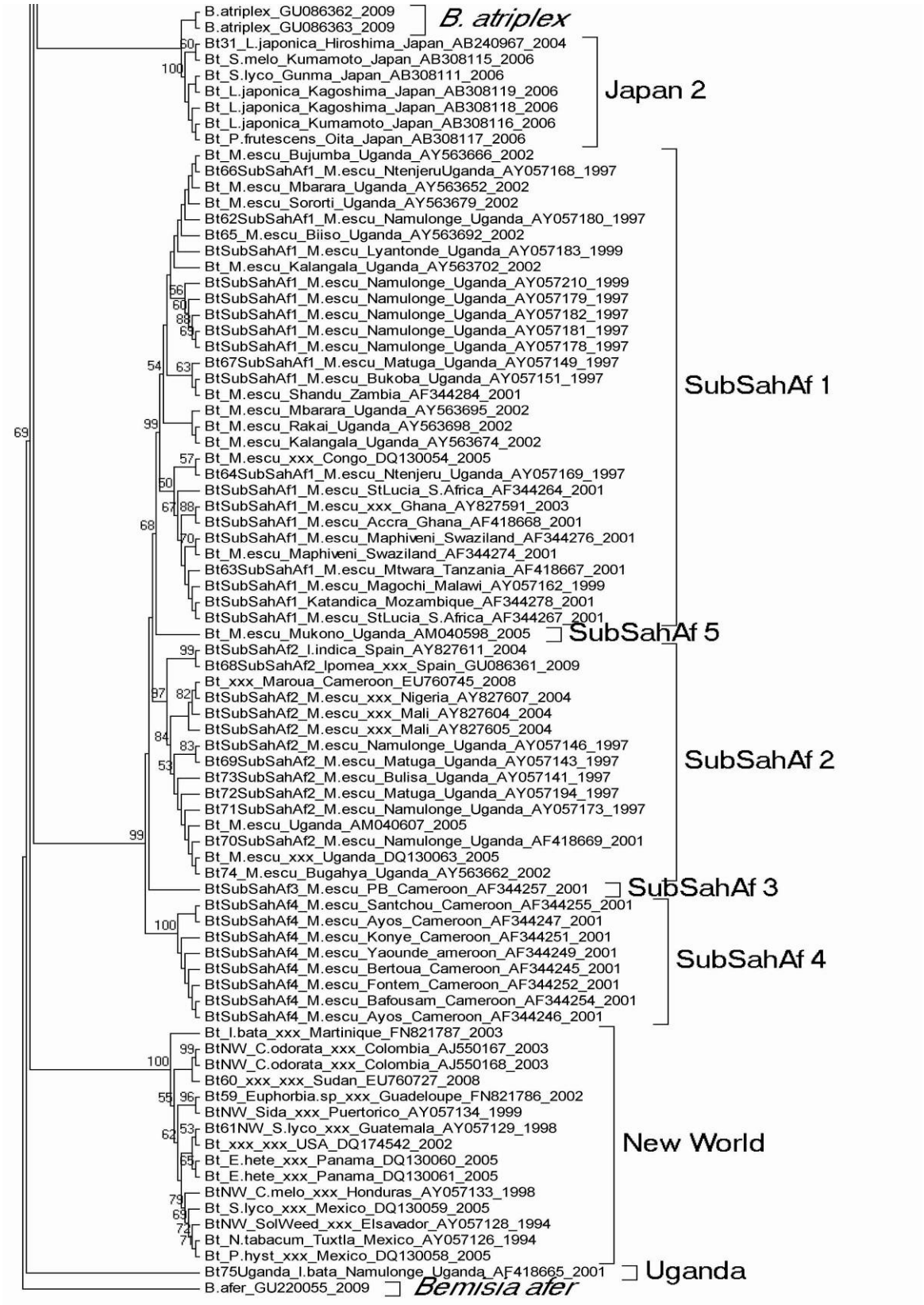

Supplementary Table 4.1 Means of nymphal density in whitefly resistance screening of tomato accessions in free-choice condition.

No	Accession Name	Observation time		
		1	2	3
1	*S. galapagense* PRI95004/PY-8027	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]
2	*S. galapagense* PRI95004/PY-8030	2.9 (bcde) [a]	6.1 (g) [b]	6.6 (fg) [b]
3	*S. cheesmaniae* CGN15916	10.1 (fg) [a]	14.1 (i) [ab]	17.9 (i) [b]
4	*S. cheesmaniae* CGN24039	3.0 (bcde) [a]	44.1 (mn) [b]	30.6 (jkl) [b]
5	*S. cheesmaniae* CGN17086	29.4 (i) [b]	5.2 (fg) [a]	3.4 (c) [a]
6	*S. arcanum* CGN14355	4.5 (de) [a]	19.9 (j) [b]	12.1 (h) [b]
7	*S. arcanum* CGN15877	3.3 (cde) [a]	9.9 (h) [b]	10.2 (h) [b]
8	*S. glandulosum* CGN15803	19.3 (hi) [b]	5.7 (g) [a]	5.2 (defg) [a]
9	*S. glandulosum* CGN14357	2.5 (bcde) [a]	16.4 (ij) [b]	17.8 (i) [b]
10	*S. glandulosum* CGN14358	1.6 (bc) [a]	1.9 (c) [a]	2.1 (b) [a]
11	*S. habrochaites* f. *glabratum* CGN24035	0.0 (a) [a]	4.3 (ef) [b]	6.7 (fg) [b]
12	*S. habrochaites* f. *glabratum* PRI921237	0.0 (a) [a]	0.5 (b) [b]	5.1 (def) [c]
13	*S. habrochaites* CGN15391	2.2 (bcd) [a]	53.3 (n) [b]	53.2 (m) [b]
14	*S. habrochaites* LA1777	1.5 (bc) [a]	19.8 (j) [b]	23.5 (j) [c]
15	*S. habrochaites* LA1033	0.0 (a) [a]	43.3 (m) [b]	39.9 (l) [b]
16	*S. lycopersicoides* CGN23973	5.5 (de) [a]	28.4 (k) [b]	29.9 (jk) [b]
17	*S. lycopersicum* PRI91117	2.1 (bc) [a]	37.0 (lm) [b]	39.6 (l) [b]
18	*S. lycopersicum* EWSI24294	0.2 (a) [a]	38.3 (lm) [b]	34.7 (kl) [b]
19	*S. lycopersicum* EWSI49444	2.7 (bcde) [a]	4.0 (e) [ab]	5.7 (efg) [b]
20	*S. neorickii* CGN15816	4.6 (de) [a]	4.0 (e) [a]	4.6 (de) [a]
21	*S. neorickii* CGN15815	9.9 (fg) [b]	3.1 (d) [a]	3.9 (cd) [a]
22	*S. pennellii* CGN23952			
23	*S. peruvianum* CGN17052	1.3 (b) [a]	10.5 (h) [b]	11.9 (h) [b]
24	*S. peruvianum* CGN17047	11.1 (gh) [a]	31.2 (kl) [b]	40 (l) [b]
25	*S. pimpinellifolium* CGN14401	5.1 (ef) [a]	31.4 (kl) [b]	29.7 (jk) [b]
26	*S. pimpinellifolium* PRI91059	5.8 (ef) [a]	6.2 (g) [a]	7.0 (g) [a]

The mean followed by different letters in the parenthesis within columns are different according to Duncan's multiple range test and different letters in the brackets within lines are different according to Fisher's student test in $p = 0.05$.

Supplementary Table 4.2 Mean of honeydew score (0 = no honeydew to 4 = much honeydew) in whitefly resistance screening of tomato accessions in free-choice condition.

No	Accession Name	Observation time		
		1	2	3
1	*S. galapagense* PRI95004/PY-8027	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]
2	*S. galapagense* PRI95004/PY-8030	0.0 (a) [a]	0.0 (a) [a]	3.0 (ab) [b]
3	*S. cheesmaniae* CGN15916	0.0 (a) [a]	2.7 (ab) [b]	2.0 (ab) [b]
4	*S. cheesmaniae* CGN24039	0.0 (a) [a]	2.7 (ab) [b]	2.0 (ab) [b]
5	*S. cheesmaniae* CGN17086	1.7 (a) [a]	3.3 (ab) [ab]	4.0 (b) [b]
6	*S. arcanum* CGN14355	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]
7	*S. arcanum* CGN15877	0.0 (a) [a]	2.0 (ab) [b]	3.0 (ab) [c]
8	*S. glandulosum* CGN15803	1.3 (a) [a]	2.7 (ab) [a]	2.3 (ab) [a]
9	*S. glandulosum* CGN14357	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]
10	*S. glandulosum* CGN14358	0.0 (a) [a]	0.7 (ab) [a]	0.7 (ab) [a]
11	*S. habrochaites* f. *glabratum* CGN24035	0.0 (a) [a]	4.0 (b) [b]	4.0 (b) [b]
12	*S. habrochaites* f. *glabratum* PRI921237	0.0 (a) [a]	0.3 (ab) [a]	3.0 (ab) [b]
13	*S. habrochaites* CGN15391	2.3 (a) [a]	4.0 (b) [b]	4.0 (b) [b]
14	*S. habrochaites* LA1777	0.0 (a) [a]	3.7 (ab) [b]	4.0 (b) [b]
15	*S. habrochaites* LA1033	0.0 (a) [a]	3.7 (ab) [b]	4.0 (b) [b]
16	*S. lycopersicoides* CGN23973	0.7 (a) [a]	4.0 (b) [b]	4.0 (b) [b]
17	*S. lycopersicum* PRI91117	1.7 (a) [a]	4.0 (b) [b]	4.0 (b) [b]
18	*S. lycopersicum* EWSI24294	0.0 (a) [a]	3.7 (ab) [b]	3.7 (ab) [b]
19	*S. lycopersicum* EWSI49444	0.3 (a) [a]	1.7 (ab) [a]	1.0 (ab) [a]
20	*S. neorickii* CGN15816	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]
21	*S. neorickii* CGN15815	0.0 (a) [a]	0.3 (ab) [a]	0.7 (ab) [a]
22	*S. pennellii* CGN23952			
23	*S. peruvianum* CGN17052	0.0 (a) [a]	2.7 (ab) [b]	3.0 (ab) [b]
24	*S. peruvianum* CGN17047	0.0 (a) [a]	3.3 (ab) [b]	3.0 (ab) [b]
25	*S. pimpinellifolium* CGN14401	0.3 (a) [a]	2.7 (ab) [b]	3.0 (ab) [b]
26	*S. pimpinellifolium* PRI91059	0.0 (a) [a]	3.7 (ab) [c]	2.0 (ab) [b]

The mean followed by different letters in the parenthesis within columns are different according to Dunn's test with Bonferroni's alpha correction and different letters in the brackets within lines are different according to Wilcoxon test in $p = 0.05$.

Supplementary Table 4.3 Mean of sooty-mold score (0 = no sooty mold to 4 = heavy sooty mold) in whitefly resistance screening of tomato accessions in free-choice condition.

No	Accession Name	Observation time		
		1	2	3
1	*S. galapagense* PRI95004/PY-8027	0.0 (a) [a]	1.0 (a) [b]	1.0 (a) [b]
2	*S. galapagense* PRI95004/PY-8030	1.0 (a) [a]	2.0 (ab) [b]	3.0 (ab) [c]
3	*S. cheesmaniae* CGN15916	2.0 (ab) [a]	2.0 (ab) [a]	3.0 (ab) [b]
4	*S. cheesmaniae* CGN24039	2.0 (ab) [a]	2.7 (ab) [b]	3.0 (ab) [b]
5	*S. cheesmaniae* CGN17086	2.3 (ab) [a]	2.7 (ab) [a]	3.0 (ab) [b]
6	*S. arcanum* CGN14355	3.0 (b) [c]	2.0 (ab) [b]	1.0 (a) [a]
7	*S. arcanum* CGN15877	3.0 (b) [a]	3.0 (b) [a]	3.0 (ab) [a]
8	*S. glandulosum* CGN15803	2.0 (ab) [a]	3.0 (b) [b]	3.0 (ab) [b]
9	*S. glandulosum* CGN14357	2.0 (ab) [b]	2.0 (ab) [b]	1.0 (a) [a]
10	*S. glandulosum* CGN14358	2.0 (ab) [b]	1.0 (a) [a]	2.0 (ab) [b]
11	*S. habrochaites* f. *glabratum* CGN24035	1.0 (a) [a]	2.0 (ab) [b]	2.7 (ab) [b]
12	*S. habrochaites* f. *glabratum* PRI921237	2.0 (ab) [b]	1.0 (a) [a]	1.0 (a) [a]
13	*S. habrochaites* CGN15391	2.0 (ab) [a]	1.7 (ab) [a]	3.0 (ab) [b]
14	*S. habrochaites* LA1777	1.0 (a) [a]	1.0 (a) [a]	3.0 (ab) [b]
15	*S. habrochaites* LA1033	1.0 (a) [a]	1.0 (a) [a]	2.0 (ab) [b]
16	*S. lycopersicoides* CGN23973	1.3 (ab) [a]	2.0 (ab) [a]	3.0 (ab) [b]
17	*S. lycopersicum* PRI91117	2.0 (ab) [a]	3.0 (b) [b]	3.3 (b) [b]
18	*S. lycopersicum* EWSI24294	1.0 (a) [a]	1.0 (a) [a]	1.3 (ab) [a]
19	*S. lycopersicum* EWSI49444	2.0 (ab) [a]	3.0 (b) [b]	2.0 (ab) [a]
20	*S. neorickii* CGN15816	2.0 (ab) [a]	4.0 (b) [b]	2.3 (ab) [a]
21	*S. neorickii* CGN15815	2.0 (ab) [a]	2.7 (ab) [ab]	3.0 (ab) [b]
22	*S. pennellii* CGN23952	3.0 (b) [a]	3.0 (b) [a]	3.0 (ab) [a]
23	*S. peruvianum* CGN17052	2.0 (ab) [a]	2.0 (ab) [a]	3.0 (ab) [b]
24	*S. peruvianum* CGN17047	2.0 (ab) [a]	2.0 (ab) [a]	2.0 (ab) [a]
25	*S. pimpinellifolium* CGN14401	2.0 (ab) [a]	2.0 (ab) [a]	2.0 (ab) [a]
26	*S. pimpinellifolium* PRI91059	2.0 (ab) [a]	2.0 (ab) [a]	2.0 (ab) [a]

The mean followed by different letters in the parenthesis within columns are different according to Dunn's test with Bonferroni's alpha correction and different letters in the brackets within lines are different according to Wilcoxon test in $p = 0.05$.

Supplementary Table 4.4 Mean of damage score (0 is no damage to 4 is severe damages) in whitefly resistance screening of tomato accessions in free-choice condition.

No	Accession Name	Observation time		
		1	2	3
1	*S. galapagense* PRI95004/PY-8027	0.0 (a) [a]	0.0 (a) [a]	0.0 (a) [a]
2	*S. galapagense* PRI95004/PY-8030	1.0 (ab) [a]	1.3 (abc) [ab]	2.3 (abc) [b]
3	*S. cheesmaniae* CGN15916	1.0 (ab) [a]	2.3 (abc) [b]	3.0 (bc) [b]
4	*S. cheesmaniae* CGN24039	1.0 (ab) [a]	3.0 (bc) [b]	3.0 (bc) [b]
5	*S. cheesmaniae* CGN17086	2.0 (b) [a]	2.0 (abc) [a]	3.0 (bc) [b]
6	*S. arcanum* CGN14355	1.0 (ab) [a]	1.3 (abc) [ab]	2.3 (abc) [b]
7	*S. arcanum* CGN15877	1.0 (ab) [a]	2.0 (abc) [b]	3.0 (bc) [c]
8	*S. glandulosum* CGN15803	1.0 (ab) [a]	2.0 (abc) [b]	3.0 (bc) [b]
9	*S. glandulosum* CGN14357	1.0 (ab) [a]	1.7 (abc) [ab]	2.0 (abc) [b]
10	*S. glandulosum* CGN14358	1.0 (ab) [a]	1.7 (abc) [ab]	2.0 (abc) [b]
11	*S. habrochaites* f. *glabratum* CGN24035	1.0 (ab) [a]	1.0 (ab) [a]	2.0 (abc) [b]
12	*S. habrochaites* f. *glabratum* PRI921237	1.0 (ab) [a]	1.0 (ab) [a]	2.0 (abc) [b]
13	*S. habrochaites* CGN15391	1.7 (b) [a]	3.3 (bc) [b]	4.0 (c) [b]
14	*S. habrochaites* LA1777	1.0 (ab) [a]	1.0 (ab) [a]	2.0 (abc) [b]
15	*S. habrochaites* LA1033	1.0 (ab) [a]	1.0 (ab) [a]	2.0 (abc) [b]
16	*S. lycopersicoides* CGN23973	1.0 (ab) [a]	1.7 (abc) [b]	2.3 (abc) [b]
17	*S. lycopersicum* PRI91117	1.7 (b) [a]	3.0 (bc) [b]	3.0 (bc) [b]
18	*S. lycopersicum* EWSI24294	0.0 (a) [a]	1.0 (ab) [b]	1.3 (ab) [b]
19	*S. lycopersicum* EWSI49444	1.0 (ab) [a]	1.0 (ab) [a]	2.0 (abc) [b]
20	*S. neorickii* CGN15816	1.0 (ab) [a]	3.0 (bc) [b]	3.0 (bc) [b]
21	*S. neorickii* CGN15815	1.0 (ab) [a]	3.0 (bc) [b]	3.3 (bc) [b]
22	*S. pennellii* CGN23952	3.0 (b) [a]	4.0 (c) [b]	4.0 (c) [b]
23	*S. peruvianum* CGN17052	1.0 (ab) [a]	2.0 (abc) [b]	2.0 (abc) [b]
24	*S. peruvianum* CGN17047	1.0 (ab) [a]	2.0 (abc) [b]	2.0 (abc) [b]
25	*S. pimpinellifolium* CGN14401	1.0 (ab) [a]	2.0 (abc) [b]	2.0 (abc) [b]
26	*S. pimpinellifolium* PRI91059	1.0 (ab) [a]	2.3 (abc) [ab]	3.0 (bc) [b]

The mean followed by different letters in the parenthesis within columns are different according to Dunn's test with Bonferroni's alpha correction and different letters in the brackets within lines are different according to Wilcoxon test in $p = 0.05$.

Supplementary Table 4.5 Coefficient correlation between observation time points of adult, egg and nymphal density as well as honeydew, sooty mold and plant damages.

Parameters	R correlation[1)]		
	r_{1-3}	r_{1-5}	R_{3-5}
Whitefly density	0.454* (75)	0.413** (75)	0.931** (75)
Egg density	0.509** (75)	0.377* (75)	0.884** (75)
Nymphal density	0.159 (75)	0.042 (75)	0.939** (75)
Honeydew	0.313* (75)	0.402** (75)	0.788** (75)
Sooty mold	0.535** (78)	0.113 (78)	0.460* (78)
Plant damage	0.557** (78)	0.642* (78)	0.835** (78)

[1)] Pearson r correlation for adult, egg and nymphal density, Spearman correlation for honeydew, sooty mold and plant damage; *) Correlation is significant at the 0.05 level (2-tailed); **) Correlation is significant at the 0.01 level (2-tailed);

Supplementary Table 4.6 Mean of whitefly resistance parameters in clip-on and leaf disc tests.

No	Accession Name	Leaf disc test		Clip-on cage test	
		Adult survival	**Oviposition rate**	**Adult survival**	**Oviposition rate**
1	*S. galapagense* PRI95004/PY-8028	0.92 e	5.30 d	0.98 d	6.93 d
2	*S. habrochaites* f. *glabratum* PI134417	0.0 a	0.0 a	0.72 b	0.28 a
3	*S. habrochaites* f. *glabratum* PI134418	0.0 a	0.02 a	0.0 a	0.19 a
4	*S. peruvianum* PI126928/PY-8038	0.97 e	9.08 f	0.99 d	11.39 e
5	*S. pimpinellifolium* LA1584/PY-8040	0.53 b	0.07 a	0.71 b	0.62 b
6	*S. habrochaites* LA1718	0.84 d	2.12 c	0.78 c	0.27 a
7	*S. habrochaites* f. *glabratum* CGN15879	0.67 c	0.22 b	0.72 b	1.76 c
8	*S. galapagense* PRI95004/PY-8027	0.0 a	0.0 a	0.0 a	0.10 a
9	*S. lycopersicum* Moneymaker	0.97 e	8.51 e	1.00 d	6.32 d

The mean followed different letters within columns are different by Duncan's multiple range test in 0.05 *p*-significance.

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Syarifin Firdaus**
Date: **12 September 2012**
Group: **Plant Breeding, Wageningen University**

1) Start-up phase	*date*	*cp*
► **First presentation of your project**		
Components of whitefly resistance on tomato and pepper	May 27, 2008	1.5
► **Writing or rewriting a project proposal**		
Tomato defense against whiteflies	2008	6.0
► **Writing a review or book chapter**		
► **MSc courses**		
► **Laboratory use of isotopes**		
Subtotal Start-up Phase		7.5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD Student Days**		
EPS PhD student day 2009, Leiden University	Feb 26, 2009	0.3
EPS PhD student day 2011, Wageningen University	May 20, 2011	0.3
► **EPS Theme Symposia**		
EPS theme 2 'Interaction between plants and biotic agents', Utrecht University	Nov 16, 2009	0.3
EPS theme 5 'Plant-Insect Interactions workshop', Wageningen University	Nov 11, 2010	0.3
EPS theme 2 'Interaction between plants and biotic agents', University of Amsterdam	Feb 03, 2011	0.3
EPS theme 5 'Plant-Insect Interactions workshop' University of Amsterdam	Nov 23, 2011	0.3
EPS theme 2 'Interaction between plants and biotic agents', Wageningen University	Feb 10, 2012	0.3
► **NWO Lunteren days and other National Platforms**		
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07 - 08, 2008	0.6
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06 - 07, 2009	0.6
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19 - 20, 2010	0.6
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04 - 05, 2011	0.6
► **Seminars (series), workshops and symposia**		
The First Annual INDOSOL Symposium, Bogor, Indonesia	Nov 06, 2008	0.3
Plant Breeding research day	Jun 17, 2008	0.3
Plant Breeding research day	Mar 03, 2009	0.3
One Day Seminar: Food Security issues in Asia-Africa	Oct 19, 2009	0.3
The Second Annual INDOSOL Symposium, Wageningen, The Netherlands	May 31 - Jun 01, 2010	0.6
Mini-symposium " How to write a world-class paper" Wageningen University	Oct 26, 2010	0.2
Mini-symposium " Plant breeding in the genomic era" Wageningen University	Nov 25, 2011	0.3
23rd Dutch Entomology day, Ede	Dec 16, 2011	0.3
► **Seminar plus**		
► **International symposia and congresses**		
XVIth EUCARPIA Symposium of the Tomato Working Group. Wageningen University	May 12 - 15 2008	1.2
Eusol-Latsol-Indosol joint conference, Natal, Brazil	Nov 14 - 16, 2010	0.9
► **Presentations**		
The First Annual of INDOSOL programme Symposium, Bogor, Indonesia (oral	Nov 06, 2008	1.0
EPS PhD student day 2009, Leiden University (poster)	Feb 26, 2009	1.0
EPS 'Plant-Insect Interactions workshop', Wageningen University (poster)	Nov 11, 2010	1.0
Eusol-Latsol-Indosol joint conferences, Natal, Brazil (poster)	Nov 14 - 16, 2010	1.0
Yearly workshops of Indosol, Wageningen, The Netherlands (oral presentation)	Jun 01, 2010	1.0
23rd Dutch Entomology day, Ede (oral presentation)	Dec 16, 2011	1.0
EPS theme 2 'Interaction between plants and biotic agents', Wageningen University (oral presentation)	Feb 10, 2012	1.0

►	**IAB interview**	Jan 21, 2011	0.7
►	**Excursions**		
	Excursion to PT East West Seed Indonesia and Indonesian vegetables research Institut	Nov 07, 2008	0.3
	Excursion to Solanaceous seed bank at Radboud University, Nijmegen	May 31, 2010	0.3
	Excursion to seed companies in seed valley	Jun 23, 2011	0.3
	Subtotal Scientific Exposure		17.8

3) In-Depth Studies		*date*	*cp*
►	**EPS courses or other PhD courses**		
	EPS PhD Course "System Biology: Statistical Analysis of ~omics Data"	Dec 13 - 17, 2010	1.5
	EPS PhD workshop "Natural variation on plant"	Aug 26 - 29, 2008	1.2
►	**Journal club**		
	Literature studies in plant science group	2008 - 2011	3.0
►	**Individual research training**		
	Applications of molecular markers in breeder's rights issues and phylogenetic studies	Nov 10 - 13, 2008	1.2
	Open Science Meeting 2009, Programme Master Class Agriculture Beyond Food	Nov 18 - 20, 2009	1.2
	Subtotal In-Depth Studies		8.1

4) Personal development		*date*	*cp*
►	**Skill training courses**		
	PhD course Techniques for Writing and Presenting Scientific Papers	Apr 19 - 22, 2011	1.2
	Writing a grand proposal course, RUG	Oct 21 - Nov 18, 2011	2.5
►	**Organisation of PhD students day, course or conference**		
►	**Membership of Board, Committee or PhD council**		
	Subtotal Personal Development		3.7

TOTAL NUMBER OF CREDIT POINTS*	**37.1**

Herewith the Graduate School declares that the PhD candidate has complied with the

** A credit represents a normative study load of 28 hours of study. The whole programme must contain a minimum of 30 ECTS*

Thesis lay-out: By the author
Cover design: Trapped whiteflies in a resistant tomato leaf painted by the author

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