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Identification of Sesquiterpenes in Wild Tomato Accessions with Activities Against the Potato Aphid and Their Tissue-Specific Engineering in Cultivated Tomato

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Identification of Sesquiterpenes in Wild Tomato Accessions with Activities Against the Potato Aphid and Their Tissue-Specific Engineering in Cultivated Tomato

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ABSTRACT

Identification of Sesquiterpenes in Wild Tomato Accessions with Activities Against the Potato Aphid and Their Tissue-Specific Engineering in Cultivated Tomato

Fumin Wang

The potato aphid (*Macrosiphum euphorbiae* Thomas) poses a serious problem in the commercial production of horticultural crops including tomato, since it causes damage by stylet feeding and the transmission of viruses for which it serves as the vector. Application of conventional pesticides being the fundamental tactic in the control of the aphid is increasingly considered insufficient and problematic due to emerging pest resistances and biosafety issues, highlighting the continuing need to develop new efficient and sustainable approaches. Alternatively, glandular trichomes of plants are well-known epidermal hairy tissues producing various secondary metabolites that involve in plant-insect interactions, while the terpene compounds produced from type VI glandular trichomes were frequently referred to as antixenotic or antibiotic resistant against both piercing-sucking and chewing-biting herbivores. Recent studies of terpene production in glandular trichomes of tomato, found on leaves and stems, demonstrated significant differences between cultivated (*Solanum lycopersicum* L.) and wild tomatoes (*Solanum habrochaites* S.Knapp & D.M.Spooner), as well as quantitative and qualitative variation of sesquiterpenes among the wild tomato accessions. Thus, the current research aimed to explore (1) if certain sesquiterpene chemotypes from the wild accessions support resistance against the potato aphid by affecting its pre- and postlanding behaviors during their interaction, and (2) would the resistance in cultivated tomato be improved if defensive sesquiterpenes were produced in a different tissue along the aphid stylet pathway by metabolic engineering.

To determine the effects of glandular trichome derived sesquiterpenes produced from wild tomato, five chemotypes (I-V) from a collection of *S. habrochaites* accessions were re-confirmed by gas chromatography-mass spectrometry (GC-MS). The performance (longevity and fecundity) of wingless aphids on tomato accessions, their feeding behaviors on an artificial diet, and the choice behaviors of winged aphids in an open Y-track olfactometer were analyzed. The results suggested that chemotype IV and V accessions which respectively produce mixtures of caryophyllene/α-humulene and santalene/bergamotene significantly reduced aphid longevity and fecundity in clip-cages while they were significantly repellent to winged aphids in the olfactometer. The trichome extracts from the two groups significantly affected aphid survivorship, gel saliva investment, and honeydew production in artificial diets, and significantly reduced the attraction of winged aphids to cultivated tomato. The same effects on feeding and choice behaviors were also observed by using pure caryophyllene/α-humulene as well as the trichome extract from one introgression line LA3935 which has santalene/bergamotene isomers predominantly produced in glandular trichomes.

Cultivated tomato lines generally produce a large quantity of TPS20-derived monoterpenes and low quantities of TPS9-derived δ-elemene and TPS12-derived sesquiterpenes. To explain the susceptibility of cultivated tomato plants against the potato aphid, the performance parameters (longevity and fecundity) of wingless aphids were analyzed on four tomato lines that

quantitatively differ in their terpene production, i.e., two cultivated tomato lines (Alisa Craig and Castlemart) with normal terpene production and their trichome mutants (*hairless* and *odorless-2*) producing respectively lesser amounts of sesquiterpenes and only tiny amounts of all terpene compounds. A principal component analysis (PCA) indicated that the performance parameters were negatively correlated with the production of the TPS12-derived sesquiterpenes, while no strong relationships were formed between the performance parameters and the production of TPS20-derived monoterpenes, which were further confirmed in artificial diets showing that the increasing concentration of TPS20-derived monoterpenes has little effects on aphid survivorship and production of gel saliva and honeydew. Additionally, a specific concentration of the TPS20-derived monoterpenes was significantly attractive to winged aphids in the olfactometer. Thus, the analyses explained the susceptibility of the cultivated tomato by revealing the contrasting roles of glandular trichome-derived monoterpenes and sesquiterpenes: while TPS12-derived sesquiterpenes contribute to host plant resistance against the potato aphid, TPS20-derived monoterpenes appear to be exploited as a cue for host plant orientation by the species.

Since the two sesquiterpene mixtures produced in glandular trichomes of wild tomato accessions affect aphid feeding, two multicistronic expression constructs were developed to engineering their production in epidermal cells, as their epidermal-specific formation was presumed to reduce tissue penetration by aphids and subsequent performance. Both constructs contained sequences encoding a prenyl transferase (TPS), a respective terpene synthase (TPS), and an enhanced green fluorescent protein (GFP) as visible marker. All three coding sequences were linked by short nucleotide sequences encoding the foot-and-mouth disease virus 2A self-processing oligopeptide which allows their co-expression under the control of an epidermis-specific Arabidopsis *CER5*-promoter. Transient expression of both constructs by infiltrating *odorless-2* leaves leads to the formation of the two sets of defensive sesquiterpenes, β-caryophyllene/α-humulene and santalene/bergamotene. The epidermis-specific transgene expression and terpene formation were verified by fluorescence microscopy and tissue fractionation with subsequent analysis of terpene profiles, respectively. In addition, the longevity and fecundity of the potato aphid feeding on infiltrated leaves were significantly reduced.

This study overall identified two groups of sesquiterpenes in glandular trichomes of *S. habrochaites* accessions that support antixenotic resistance against the potato aphid by negatively affecting their performance and choice behavior. The defensive traits engineered in epidermal cells from the wild tomato also improved the resistance in susceptible tomato plants, suggesting a novel and sustainable non-pesticide strategy for managing the aphid species. Further studies need to be conducted to test whether the defensive sesquiterpenes are also antibiotic resistant with effects on aphid physiology and biology, and to produce the sesquiterpenes in other tissues, such as companion cells, along aphid stylet pathway, to evaluate tomato plant resistance to the potato aphid.

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1.1 Introduction

1.1.1 Background: Plant and insect interaction

Plants are sessile organisms that inevitably face changing biotic challenges and other dynamic environmental conditions in their natural habitat. As part of biodiverse communities in which multiple organisms interact multidimensionally, plants drive population dynamics, ecosystem nutrient cycling, environmental changes as well as their co-evolution with arthropods (Boyer 1982; Stotz et al. 1999; Lu et al. 2014). Particularly the plant-insect interrelation has received much attention because insects are the most speciose group in Arthropoda, comprising an estimated 6 million species of which 50% are herbivorous, while insects and plants together make up approximately half of all known species of multicellular organisms. Plants started to interact with chewing detritivore dating back to the Devonian period, about 420 million years ago, when primitive rooted plants were well underway in their colonization of the land (Chaloner et al. 1991). The interaction became more intense during the Upper Carboniferous period, about 320 million years ago, when insects including simple plant piercers dominated invertebrate communities in above-ground environments (Scott and Taylor 1983). The interaction then became more diverse following the emergence of pollen/nectar consumers and entomophily in the early Cretaceous period as well as the appearance of flowering plants (angiosperms) in the late Cretaceous period (Grimaldi 1999). As of today, knowledge in plant-insect interaction involves structural and functional complexity at different hierarchies: starting from subcellular molecular modifications (Shinya et al. 2016), functional orchestration of behavioral responses (Guillet et al. 1995), to the interaction of communities at an ecological scale (Zhu et al. 2015). Such knowledge not only improved our understanding of the biosphere but also, more practically, provided solutions that ameliorate agricultural production under herbivore stress.

Insects may act as protectors, dispersers, or fertilizers for plants while plants may be food/energy resources or nest locations for insects. Thus, types of their interaction are classically viewed as mutualistic, antagonistic and commensalistic. Mutualism is established when plant and insect benefit each other, and neither is harmed. Mutualisms include pollination (e.g., flowering plant/insect pollinator systems), plant guarding and seed dispersal (e.g., plant/ant systems). For

example, butterflies and bees are flower pollinators that benefit plant reproduction while they acquire nutrition for growth during flower visitation (Kearns et al. 1998). Ants protect host trees from being crowded out by other plants and in exchange they live in hollow spaces of tree branches (Milius 2005). Ants are also attracted to seeds which have nutritional appendages (elaiosomes) attached, thereby mediating seed dispersal by carrying seeds (Gorb and Gorb 2003). In antagonistic relationships, one counterpart benefits and the other is harmed; insects feed on plants or utilize them as shelter or egg-laying sites while plants can be insectivorous (e.g., carnivorous plants). In a commensal relationship, one counterpart benefits while the other is not harmed. Relationships between plants and insects are flexible in different consumer-resource systems. In some moth-plant or ant-plant systems the relationship of mutualism, commensalism or antagonism varies according to insect density, environment parameters and their feeding habits (Thompson and Fernandez 2006; Chamberlain and Holland 2008).

1.1.2 Antagonistic interaction: insect herbivory

Phytophagous insects and green plants make up 26% and 22% of the major taxa excluding fungi, algae and other microbes, making their antagonistic relationship an important topic (Strong et al. 1984). Plant tissues (roots, stems, leaves, flowers and fruits) contain ample primary metabolites including amino acids, carbohydrates and lipids (Zhou et al. 2015). These primary metabolites are synthesized from independent or related biochemical pathways, subsequently being allocated, and then accessed as nutrition by phytophagous insects with adapted mouthparts. The main insect groups of feeding guilds are leaf and tissue chewers and piercing-sucking phloem and cell content feeders. It is generally known that mouthparts of piercing-sucking herbivores are typically evolved to absorb intracellular or extracellular fluids which are rich in carbohydrates as an energy source but lack balanced composition of amino acids (Galun and Fraenkel 1957; Canavoso et al. 2003). In contrast, chewing or biting insects swallow larger quantities of plant tissues, acquiring carbohydrates and amino acids in a balanced ratio, which is optimal for larvae development (Chapman 1998).

1.1.3 Antagonistic interaction: plant defense strategies

Plants have adapted various mechanisms/traits to resist the access of primary metabolites allocated in tissues by phytophagous insects, which are defined in different types. Above all, plants use mechanical/structural adaptations to discourage consumption by herbivores, as well as

a variety of defensive chemicals that are produced to affect herbivore behaviors and biology (Barah and Bones 2015). Plant defenses are also constitutive or inducible, depending on whether responses are present regardless of ambient conditions or are induced by biotic and abiotic factors. Secondary metabolites produced from plants either have direct (repellent or toxic) effects on herbivores, or indirect effects by acting attractive to carnivores and parasitoids such that an herbivore population is indirectly suppressed. Furthermore, plants affect insects in different resistance manners (e.g., antibiosis, antixenosis or non-preference, and tolerance) (Teetes 1996). Antibiosis resistance usually refers to plant characteristic that affects pest biology in a deleterious manner, resulting in increased mortality, reduced longevity and reproduction, and subsequently less damage to plants. Antixenosis occurs when there is non-preference for the resistant plant compared to a susceptible one, usually defined with characteristics that direct a pest away from approaching. Tolerance is a type of resistance that causes the plant to withstand or recover from damage caused by insect pests to a degree exceeding non-tolerant plants.

Plant defense mechanisms operate at different stages of an herbivore attack. Prior to landing, herbivores assess the quality of plants by detecting a mixture of volatile organic compounds (VOCs) emitted from the plants (Bernays and Chapman 2007). The detection of plant odorants by olfactory neurons triggers neuronal activates in higher centers before behavioral responses are initiated (Kaupp 2010). Upon acceptance, herbivores have to deal with both physical and chemical factors that interfere with feeding behaviors. Plants have external protective structures, including an impenetrable cuticle as well as epidermal outgrowths (appendages) that keep typically small insects away from the plant surface. The plant cuticle is a bilayer that consists of epicuticular lipids and a cutin matrix, which affects herbivores due to its physicochemical properties (Bargel et al. 2006; Jeffree 2006). It is well-known that the attachment of insect eggs and tarsal locomotion is affected by the structure and loading of epicuticular waxes (Peter and Shanower 2001), while the movement and feeding of neonate larvae are influenced by the special constitution in cuticles (Zalucki et al. 2002). Plant epidermal hairs or trichomes can entrap insect locomotion and some of them have secretory cells releasing sticky and toxic compounds that make plants unpalatable and harmful (Fordyce and Agrawal 2001; Cardoso 2008). In addition to the external structures, local and systemic responses can be elicited in plant tissues in response to the feeding activities of herbivores, from which the production of more

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specialized metabolites are induced to retard insect growth or recruit predators for indirect control (Bodenhausen and Reymond 2007; Bruinsma and Dicke 2008).

1.1.4 Pest issues in greenhouse production

In a greenhouse environment, cultivated plants are well-protected from harsh abiotic conditions for year-round production. However, the pest population in a greenhouse could amplify rapidly due to the warm and humid atmosphere, the absence of wind and rainfall that remove pest insects from plants, as well as the lack of factors that disrupt pest life cycles (Schoonhoven et al. 2005). Compared with an open field with a multitude of plant species, pests are much easier to locate host plants under greenhouse conditions, since the volatile compounds emitted from host plants in a greenhouse failed to be 'masked' or 'diluted' (Collier et al. 2001). Additionally, predators and parasitoids are normally absent in a greenhouse due to their monocultural planting patterns (Tscharntke et al. 2007; Gardiner et al. 2009). For these reasons, pest situations often develop in controlled 'indoor' environments more rapidly and with greater severity than outdoors.

1.1.5 Piercing-sucking pests are problematic in greenhouse production

Piercing-sucking herbivores can lead to serious economic loss in the greenhouse, although they are normally tiny, consume little to no tissues and do not immigrate frequently. Most piercing-sucking herbivores are *r*-selected species, meaning that they have overlapping generations because of their short premature period, short lifespan, and high fecundity. Unlike chewing pests that cause evident feeding injury, the feeding of sucking insects- such as aphids, leafhoppers, thrips, and whiteflies- cause relatively little immediate harm to a plant, making it difficult to have a timely recognition and management. In addition, sap suckers transmit plant viruses during salivation and frequent stylet probing. Those viruses are spread mechanically or they are absorbed and retransmitted through stylet movement in a circulative manner (Jones 2003; Harris and Maramorosch 2014).

1.1.6 Cultivated tomato fruits are commercially important

The genus *Solanum* contains a lot of intercrossable tomato species widely distributed in temperate climates across the world. The cultivated tomato, *Solanum lycopersicum*, is easy to breed, self-compatible, and provides edible fruits annually that are consumed in diverse ways.

Tomato fruits provide excellent or good amounts of a variety of micronutrients, including vitamins and minerals, which make the production of tomato fruits in tons ranked 11ths of all agricultural products consumed in the USA (FAOSAT, http://www.fao.org/). Compared with the early 1960s, the tomato industry in 2017 had more than a 20-fold increase in harvested area, whereas only a 9-fold increase in production was achieved (FAOSAT, http://www.fao.org/). In North America greenhouse tomato farming has grown rapidly since the early 1990s and now spans most US states, playing a major role in the fresh tomato industry. Accordingly, more attention and efforts have been paid to the development of innovative technologies used for pest control in greenhouse tomato production.

1.1.7 Aphid issue in tomato production

Reproduction of aphids can alternate between sexual and asexual, depending on the coordination of day length, temperature, parent type and genetic factors (Kenten 1955; Blackman 1971). Under favorable conditions, apterous viviparous females are constantly reproduced by parthenogenetic viviparous females without eggs being laid (Vilcinskas 2016). Apterae can have a premature period as short as a few days, such that tomatoes are often attacked by overlapping generations before harvest (Wyatt and White 1977). As the season changes and temperatures fall, viviparous females begin producing alate females which then reproduce oviparous (sexual) females and males that can mate thereafter. Semiochemicals play a vital role in sexual reproduction since volatiles from host plants can guide aphid immigration, while males are attracted by sex pheromones emitted from the hind legs of oviparae of females (MacGillivray and Anderson 1964).

Macrosiphum euphorbiae (Thomas), the potato aphid, is a sap-sucking pest in the family Aphididae and it infests many commercially important *Solanum* plants such as tomatoes and potatoes. The potato aphid feeds preferentially on the leaves, stems, buds, and flower parts of tomato plants, absorbing phloem sap after the stylet passes through epidermis, mesophyll, and companion cells. Although feeding by a low number of aphids usually does not damage leaves intensively, it takes only a few minutes for them to transmit viruses into plant tissues. The potato aphid is a vector for transmitting a number of viruses that include lettuce mosaic virus, bearded iris mosaic virus, narcissus yellow stripe virus, tulip breaking virus, potato leafroll virus, potato virus Y, beet mild yellowing virus and beet yellows virus. Plant viruses not only mottle, yellow,

or curl leaves and stunt plant growth but also affect the performance of aphid endoparasitoids (Ng and Perry 2004; Calvo and Fereres 2011). Without timely monitoring and management, a large population of potato aphid can cause distortion and falling of tender leaves and flowers and eventually destroy the whole plant (Cannon et al. 2017).

There have been multiple explanations for how constitutive and inducible plant defensive traits are insufficient to resist aphid herbivory. Above all, the feeding of some aphid species has extracellular stylet-probing behaviors beyond the epidermis level (Walling 2008; Will et al. 2013). The "stealth" feeding manner not only reduces the exposure of stylet to constitutive defensive chemicals localized in mesophyll cells but also diminishes the induction of phytohormonal signaling (Molyneux et al. 1990). Aphids have adapted manifold tolerance against phytotoxin, including 1) avoidance of toxins uptake by the gut, 2) toxin degradation by detoxifying enzymes, 3) development of insensitive target sites for toxins, as well as 4) their active elimination from the body (Wink 1998). Moreover, some aphid species actively invest proteinaceous elements during salivation which can be utilized as "effectors" to modulate and suppress the phytohormonal pathway that occurred in plants (Will et al. 2007; Will and van Bel 2008; Elzinga and Jander 2013). For example, saliva elements injected by *Myzus persicae* were used to induce the SA-signaling, while the JA-induced signaling which affected the aphid was downregulated by the "cross-talk" effect (Mewis et al. 2005).

Key components in the control of potato aphids include organic, biological, and chemical control. Water, flour, insecticidal soap, and essential oils can be applied to deter aphids, but such application is not effective for a large population, while frequent applications may negatively affect plant physiology. Parasites and carnivores (e.g., lady beetles and lacewings) can be used for controlling the potato aphid but the performances of the natural enemies are limited. In addition to safety concerns, applying pesticides might not be effective considering the biochemical, physiological, and behavioral resistance mechanisms developed in aphids. For instance, some aphid genes encode enzymes to overexpress carboxylesterases to hydrolyze or sequester pesticides belonging to organophosphate (OP), (monomethyl) carbamate and pyrethroid classes before the pesticides reach target sites in the nervous system. The structure of acetylcholinesterase enzyme (AChE) in some aphid clones has been modified causing the insensitivity to dimethylcarbamates pirimicarb and triazamatem (Moores et al. 1994). Some

aphid clones have the ability to upregulate cytochrome P450 gene *CYP6CY3*, which is closely related to the detoxification of nicotine and neonicotinoid insecticides (Moores et al. 1994; Puinean et al. 2010). Resistant aphid clones sometimes evade contacting pesticides while spending a greater proportion of time on insecticide-free plants than neonicotinoid-treated tissues (Fray et al. 2014).

1.1.8 Trichomes are important defensive traits

Trichomes are epidermal outgrowth (appendage) localized on the leaf, stem, and other organs of plants. Both glandular and non-glandular trichomes are presented on *Solanum* species and they vary in size and morphologies. Non-glandular trichomes are thought to act as a protective physical barrier against herbivore attack, preventing mouthpart penetration as well as restricting their locomotion (Van et al. 1998; Pott et al. 2012). In many plant species, the density of non-glandular trichomes is negatively correlated with the performance (e.g., feeding and oviposition) of sap-suckers (Khan et al. 2000; Dalin et al. 2008). Glandular trichomes have additional secretory cells that support the biosynthesis of different secondary metabolites (Glas et al. 2012). There are four types (I, IV, VI, VII) of glandular trichomes classified throughout *Solanum* species and, through analysis of transcriptomes and metabolomes, those glandular trichomes are attributed to the production of terpenes, phenylpropenes, flavonoids, methyl ketones, acyl sugars, polyphenol oxidases and protease inhibitors (Glas et al. 2012).

Specialized metabolites derived from glandular trichomes have been well-known for conferring chemical resistance against chewing and piercing-sucking herbivores. Terpenes accumulated in type VI trichomes intoxicate herbivores and attract their natural enemies, including predators and parasitoids (De Moraes et al. 1998). The phenylpropenes and flavonoids that originate from the phenylpropanoid pathway are abundant in type I and IV glandular trichomes, and these chemicals affect the growth of Coleopteran pest and lepidopteran larvae (Duffey and Isman 1981; Obeng-Ofori and Reichmuth 1997). In some plant species, type I and IV trichomes have the ability to secrete sticky acyl sugars that not only suffocate small herbivores and subsequently influence their feeding and oviposition (Hawthorne et al. 1992; Puterka et al. 2003), but also deter probing of some sap-suckers (Goffreda et al. 1989). Type VII glandular trichomes generally store many defensive proteins such as polyphenol oxidases and peroxidases. Following insect-mediated rupture of the glandular cuticle, the foliar oxidases are

exposed to a trichome-born phenolic substrate to generate toxic orthoquinones, therefore reducing the availability and digestibility of essential amino acids to herbivores (Felton et al. 1989).

1.1.9 Glandular trichomes of wild tomato plants are resources for defensive traits

Cultivated tomato *S. lycopersicum* has abundant type VI trichomes and less abundant type I trichomes on leaves and stems, as well as a few other trichome types on stems, veins, and leaflet edges (Glas et al. 2012). In glandular trichomes on leaves and stems of cultivated tomato, the characteristic volatiles are primarily a mixture of monoterpenes and a few less abundant sesquiterpenes (Bleeker et al. 2009; Tian et al. 2012). Despite a large quantity of monoterpenes, cultivated tomato plants are highly susceptible to herbivore infestation and biosynthesis of herbivore-defensive chemicals was thought to be lost during the long-term domestication. In contrast, glandular trichomes of wild relatives of cultivated tomato can produce different terpenes compounds that are broadly resistant to different herbivores (Kowalski et al. 1992; Antonious 2001). For example, some *S. pennellii* accessions were repellent to whiteflies due to the accumulation of monoterpenes (*p*-cymene, α-terpinene, and α-phellandrene) and sesquiterpenes (zingiberene and curcumene) in glandular trichomes (Bleeker et al. 2009). The mixture of zingiberene, curcumene and bisabolene extracted from *S. habrochaites* accessions had a low level of median lethal dose (LD_{50}) to Colorado potato beetles (Carter et al. 1989). Methyl undecyl ketone (2-tridecanone) accumulated in trichomes of an *S. habrochaites* accession were lethal to tobacco hornworm (*Manduca sexta*) and the cotton aphid (*Aphis gossypii*) when tested at a large amount (Williams et al. 1980; Dimock and Kennedy 1983).

1.1.10 Research focus and objectives

To study whether trichome-derived specialized metabolites play an antagonistic role in the interaction of tomato plants with the potato aphid, antixenosis (preference) and antibiosis (performance) from a collection of different *S. habrochaites* accessions will be evaluated against the aphid species. The *S. habrochaites* accessions are separated into different chemotypes based on the diversity of major sesquiterpenes produced in glandular trichomes (Gonzales‑Vigil et al. 2012). The first objective of this project is to identify any chemotype that can influence the performance and preference of the potato aphid by testing plant leaf materials, trichome extracts,

as well as pure sesquiterpenes identified in the extracts. The second part of the project aims to improve the resistance in cultivated tomato plants by introducing the biosynthesis of defensive sesquiterpenes from the wild tomato accessions. The production of defensive sesquiterpenes will be engineered in glandular trichomes and other tissues of cultivated tomato leaves along the stylet pathway of the potato aphid, by guiding the expression of genes encoding enzymes involved in the biosynthesis of the defensive sesquiterpenes by promoters with tissue-specific activities. The feasibility of the approach is that 1) genes encoding prenyl transferases and terpene synthases have been characterized in *S. habrochaites* accessions, and 2) promoters that have specific activities in glandular trichome, epidermal cells, or companion cells have been identified (see next chapter). The last objective of the project is to assess the antixenosis and antibiosis resistance in transgenic tomato plants which have defensive sesquiterpenes produced in different tissues (glandular trichomes, epidermal cells and companion cells). Stylet activities of the potato aphid will also be monitored and analyzed in order to determine an optimal metabolic engineering strategy for further sustainable management of the piercing-sucking aphid species in the greenhouse.

1.2. Literature review

1.2.1 Aphid olfaction and their attraction to plant volatiles

Aphid populations can grow rapidly under greenhouse conditions by the parthenogenetic reproduction of apterae that produce abundant apterous viviparae. Increases in population density result in the production of alate virginoparae, the sexual generation that migrates for host-alternation and subsequently develops viviparae offspring. During the host-alternation process volatile emissions from the host and non-host plants are perceived by the olfactory system of alate virginoparae to differentiate between these (Webster 2012; Döring 2014). Aphids have receptor neurons housed in antennal rhinaria, known as circular openings positioned on the membrane of antennae (Nichols 1989). Primary rhinaria are plate-like sensory patches localized on the 5th to 6th antennal segment, and most aphid species use sensilla in the primary rhinaria to respond to biologically active plant volatiles, including host plant and non-host plant volatiles. Secondary rhinaria are small patches on the 3rd, 4th and 5th antennal segment of aphids, containing receptors that respond to sex pheromone components (Marsh 1975; Dawson et al. 1987).

Previous choice-behavior experiments concluded that aphids were attractive to a unique mixture of multiple volatile compounds commonly found in plants, or certain compounds exclusively emitted from a plant species or genus. For example, the volatile emission of faba bean (*Vicia faba*) was attractive to black bean aphid (*Aphis fabae*), while none of the volatile components of faba bean are species-specific (Knudsen et al. 1993; Webster et al. 2008a). By olfactometry testing with each compound it was shown that large amounts of (Z)-3-hexen-1-ol were only slightly attractive, whereas the effects of other compounds were all neutral (Webster et al. 2008b, 2010). On the other hand, alate virginoparae of several species including *Lipaphis erysimi*, *A. fabae*, and *Brevicoryne brassicae*, which all feed on Brassicaceae plants, are known to be attracted by isothiocyanates (V.K. and A.S. 1989), a taxonomically-specific compound found only in this crop family (Fahey et al. 2001).

Unlike host-plant cues, non-host plant volatile compounds (NHPVs) are widely known to disrupt aphid attraction toward plants. The presence of NHPVs is thought to mask the information of host plant volatiles or change the ratio of concentrations of volatile compounds, thereby affecting the prelanding of aphid alatae as an antixenosis (Hori 1998). For example, methyl salicylate, a major volatile compound identified from a wide range of non-host plant species for *A. fabae*, inhibits the orientational flying and settling behaviors of alate *A. fabae* (Nottingham et al. 1991; Hardie et al. 1994). Aphid specialists that feed on non-Brassicaceae plants are evasive to the isothiocyanate extracted from non-host Brassicaceae essential oils (Verheggen et al. 2013). Additionally, a mixture of terpenoids distilled from essential oils of non-host plants had strong activity in repelling the landing of *Rhopalosiphum maidis* alatae (Halbert et al. 2009).

1.2.2 Within-species diversity of plant genotype and phenotype affect aphid performance

Following landing behaviors, aphids access plants immediately through a number of pre- and post-stylet probing (Powell et al. 2006). The host acceptance for aphids can be quantified in the greenhouse at an individual level by measuring different performance parameters (e.g., longevity and fecundity) and intrinsic growth rate (r_m). A favorable host plant for aphids is defined by a short premature period, and a high level of longevity and fecundity. The performance parameters

are correlated with the initial exponential growth rate of an aphid population according to the Malthusian growth model (Wyatt and White 1977).

Intraspecific diversity of genotype and phenotype in plants is a factor that results in varying levels of aphid performance, as observed from many experiments under controlled conditions (Linhart et al. 2005; Staudt et al. 2010; Utsumi et al. 2011; Shoffner and Tooker 2013; Williams and Avakian 2015). Different plant cultivars are assumed to differ in their ability to express resistance genes and to elicit systemic resistance responses upon aphid herbivory (Smith and Clement 2012). For instance, a semi-dominant gene *AIN* (*Acyrthosiphon*-induced necrosis) in *Medicago truncatula* was related to a hypersensitive response induced by bluegreen aphid (*Acyrthosiphon kondoi*). The hypersensitive response, defined as a host plant resistance, refers to the localized cell death of plant cells at the point of pathogen infection (Klingler et al. 2009). It was found that the response differs in *Medicago truncatula* accessions due to the varied expression level of the gene (Klingler et al. 2009).

Heritable physiological characteristics of plants that affect aphid performance include leaf water content, accessible nitrogen in the phloem, and distribution of trichomes (Johnson 2008). Water-soluble carbohydrates (WSCs) in phloem have a positive impact on aphid survivorship because abundant WSCs facilitate the process of osmotic regulation during aphid feeding (Johnson 2008; Alkhedir et al. 2013). Abundant leaf nitrogen increases the aphid growth rate by supporting amino acid biosynthesis in aphids (Agrawal 2004). The influences of trichomes on aphid performance are manifold, but mainly negative. Generally, the density of non-glandular trichomes negatively correlated with aphid feeding and oviposition responses (Levin 1973), but they might help increase aphid population size by protecting aphid nymphs from natural enemies (Johnson 2008). Intraspecific chemical diversity of glandular trichomes is an additional factor related to the variation of aphid performance. For example, multiple aphid species exhibited a similar occurrence pattern on tansy (*Tanacetum vulgare*) plants with different chemotypes, which correlated with the concentration of camphor produced in trichomes (Kleine and Müller 2011). The abundance of *A. serpylli* also varied on thyme plants with different terpene compositions, whereas the abundance of insects was low on plants with high levels of linalool and other terpenoids (Linhart et al. 2005).

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1.2.3 Aphid feeding activity in aphid-plant interaction

Similar to the feeding behavior of other phloem suckers, aphids extend a specialized mouthpart (stylet) to uptake photosynthetic products from the phloem by penetrating epidermal cells, mesophyll, parenchyma, and companion cells. During regular penetration, ingesting small quantities of cell sap before the stylet reaches the phloem is inevitable (Wensler and Filshie 1969). The stylet activities in the different plant tissues are both inter- and intracellular, and the stylet route can be circuitous, involving dead-ends, direction reversal, and aborted sieve-element punctures (Tjallingii and Esch 1993).

An electrical penetration graph (EPG) is a popular system employed to track stylet activity and feeding behaviors of aphids. A standard EPG system consists of one voltage source, one fixed input resistor, and two variable resistors represented respectively by the connection of a wired aphid and by a plant which settles the aphid with copper wire (van Helden and Tjallingii 1993). Feeding activities of aphids cause the fluctuation of conductivity/resistance in the system, generating waveform patterns recorded by the system used for distinguishing different feeding events (Walker 2000; Tjallingii 2001). For instance, fluctuation in electrical conductivity is inevitable when aphid stylet touches apoplastic fluid with varying ionic concentrations. Electrical resistances of the system also differ when an aphid produces different types of saliva (watery or gel) or when the aphid actively controls sap flow by opening and closing of its precibarial valve (Vilcinskas 2016).

Because stylet activities in different plant tissues generate different EPG waveform patterns (see next chapter for detail), the EPG parameters can be utilized to verify plant resistance as well as its localization (van Helden and Tjallingii 1993). For instance, an "E2" waveform indicates the ingestion behavior at the phloem level, while the remarkable reduction of its duration implies the presence of sieve element-resistant substrates (Caillaud et al. 1995; Chen et al. 1997). Plant morphological structures that confer surface resistance might bias parameters of EPG waveforms that present stylet activities at the epidermis or in deeper tissues. It was shown that both the "probing initiation" (time to the first waveform) on the tissue surface and "probing duration" in the phloem (represented by waveform C) by *M. persicae* were significantly affected on two wild potato accessions which have high densities of glandular trichomes (Alvarez et al. 2006).

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1.2.4 The function of saliva in aphid feeding

During stylet penetration, two types of saliva are produced from a pair of secretory organs (salivary glands) in the dorsal metathorax: a large principal gland that contains gelling (gel) saliva while an accessory gland that produces watery saliva (Ponsen 1972; Gray and Gildow 2003). Generally, the secretion and positioning of gelling saliva (i.e., small proteinaceous droplets) occurs at the early stage of stylet insertion into leaf tissue, forming a "flange" or "salivary sheath" structure which benefits penetration and feeding in multiple ways (Miles 1999). The "sheath" structure prevents the leaking of cell sap from stylet and the ingress of unwanted plant material into the feeding channel. Miles (1999) proposed that the hardening gel saliva lubricates the prolongation and refraction of the stylet against mechanical forces. This was supported by the knockdown of genes encoding sheath structural proteins that lead to the insufficient hardening of the salivary sheath and subsequent unsustainable feeding (Abdellatef et al. 2015). In addition, by sealing the stylet piercing site in the plasma membrane of sieve elements, salivary sheath prevents the induction of some defense responses in sieve elements (Will and van Bel 2006). Unlike gel saliva, watery saliva is mainly produced intracellularly during the penetration stage (Tjallingii 2006), which is thought to aid in lubrication, the suppression of host plant defense mechanism, the digestion of external and ingested food material, and the excretion of certain metabolites (Miles 1972, 1999; Taylor and Miles 1994; Urbanska et al. 1998; Will et al. 2007; Will and van Bel 2008; Carolan et al. 2009; Ma et al. 2010).

Aphids are able to detect environmental conditions by sensing their stylet-tip milieu and adjusting salivation behaviors as an adaptation. It was found that *Megoura viciae* prefers investing gel saliva under the apoplastic condition, while in the weakly alkaline sieve-tube condition the production of watery saliva was enhanced (Will et al. 2012). Notably, the pea aphid *Acyrthosiphon pisum* actively suppresses plant defense by increasing watery salivation events, while reducing gel saliva formation by inhibiting SHP (structural protein formed in gel saliva) metabolism (Will and Vilcinskas 2015). However, it remains an unanswered question whether the constitutive or inducible defense mechanisms in tomato plants could affect the salivation activities of aphids.

1.2.5 Terpene biosynthesis in cultivated and wild tomato plants

Plants produce a plethora of specialized (previously named secondary) metabolites that have specific roles in plant-arthropod interactions. Among those, terpenes (terpenoids or isoprenoids) represent the largest and most diverse class, which are well-known for their role in plant defense. Thus, understanding how terpenes are formed in tomato plants is a prerequisite for utilizing them in aphid control. In both cultivated tomato plants and their wild relatives, terpenes are mainly produced in type VI glandular trichomes which are distributed on leaves and stems. The initial substrates for terpene biosynthesis are isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), both of which are naturally produced in trichome cells through one of two pathways, i.e., the cytosolic mevalonic acid (MVA) pathway and the plastidic 2C-methyl-D-erythritol 4-phosphate (MEP) pathway (**Figure 1.1**). IPP and DMAPP undergo different head-to-tail condensations in specific ratios, which are catalyzed by a class of prenyl transferases (cis or trans isomers) that transfer allylic prenyl groups to acceptor molecules, forming a variety of larger prenyl diphosphate intermediates. The diversity of terpenes found in plants is then generated by terpene synthases (TPS) which utilize one or several prenyl diphosphate substrates, and often can produce multiple terpene products from one prenyl diphosphate substrate (Degenhardt et al. 2009b). In general, cytosolic sesquiterpene synthases use farnesyl diphosphate (FPP) while plastidic monoterpene synthases use geranyl diphosphate (GPP), respectively, as substrates, to produce monoterpenes and sesquiterpenes. However, some noncanonical biosynthetic pathways exist in cultivated and wild tomato plants. For example, in *S. lycopersicum* cultivar M82, IPP and DMAPP are used by neryl diphosphate synthase (NPPS), a plastidic cis-prenyltransferase, for generating neryl diphosphate (NPP), which serves as precursor used by phellandrene synthase 1 (PHS1) to produce β-phellandrene and other monoterpenes (Schilmiller et al. 2009). In some accessions of the wild tomato *S. habrochaites*, a sesquiterpene mixture of santalene and bergamotene isomers is produced in plastids from Z,Z-farnesyl diphosphate (z-FPP), a product generated by cis-farnesyl pyrophosphate synthase (z-FPPS), which is another plastidic cis-prenyl diphosphate (Sallaud et al. 2009).

The wild tomato *S. habrochaites* consisting of multiple accessions is native to Peru and southern parts of Ecuador and has small, green, hairy fruits. This species is known as a source of cold tolerate and pathogen resistance (Perring et al. 2018). More importantly, *S. habrochaites* has

been noted for its several chemical resistances to pests. For example, *S. habrochaites* contains a high concentration of a naturally occurring pesticide called 2-tridecacone, which provides a high degree of protection against spite mites, leaf miners, and caterpillars (Aragão et al. 2000; Oliveira et al. 2012; Silva et al. 2018). The produced terpenes are distinct between cultivated tomato and *S. habrochaites* accessions. While β-phellandrene is the major monoterpene compound in cultivated tomato, type VI glandular trichomes of *S. habrochaites* accessions have high intraspecific variability in their terpene composition. A systematic screen of volatile terpenes produced in glandular trichomes of different accessions of *S. habrochaites*, revealed a couple of chemotypes characterized by the dominant production of distinct sesquiterpene compounds (Gonzales‑Vigil et al. 2012), of which most are not found in *S. lycopersicum*. Multiple behavioral assays demonstrated that glandular trichome derived sesquiterpenes in *S. habrochaites* have repellent or toxic activity against tomato pinworm (*Keiferia lycopersicella*), Colorado potato beetle (*Leptinotarsa decemlineata*), and whitefly (*Bemisia tabaci*) (Carter et al. 1989; Bleeker et al. 2011; Zanin et al. 2021).

1.2.6 Metabolic engineering of terpenes in glandular trichomes of tomato plants

Glandular trichomes are excellent tissues for studying terpene biosynthesis and are especially suitable targets for metabolic engineering. Both plastidic- and cytosolic IPP and DMAPP are accumulated in glandular trichome cells, while a lot of genes that encode prenyl transferases and terpene syntheses have also been characterized (Falara et al. 2011; Akhtar et al. 2013). Trichome-specific production of terpenes can be achieved by guiding the expression of genes by promoters having trichome cell-specific activity (Rontein et al. 2008; Sallaud et al. 2012). Using trichome-specific promoters rather than constitutive promoters has the advantage that possibly toxic byproducts are not produced outside of these target tissues and that perturbation of metabolic pathways in the rest of the plant is avoided (Tissier 2012). Production of the sesquiterpene compounds identified from *S. habrochaites* has been engineered in type VI glandular trichomes of cultivated tomato background, aiming to improve its resistance against pests. For example, two genes that encode respectively farnesyl diphosphate synthase (z-FPPS) and 7-epizingiberene synthase (ShZIS) from wild tomato *S. habrochaites* have been expressed under the control of a *S. habrochaites* Methyl Ketone Synthase (ShMKS1) promoter and a *S. lycopersicum* Monoterpene Synthase (ShMTS1) promoter, respectively, in the cultivated tomato

c.v. Moneymaker (Bleeker et al. 2012). The transgenic plants that have ShZIS and z-FPP synthase specifically produced in glandular trichomes showed production of 7-epizingiberene in glandular trichome cells. The transgenic plants exhibited significant antixenosis against spider mites and whitefly by affecting their fecundity and populational development.

1.2.7 Metabolic engineering of terpenes in different tissues along the stylet pathway of aphids

Despite the defensive specialized metabolites accumulated in glandular trichomes, factors contributing to aphid resistance could be localized also in other tissues. For instance, host selection of aphids is also influenced by plant surface layers (cuticle and epidermis). An important protective barrier against aphid feeding is represented by morphological structures of the epidermal layer, epicuticular lipids and waxes, as well as epidermal cell sap components that together abate stylet activities (Powell et al. 1999; Powell and Hardie 2000; Reynolds et al. 2009). Because aphid feeding involves intra- and extracellular stylet-penetration across epidermis, mesophyll and phloem companion cells, the susceptibility of cultivated tomato plants to aphid infestation might indicate the absence of defensive phytochemicals in those tissues. Therefore, it is hypothesized that the metabolic engineering of defensive sesquiterpenes specifically in the epidermis or phloem companion cells would also improve the resistance of cultivated tomato background to aphids.

To date, promoters guiding the expression of fluorescent protein genes respectively in glandular trichomes, epidermal cells, mesophyll cells and companion cells have been all identified. The *CER5* gene in *Arabidopsis* encodes an ABC transporter localized exclusively in the plasma membrane of epidermal cells, and it was found that the 2.6kb genomic DNA upstream of the *CER5* start codon guides the epidermis-specific expression of GUS in stem cells (Pighin et al. 2004). In *Arabidopsis* a nonspecific lipid transfer protein (LTP1) has the ability to stimulate phospholipid transfer between membranes. The gene that encodes this protein is expressed in epidermal cells of young leaves and the stem, and it has a 1237bp promoter region upstream of the translation initiation codon (Thoma et al. 1994). *AtSUC2* encodes a plasma-membrane sucrose symporter which is responsible for the loading and unloading of sucrose between the sieve elements and companion cells in the phloem. The ~940 bp DNA region upstream of the start codon was shown to direct the expression of *AtSUC2* in the vascular

bundles of leaves (Truernit and Sauer 1995). In a recent integrative study, by using promoters of *AtLTP1*, *AtSUC2* and *AtRbcS*, the expression of genes for linalool/nerolidol synthases (*FaNES1*) in strawberry have been specifically guided into epidermis, mesophyll and companion cells of *Nicotiana benthamiana* leaves. In transgenic plants linalool and derivates were detected in different tissues via GC-MS analysis (Juneidi et al. 2014).

1.3 Significance and novelty

1.3.1 Exploiting defensive traits from wild relatives with diverse sesquiterpene productions

Unlike many studies trying to understand the phytohormonal signaling of plants in response to aphid feeding (Mewis et al. 2005; Elzinga and Jander 2013), this project aims to screen and utilize the defensive traits from wild tomato accessions which are presumed to have been lost in cultivated tomato during domestication and breeding.

Although some wild tomato accessions have been found to be pest-resistant (Goffreda et al. 1988; Le Roux et al. 2007; Sarria et al. 2010), comprehensive approaches, as proposed in this project, are required to characterize the resistant traits as well as the roles they play in different stages of aphid-tomato interaction including pre-landing and feeding stage. Additionally, because type VI glandular trichomes, as well as the terpene diversity, are also present in wild accession of other *Solanaceae* species, the approaches used in this project may also apply to other plant-insect models.

1.3.2 Engineering of terpene biosynthesis in different tissues along the stylet pathway of aphids

Traditional approaches for metabolic engineering of terpenes to improve pest resistance mainly attempt to introduce new terpene synthase genes, or to overexpress existing terpene synthases genes and upstream pathway genes (Degenhardt et al. 2009a; Orlova et al. 2009; Maeda et al. 2010). Using these approaches the expression of introduced genes is generally controlled by constitutive promoters (such as CaMV 35S promoter) without tissue-specific activities (Hilder et al. 1995; Zhou et al. 2009). In a recent study, to improve tomato resistance to spider mites, two trichome-specific promoters were used to express two genes encoding respectively a cis-prenyl transferase and a sesquiterpene synthases, however, the two respective

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expression cassettes with the promoter sequences were introduced by two independent transformations (Bleeker et al. 2012). The proposed project differs from previous approaches in that it will use tricistronic constructs to achieve the co-expression of all required pathway genes under the control of one promoter in specific tissues, thus avoiding the need to subsequently stack multiple independent transgenes in one plant line through crossings. In addition, the proposed tissue-specific engineering approach will also result in the sesquiterpene production along the stylet pathway of aphids, influencing different stages of stylet activities for an optimal aphid-resistance strategy. Therefore, the results of this project will provide valuable information for other studies dedicated to improving plant resistance to different piercing-sucking herbivores.

1.4 Summary

Overall, the whole project will be mainly divided into three parts: 1) The screening of repellent and deterrent terpenes from glandular trichomes of a collection of wild tomato *S. habrochaites* accession, by evaluating important behaviors of wingless and winged *M. euphorbiae*. 2) Cloning of different tricistronic constructs and their introduction into the tomato line MP-1. 3) A series of bioassays including non-choice assays, feeding experiments, choice behavior tests and EPG recording for evaluating different tissue-localized resistances in transgenic tomato plants.

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1.6 Figures
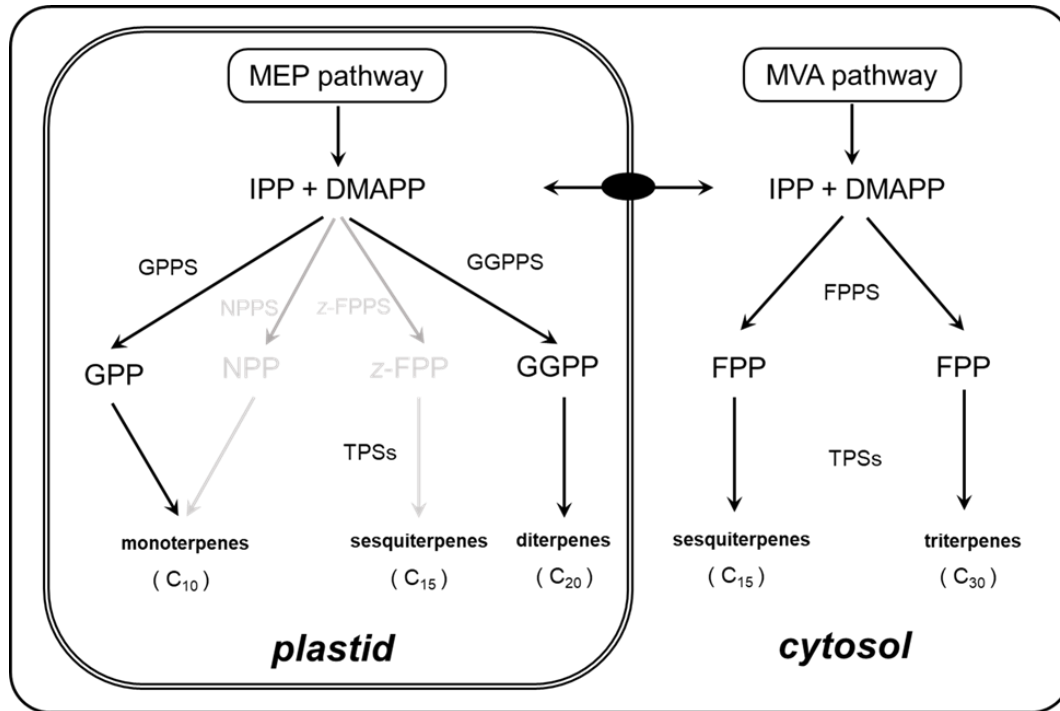
Figure 1.1 Schematic representation of common (black) and noncanonical (gray) terpene synthetic pathways and their subcellular localization in plant cells. Monoterpenes, diterpenes and a few sesquiterpenes are derived from IPP and DMAPP from the plastidic MEP pathway. Triterpenes and most sesquiterpenes are synthesized in the cytosol by IPP and DMAPP from the MEV pathway. IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; GPPS: geranyl diphosphate synthase; GGPPS: geranylgeranyl diphosphate synthase; NPPS: neryl diphosphate synthase; z-FPPS: cis-farnesyl diphosphate synthase; FPPS: trans-farnesyl diphosphate synthase; GPP: geranyl diphosphate; NPP: neryl diphosphate; z-FPP: cis-farnesyl diphosphate; GGPP: cis-geranylgeranyl diphosphate; FPP: cis-farnesyl diphosphate; TPSs: terpene synthases.

Chapter 2: Glandular trichome-derived sesquiterpenes of wild tomato accessions (*solanum habrochaites*) affect aphid performance and feeding behavior

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Abstract: Glandular trichomes of tomato produce a number of secondary metabolites including terpenes that contribute to host plant resistance against pests. While glandular trichomes of cultivated tomato *Solanum lycopersicum* primarily accumulate a monoterpene blend, those of wild tomato species like *Solanum habrochaites* produce various sesquiterpenes. Previous studies have shown that glandular trichome derived terpenes in cultivated and wild tomato species have repellent and toxic activity against multiple biting-chewing herbivores. In contrast, considerably less is known about the effect of these glandular trichome derived terpenes on piercing-sucking herbivores such as aphids. Here, we have screened a collection of *S. habrochaites* accessions representing five chemotypes that produce distinct sets of sesquiterpenes to identify those affecting the potato aphid (*Macrosiphum euphorbiae*). Non-choice assays demonstrated that the longevity and fecundity of *M. euphorbiae* was significantly reduced when kept on the leaf surface of *S. habrochaites* accessions producing β-caryophyllene and α-humulene, or α-santalene, α-bergamotene, and β-bergamotene, respectively. When *M. euphorbiae* apterae were feeding on artificial diets with added terpene containing leaf dip extracts, the same β-caryophyllene/α-humulene and α-santalene/α-bergamotene/β-bergamotene producing *S. habrochaites* accessions were found to affect aphid survivorship and feeding behavior as indicated by gel saliva investment and honeydew production. Olfactometer assays revealed that the sesquiterpenes emitted from these *S. habrochaites* accessions also have repellent activity against *M. euphorbiae* alatae affecting their choice behavior prior to landing on host plants. Assays performed with pure sesquiterpene compounds and an introgression line carrying respective *S. habrochaites* terpene biosynthetic genes in the *S. lycopersicum* background confirmed that β-caryophyllene/α-humulene and α-santalene/α-bergamotene/β-bergamotene were responsible for the observed effects on performance, feeding and choice behavior of *M. euphorbiae*.

Keywords: *Solanum habrochaites*; *Solanum lycopersicum*; Solanaceae; tomato; *Macrosiphum euphorbiae*; potato aphid; glandular trichomes; sesquiterpenes

2.1 Introduction

In nature plants are integrated in a complex system of biotic interactions and, due to their sessile lifestyle, have evolved specific strategies to defend themselves against attacking organisms such as pests. In this context plants produce a wide variety of secondary metabolites including volatile organic compounds that contribute directly to the defense against herbivores by acting repellent and/or toxic, and indirectly by attracting natural enemies (Dudareva et al. 2013; Unsicker et al. 2009). Plant volatile organic compounds primarily belong to three classes of secondary metabolites: phenylpropanoids/benzenoids, fatty acid derivatives, and terpenoids (Dudareva et al. 2013). Terpenoids are a large and diverse class of plant metabolites that include vital molecules such as sterols, chlorophylls, carotenoids, and several hormones involved in basic plant processes, as well as in addition volatile monoterpenes and sesquiterpenes. All terpenoids originate from the building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP are used by prenyl transferases to form larger prenyl diphosphate intermediates. While geranyl diphosphate and neryl diphosphate serve as precursors for monoterpene formation, *trans-* and *cis-*farnesyl diphosphate are utilized for sesquiterpene formation. The diversity of terpenes found in plants is then generated by terpene synthases which utilize one or several prenyl diphosphate substrates, and often have the ability to produce multiple terpene products from one prenyl diphosphate substrate (Degenhardt et al. 2009). To facilitate their role in the antagonistic interaction with herbivores (Gershenzon and Dudareva 2007) plant terpenes are often produced in specific plant tissues like glandular trichomes or internal ducts located in different tissues including parenchyma and vascular tissues. In wild plants these defensive volatile terpene traits are constantly under positive selection pressure to increase survival and reproduction. In contrast, it appears that they have been compromised in crop plants since selective breeding has favored other agronomic traits (e.g. Köllner et al. 2008).

Glandular trichomes are present on the vegetative tissues of cultivated and wild tomato species. Type VI glandular trichomes have four glandular cells on top of one intermediate cell and one stalk cell, and are highly abundant on these tomato plants and produce volatile terpenes (Bergau et al. 2015; Besser et al. 2009; Schilmiller et al. 2009). In cultivated tomato *Solanum lycopersicum* L. (Solanaceae) these primarily produce a blend of monoterpenes and only small

amounts of a few sesquiterpenes (Schilmiller et al. 2009). In addition, trace amounts of acyl sugars and methyl ketones are found in cultivated tomato (Kennedy 2003), suggesting that production of these compounds was lost or downregulated during domestication and breeding. In contrast, glandular trichomes of wild tomato species are characterized by the abundant production of a number of secondary metabolites involved in plant defense, including terpenes (Simmons and Gurr 2005). A systematic screen of volatile terpenes produced in glandular trichomes of different accessions of *Solanum habrochaites* S. Knapp & D.M. Spooner (Solanaceae), previously *Lycopersicon hirsutum* Dunal, revealed a number of subgroups characterized by the dominant production of distinct blends of sesquiterpenes (Gonzales-Vigil et al. 2012), of which most are not found in *S. lycopersicum*.

Multiple studies demonstrated that glandular trichome derived volatile terpenes in *S. lycopersicum* and *S. habrochaites* have repellent and toxic activity against biting-chewing herbivores. The terpenes present in *S. lycopersicum* were shown to affect Colorado potato beetle (*Leptinotarsa decemlineata*), tobacco hornworm (*Manduca sexta*) and tomato fruitworm (*Helicoverpa zea*) (Gutensohn et al. 2014; Kang et al. 2010a; b; Tian et al. 2012). Likewise, *S. habrochaites* accessions producing the sesquiterpenes zingiberene or santalene/bergamotene were found to affect the performance of *L. decemlineata* (Carter et al. 1989a, b), or *H. zea* and beet armyworm (*Spodoptera exigua*) (Frelichowski and Juvik 2001), respectively. In contrast, considerably less is known about how piercing-sucking herbivores such as whiteflies, spider mites and aphids are affected by glandular trichome derived terpenes in cultivated and wild tomato species. Only the sesquiterpenes 7-epizingiberene and *R*-curcumene produced in the trichomes of some *S. habrochaites* accessions were so far shown to have repellent activity against the silverleaf whitefly (*Bemisia tabaci*) thus affecting their host plant choice (Bleeker et al. 2011a). However, piercing-sucking herbivores such as aphids pose a serious problem in the production of horticultural crops including tomato that are often grown in controlled environments like greenhouses that exclude natural enemies. While direct feeding by aphids generally does not cause severe damage, significant loss of yield and crops can be caused indirectly through the transmission of viruses for which these herbivores serve as vectors (van Emden and Harrington 2007), and the secretion of honeydew promoting the development of sooty mold on foliage and fruits. Sufficient pest control measures are required since virus transmission can occur even at low levels of aphid infestation. Current control strategies utilizing

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synthetic insecticides are increasingly ineffective due to emerging resistances and avoidance behavior (Bass et al. 2014; Fray et al. 2014; Silva et al. 2012). In this study we have screened a collection of *S. habrochaites* accessions producing distinct sets of sesquiterpenes in their glandular trichomes to identify those that affect the potato aphid (*Macrosiphum euphorbiae*). Additional assays were performed with pure terpene compounds as well as with a tomato introgression line carrying respective *S. habrochaites* terpene biosynthetic genes in the *S. lycopersicum* background. These further confirmed that two groups of sesquiterpenes, β-caryophyllene/α-humulene and α-santalene/α-bergamotene/β-bergamotene, found in large quantities in some *S. habrochaites* accessions have the potential to affect the performance, feeding and choice behavior of *M. euphorbiae*.

2.2 Experimental

2.2.1 Plant material and growth conditions

Seeds of *Solanum lycopersicum* L. (Solanaceae) (cv. M82 and cv. Moneymaker), 10 accessions of *Solanum habrochaites* S. Knapp & D.M. Spooner (Solanaceae) (LA1691, LA2650, LA1721, LA1927, LA1978, LA2155, LA1775, LA1779, LA1624, LA2860), as well as the introgression lines (LA3934, LA3935, LA3936, LA3937) and the respective *S. lycopersicum* cv. E−6203 (LA4024) and *S. habrochaites* (LA1777) parental lines were obtained from the Tomato Genetics Resource Center, University of California Davis. All tomato plants used for leaf dip extractions, head space collections and aphid bioassays were grown from seeds in Sungro® soil mixture (Sun Gro Horticulture, Agawam, MA, USA) under a 16 h photoperiod in standard greenhouse conditions (23–25 °C, 50–60% relative humidity) without pesticide application.

2.2.2 Aphid cultivation

An aphid colony was established from apterae collected in the WVU Evansdale Greenhouse (Morgantown, WV, USA). To avoid experience on tomato plants prior to any of the assays, aphids were allowed to reproduce parthenogenetically on potted potato plants in an insect rearing room under a 16 h photoperiod at 20–22 °C. The established colony was identified as potato aphid (*Macrosiphum euphorbiae*) through barcode sequencing. An alatae population was developed from confining apterae on potato leaves by applying vaseline to the leaf petioles.

2.2.3 Extraction, collection, and analysis of glandular trichome derived terpenes

To extract terpenes from *S. lycopersicum* and *S. habrochaites* leaf glandular trichomes, fresh leaves of 6- to 8-week-old plants were dipped and gently shaken for 30 s in 30 mL of methyl *tert*-butyl ether (MTBE). The extracts were concentrated under a gentle stream of nitrogen gas and centrifuged. For the analysis of terpene profiles extracts were transferred into GC vials and supplemented with 3.33 μg of naphthalene as an internal standard. Terpenes emitted from tomato leaves were collected using a closed-loop stripping method (Dudareva et al. 2005). Headspace collections from detached tomato leaves supplemented with 20% (w/v) sucrose were performed for 24 h using Porapak-Q traps (Volatile Collection Trap (VCT) LLC, Gainesville, FL, USA). After the collection traps were immediately eluted with dichloromethane and 3.33 μg of naphthalene added to the eluate as internal standard.

Samples from leaf extractions and headspace collections were analyzed by combined GC-MS using a TRACE 1310 gas chromatograph system linked to a TSQ 8000 Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). 2 μL of each sample were injected, volatilized at 220 °C and then separated on a TraceGOLD TG-5MS GC column (30 m length, 0.25 mm I.D., 0.25 μm film; Thermo Fisher Scientific, Pittsburgh, PA, USA). The initial column temperature was held at 40 °C for 3 min and then ramped at 5 °C/min to 120 °C, 10 °C/min to 180 °C and 20 °C/min to 300 °C which was maintained for 2 min. The helium carrier gas flow was 1.3 mL/min. All samples from leaf dip extractions and headspace collections were separated and analyzed by combined GC-MS using the total ion chromatogram (TIC) mode. Individual terpene compounds were identified by comparing their mass spectra with those deposited in the NIST database. Representative monoterpene (α-pinene, δ-2-carene) and sesquiterpene (β-caryophyllene, α-humulene) standards were used to determine average response factors for both compound classes (monoterpenes RF = 0.551, sesquiterpenes RF = 0.903), which were used in combination with the internal standard for the quantification of the analyzed compounds.

2.2.4 Aphid non-choice assays on tomato plants

The performance of *M. euphorbiae* alatae on the different *S. lycopersicum* and *S. habrochaites* accessions was determined under greenhouse conditions as described previously (Eichele-Nelson et al. 2018). At the beginning of the assay two apterae (F_0) reared on potato

plants were placed on the surface of a young leaf (2nd or 3rd fully expanded leaf) of a 6-week-old tomato plant and subsequently enclosed in a clip cage (BioQuip Products, Rancho Dominguez, CA, USA) that was attached to the leaf. Three tomato plants each with two clip cages attached (in total 6 clip cages) were used for individual experiments. After 4 days all aphids except three F_1 neonate nymphs (considered as 1 day old) were removed from each cage. In cases without initial aphid reproduction and thus absence of F_1 nymphs, F_1 nymphs which had emerged on *S. lycopersicum* were introduced into respective clip cages. Over the course of the experiment F_2 nymphs and exuviae were removed daily from the clip cages. The longevity of F_1 nymphs and their fecundity represented by the number of F_2 nymphs in each cage were recorded.

2.2.5 Aphid feeding assays

To determine the effect of tomato glandular trichome-derived terpenes on the feeding behavior of *M. euphorbiae* apterae, feeding assays on an artificial diet were performed as described previously (Mittler and Dadd 1963). 100 μL aliquots of an artificial diet were prepared by mixing 98 μL of a basal diet containing 20% (w/v) sucrose, 0.2% (w/v) neutral red, and 2 μL of tomato leaf dip extracts or pure terpenes (Sigma-Aldrich, MO, USA; (−)-trans-caryophyllene # 22075; α-humulene # 53675) dissolved in MTBE. Leaf dip extracts were prepared as described above by dipping a defined amount of tissue (4.0 ± 0.1 g) into 30 mL of MTBE and then concentrating the extract to 500 μL under a gentle stream of nitrogen gas. Upon addition of extracts the solvent MTBE was allowed to evaporate from the diet for 5 min before each assay. Clear polystyrene plastic containers (31.75 mm diameter, 19.05 mm height; PSC Products, Beverly Hills, CA, USA) were used as feeding chambers. The inner side of each chamber cap was covered by an unstretched piece of parafilm membrane and another layer of fully stretched parafilm with 100 μL of the diet spread in between. Four aphid apterae (7–8 days old) were starved for 12 h and then introduced into a feeding chamber. This assay was replicated 10 times with a total of 40 apterae being used. The survival of the apterae was examined at regular time intervals over the course of the experiment. Dead apterae and newly emerged offspring were removed immediately. In addition, immature aphids that had been accidentally included and aphids that had been physically damaged during handling were removed and recorded. After 48 h, all feeding chambers with 4 living apterae were assessed under a stereo microscope (SZ-

ST, Olympus, Tokyo, Japan) to quantify the numbers of gel saliva and honeydew droplets. Three 0.25-cm^2 areas were randomly selected on the parafilm for each feeding chamber and the number of stained gel saliva was determined. In addition, the number of honeydew droplets produced by aphids in each feeding chamber was counted.

2.2.6 Aphid olfactometer choice assays

An open Y-track system (Visser and Piron 1998) was utilized to study the choice behavior of *M. euphorbiae* alatae. The vertical Y-track was constructed from an iron rod (diameter 2.5 mm, length 130 mm) and a Y-junction that consists of a 50-mm vertical part and two 80-mm arms separated by 120°. Each arm of the Y-junction was inserted by 6 cm into a separate glass tube from which a constant air flow (0.8 L/min) carrying odors from a source located in a separate plexiglass container was driven by a 2-channel air delivery system (Analytical Research System Inc., Gainesville, FL, USA). The air flow was moistened and purified before reaching the enclosed odor source. The Y-track olfactometer was illuminated from the top by one halogen lamp.

Macrosiphum euphorbiae alatae were reared on potato plants, then collected from these potato plants and starved for 12 h prior to each experiment. For each assay an aphid was released at the bottom of the rod, and a choice was considered complete when the aphid touched either end of the Y-junction. Each aphid was observed for no more than 3 min. At least 50 alatae were tested for each pair of odor sources and the position of glass tubes relative to the Y-junction was switched after each 10 alatae tested. All glassware and tubing were rinsed with 70% ethanol and dried after testing each pair of odor sources. The preferences of alatae for specific *S. lycopersicum* and *S. habrochaites* accessions were tested by introducing 5.0 ± 0.2 g of fresh tomato leaves into either one or both of the plexiglass containers of the 2-channel air delivery system. Leaf petioles were immersed into 20% (w/v) sucrose solution before introduction. Leaf dip extracts or pure terpene compounds dissolved in MTBE were applied on round Whatman filter paper (8 cm diameter). The solvent was allowed to evaporate 2 min before the filter papers were transferred into the plexiglass containers of the 2-channel air delivery system.

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2.2.7 Statistical analysis

All statistical analyses were conducted with R (version 3.4.3, R Development Core Team, www.R-project.org) and a 0.05-significant level was used for inference. The normalized amount of each terpene identified from leaf dip extracts and headspace collections was summarized as mean ± SE. The effects of plants on the logarithmic performance parameters longevity and fecundity of aphid apterae in clip cages were analyzed in a linear model with plant accession as the main effect by using Tukey's HSD for post hoc tests. In feeding experiments, the effects of extracts or pure terpene compounds on the number of gel saliva and honeydew droplets were analyzed by using general linear models and the dependent variables were log-transformed to meet normality assumption. Dunnett's tests and Tukey's HSD were used for comparing the effects of respective extracts and pure terpenes. The survivorship of aphids on different diets were compared in the "survival" package with nonparametric Kaplan–Meier estimation (Kaplan and Meier 1958). In choice assays, the observed frequencies of choices aphid alatae made for odor sources were analyzed with Chi-Square test.

2.3 Results

2.3.1 *Solanum habrochaites* chemotypes produce distinct sets of glandular trichome derived sesquiterpenes

To identify glandular trichome derived terpene traits in tomato that have the potential to improve the plant defense against piercing-sucking pests such as the potato aphid (*M. euphorbiae*) we have assembled and screened a collection of two *S. lycopersicum* cultivars and ten *S. habrochaites* accessions that based on previous analyses (Gonzales-Vigil et al. 2012) represent five distinct chemotypes with specific terpene profiles. To test if the results of this previous characterization of glandular trichome derived terpenes could be verified for tomato plants grown under our conditions, we performed leaf dip extractions for these tomato accessions and analyzed the resulting extracts by combined gas chromatography-mass spectrometry (GC-MS). Indeed, our analysis confirmed that the blend of terpenes produced in glandular trichomes differed significantly between *S. lycopersicum* and *S. habrochaites*, as well as among different *S. habrochaites* accessions (Table S2.1). The two *S. lycopersicum* cultivars (c.v. M82 and c.v. Moneymaker) produced a mixture of monoterpenes with β-phellandrene being the most

prominent compound besides smaller amounts of α-pinene, δ-2-carene, and α-phellandrene. In addition, both cultivars produced minor amounts of the sesquiterpenes β-caryophyllene and α-humulene, while δ-elemene was only found in extracts of c.v. M82. In contrast to the *S. lycopersicum* cultivars the *S. habrochaites* accessions lack monoterpenes and can be sorted into five different chemotypes based on the profile of sesquiterpenes produced (Table S2.1). Chemotype 1 includes the *S. habrochaites* accessions LA1691 and LA2650, and primarily produces 7-epizingiberene and smaller quantities of *R*-curcumene. Leaf dip extracts from chemotype 2, represented by the accessions LA 1721 and LA1927, contained large amounts of γ-elemene in addition to smaller amounts of δ-elemene. A similar qualitative composition was found for extracts from chemotype 3, including accessions LA1978 and LA2155; however, these contained large amounts of δ-elemene and only small quantities of γ-elemene. Extracts from chemotype 4, represented by accessions LA1775 and LA1779, also contained large amounts of γ-elemene and small amounts of δ-elemene like those from chemotype 2. However, accessions of chemotype 4 are characterized by the production of substantial amounts of α-santalene, α-bergamotene and β-bergamotene as well as a few minor compounds including β-elemene and (*Z*)-α-farnesene. Extracts from chemotype 5, comprised of accessions LA1624 and LA2860, contained the two sesquiterpenes β-caryophyllene and α-humulene that were also found in *S. lycopersicum*, however, at up to 26- and 51-fold higher quantities, respectively. While δ-elemene, γ-elemene, and β-elemene were found in the extracts of many accessions, including those of chemotypes 2, 3, 4, and c.v. M82, these are likely the result of Cope rearrangements occurring upon sample injection into the hot (220 °C) GC injector port (Colby et al. 1998), thus suggesting that glandular trichomes of these accessions produce germacrene C, B, and A, respectively.

The analysis of tomato leaf dip extracts revealed the accumulation of terpenes in glandular trichomes, which can affect aphids once they are on the leaf surface. However, the behavior of aphids could already be modified prior to landing on the host plant through terpenes emitted from leaves into the atmosphere. Thus, we also performed headspace collections from leaves followed by GC-MS analysis to characterize the profile of emitted terpenes for all the tomato accessions qualitatively and quantitatively. In summary the results of this analysis of emitted terpenes (Table S2.2) are in line with the previous analysis of leaf dip extracts and further

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confirm the observed differences in the terpene profiles between *S. lycopersicum* and the five *S. habrochaites* chemotypes.

2.3.2 Identification of *S. habrochaites* chemotypes affecting longevity and fecundity of aphids

As a first step to characterize the potential of the different *S. habrochaites* chemotypes to affect *M. euphorbiae*, we performed non-choice assays utilizing all *S. habrochaites* accessions and the two *S. lycopersicum* cultivars for comparison. Newly emerged *M. euphorbiae* nymphs were reared in clip cages on the leaf surface of the different tomato accessions, and their longevity and fecundity (represented by the number of offspring) were determined. No significant differences were observed for both performance parameters between the two *S. lycopersicum* cultivars, c.v. M82 and c.v. Moneymaker (Figure 2.1). Compared to *S. lycopersicum* c.v. M82 the longevity of *M. euphorbiae* was not significantly different on leaves of the *S. habrochaites* chemotypes 1 and 3, and accession LA1721 of chemotype 2 (Figure 2.1A) characterized by the dominant production of 7-epi-zingiberene, germacrene C (δ-elemene), and germacrene B (γ-elemene), respectively (Table S2.1, S2.2). *Macrosiphum euphorbiae* kept on accession LA1927 of chemotype 2 showed only a small reduction in their longevity (Figure 2.1A). In contrast, the longevity of *M. euphorbiae* on accessions of chemotype 4 (LA1775, LA1779) and 5 (LA1624, LA2860), characterized by production of α-santalene/α-bergamotene/β-bergamotene and β-caryophyllene/α-humulene, respectively, was reduced quite significantly (24.6%–35.3% of the aphid longevity on c.v. M82) (Figure 2.1A). Similar trends as observed for the longevity of *M. euphorbiae* were also found for their fecundity on the different *S. habrochaites* accessions. The numbers of *M. euphorbiae* offspring were not significantly different on accession LA1691 (chemotype 1), LA1721 (chemotype 2), and both accessions of chemotype 3 compared to *S. lycopersicum* c.v. M82 (Figure 2.1B). Accessions LA2650 (chemotype 1) and LA1927 (chemotype 2) had a 53.6% and 45.9% reduction in fecundity, respectively. However, the most severe effect on fecundity was again observed for *M. euphorbiae* on accessions of the chemotypes 4 and 5 which almost completely lacked the emergence of nymphs (Figure 2.1B). Thus, the results of the non-choice assays suggested that the sesquiterpenes produced in *S. habrochaites* accessions of the chemotypes 4 and 5 might have the potential to significantly affect the performance of *M. euphorbiae*.

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2.3.3 Sesquiterpenes of two *S. habrochaites* chemotypes affect aphid feeding behavior

To characterize the potential effect of glandular trichome derived terpenes from different *S. habrochaites* chemotypes on *M. euphorbiae* in more detail we performed feeding assays. *M. euphorbiae* apterae were allowed to feed on an artificial diet to which aliquots of terpene containing leaf dip extracts from individual *S. habrochaites* accessions or *S. lycopersicum* c.v. M82 had been added. The survivorship of *M. euphorbiae* on these diets was determined, as well as the investment of gel saliva and the production of honeydew which serve as indicators of the aphid feeding behavior. A set of control experiments demonstrated that addition of methyl *tert*-butyl ether (MTBE), the solvent used for leaf dip extracts, as well as extracts from *S. lycopersicum* c.v. M82 to the feeding diet affected neither the survivorship of aphids (Figure 2.2A) nor the accumulation of gel saliva (Figure 2.2B) and honeydew drops (Figure 2.2C) compared to pure feeding diet without added solvent or extracts. Addition of terpene containing leaf dip extracts from accessions of the *S. habrochaites* chemotypes 1, 2, and 3 to the diet did not appear to affect *M. euphorbiae* apterae as their survivorship (Figure S2.1A) and feeding behavior measured by gel saliva investment and honeydew production (Figure S2.1B and C, respectively) were not significantly different compared to the performance of aphids feeding on a diet containing extracts from *S. lycopersicum* c.v. M82. In contrast, the performance of *M. euphorbiae* was severely affected when they were feeding on diets with added leaf dip extracts from the *S. habrochaites* chemotypes 4 and 5. The survivorship of aphids (Figure 2.2A) on diets with the sesquiterpene blend extracted from accessions LA1775 and LA1779, as well as with the β-caryophyllene and α-humulene containing extracts from accessions LA1624 and LA2860 was significantly reduced compared to that of aphids feeding on diets with *S. lycopersicum* c.v. M82 derived extracts. Likewise, the investment of gel saliva and the production of honeydew by *M. euphorbiae* was significantly lower on diets with added extracts from *S. habrochaites* chemotypes 4 and 5 compared to the *S. lycopersicum* c.v. M82 control (Figure 2.2B and C). The results of these feeding assays further confirmed the outcome of the previous non-choice assays and suggest that the sesquiterpenes produced in glandular trichomes of *S. habrochaites* chemotype 4 and 5 exhibit both antibiosis and antixenosis thus considerably affecting the performance of *M. euphorbiae* and altering their feeding behavior.

2.3.4 Some sesquiterpenes emitted by *S. habrochaites* have repellent activity against aphids

In addition to the observed effects on performance and feeding of *M. euphorbiae*, sesquiterpenes produced in glandular trichomes of different *S. habrochaites* accessions upon emission into the surrounding atmosphere could also influence the pre-landing orientation and host plant choice of *M. euphorbiae*. In order to test the behavioral response of *M. euphorbiae* alatae towards emitted tomato terpenes we performed choice assays using an open Y-track olfactometer. When the aphids were given the choice between pure air and the odors of tomato leaves (Figure 2.3A) a significant effect on their behavior was observed only with the *S. habrochaites* accesions of chemotype 5, but not with any of the other chemotypes. Only 28.3% ($\chi^2 = 8.70$, $P = 0.003$) and 32.6% ($\chi^2 = 5.23$, $P = 0.022$) of *M. euphorbiae* alatae oriented towards the odors of accession LA1624 and LA2860, respectively, suggesting that the emitted β-caryophyllene and α-humulene (Table S2) have repellent activity. Remarkably, in the same assays (Figure 2.3A) we observed that 71.8% ($\chi^2 = 7.41$, $P = 0.007$) and 68.2% ($\chi^2 = 5.82$, $P = 0.016$) of *M. euphorbiae* alatae orientated towards the odors from leaves of *S. lycopersicum* cultivars M82 and Moneymaker, respectively, suggesting that the terpenes emitted from cultivated tomato attracted *M. euphorbiae*.

Due to the observed attraction towards *S. lycopersicum*, in a second set of assays aphid alatae were given the choice between the odor of *S. lycopersicum* c.v. M82 and different *S. habrochaites* accessions (Figure 2.3B). In all but one of the pairs >50% of the *M. euphorbiae* alatae were found to orient towards c.v. M82 thus further confirming their attraction by *S. lycopersicum* odors. When LA1779, LA1624, and LA2860 were paired with c.v. M82 only 32.4% ($\chi^2 = 4.24$, $P = 0.040$), 30.2% ($\chi^2 = 6.72$, $P = 0.010$), and 30.0% ($\chi^2 = 6.40$, $P = 0.011$) of the *M. euphorbiae* alatae, respectively, oriented towards the *S. habrochaites* odors, indicating a significant repellent activity of the sesquiterpenes emitted by the accessions of the chemotypes 4 and 5 (Table S2.2).

An additional set of assays was performed to test if these sesquiterpenes produced by *S. habrochaites* could be transferred to cultivated tomato and would show a similar repellent activity towards *M. euphorbiae* in the presence of the monoterpene blend emitted by *S. lycopersicum*. *Macrosiphum euphorbiae* alatae were given the choice between the odors of *S.*

lycopersicum c.v. M82 leaves alone, and c.v. M82 leaves to which aliquots of *S. habrochaites* leaf dip extracts on filter papers had been added (Figure 2.3C). The addition of the *S. habrochaites* leaf dip extracts in general affected the orientation of *M. euphorbiae* alatae towards c.v. M82 leaves. However, only the addition of the β-caryophyllene and α-humulene containing extracts from accessions LA1624 and LA2860 significantly reduced the attraction of *M. euphorbiae* with only 33.3% (χ^2 = 4.67, *P* = 0.031) and 34.8% (χ^2 = 4.26, *P* = 0.039), respectively, of the aphids orienting towards the mixed odors of c.v. M82 leaves and added leaf dip extracts. A similar trend was observed upon the addition of extracts from accession LA1779 (chemotype 4) to c.v. M82 leaves which resulted in only 35.0% (χ^2 = 3.60, *P* = 0.058) of *M. euphorbiae* orienting towards the mixed odors. In summary, the results of the olfactometer choice assays suggested that the sesquiterpenes produced in glandular trichomes of *S. habrochaites* chemotypes 4 and 5 not only affect performance and feeding of *M. euphorbiae* but clearly also their choice behavior prior to landing on potential host plants.

2.3.5 β-caryophyllene and α-humulene affect performance, feeding and behavior of aphids

The glandular trichomes of wild tomato species such as *S. habrochaites* are known to produce not only terpenes but also a number of other secondary metabolites including acyl sugars (Kim et al. 2012; Ghosh et al. 2014), methyl ketones (Ben-Israel et al. 2009; Yu et al. 2010), and flavonoids (Schmidt et al. 2011, 2012). Therefore, to verify that the effects on performance, feeding, and choice behavior of *M. euphorbiae* observed with several of the *S. habrochaites* chemotypes and respective leaf dip extracts are indeed due to terpenes produced by these and not to other metabolites that could potentially be co-extracted, we performed additional feeding and choice assays. Since accessions of *S. habrochaites* chemotype 5 predominantly produce β-caryophyllene and α-humulene (Table S2.1 and S2.2) we utilized a 3 to 1 mixture (based on their accumulation in glandular trichomes) of these two sesquiterpenes that are both available as pure chemicals. For feeding assays increasing amounts of this mixture of pure β-caryophyllene and α-humulene were added to artificial diet resulting in sesquiterpene concentrations of 1, 2, 5, and 15 ng μL^{-1}, comparable to those previously achieved by the addition of leaf dip extracts from accessions LA1624 and LA2860 (3.2 and 5.0 ng μL^{-1}, respectively). When *M. euphorbiae* apterae were allowed to feed on these artificial diets their

survivorship was progressively reduced in the presence of increasing amounts of pure β-caryophyllene and α-humulene compared to the control (Figure 2.4A). Likewise, the investment of gel saliva by aphids was significantly lower on diets with the added mixture of pure β-caryophyllene and α-humulene (Figure 2.4B). We also observed a trend towards reduced honeydew production with increasing concentrations of the pure sesquiterpene mixture in the diet (Figure 2.4C). These results confirm that β-caryophyllene and α-humulene indeed affect the performance and feeding of *M. euphorbiae*.

Subsequently, to test the behavioral response of *M. euphorbiae* alatae towards pure sesquiterpenes we performed olfactometer choice assays using the same β-caryophyllene and α-humulene mixture. *Macrosiphum euphorbiae* alatae were given the choice between the odors of *S. lycopersicum* c.v. M82 leaves alone, and c.v. M82 leaves to which different amounts of the mixture of pure β-caryophyllene and α-humulene had been added on filter papers. The addition of pure β-caryophyllene and α-humulene reduced the attraction of *M. euphorbiae* alatae to c.v.M82 in a dose-dependent manner (Figure 2.4D). Significantly less aphids, 34.1% and 19.4% respectively, oriented towards c.v. M82 when 10 μg ($\chi^2 = 4.12$, $P = 0.042$) and 100 μg ($\chi^2 = 9.00$, $P = 0.003$) of the β-caryophyllene and α-humulene mixture were added. Thus, these assays confirmed that β-caryophyllene and α-humulene indeed have the potential to affect the choice behavior and host plant preference of *M. euphorbiae*.

2.3.6 A santalene-/bergamotene-producing tomato introgression line affects performance, feeding and behavior of aphids

While *S. habrochaites* accessions of the chemotypes 2 and 4 both form significant amounts of germacrene B (γ-elemene) and germacrene C (δ-elemene), production of α-santalene, α-bergamotene and β-bergamotene was exclusively found for accessions of chemotype 4 (Table S2.1 and S2.2). However, significant effects on the performance, feeding and choice behavior of *M. euphorbiae* were only observed with accessions of chemotype 4 (Figure 2.1, 2.2, 2.3), suggesting that these effects are due to α-santalene, α-bergamotene and β-bergamotene. Unfortunately, these sesquiterpenes are not available as pure chemicals that could be used for the further verification of the obtained results. Instead we used a genetic approach to further confirm the potential of these sesquiterpenes to affect the performance, feeding, and choice behavior of *M. euphorbiae*. Previously a collection of introgression lines had been derived from a cross

between *S. lycopersicum* LA4024 and the santalene-/bergamotene-producing *S. habrochaites* accession LA1777 (Monforte and Tanksley 2000). One of these lines, introgression line LA3935 (previously described as Near Isogenic Line TA517), contains a larger *S. habrochaites* introgression on chromosome 4 and a smaller introgression on chromosome 8, and was found to produce α-santalene, α-bergamotene and β-bergamotene (van der Hoeven et al. 2000). Subsequent analysis verified that the introgression on chromosome 8 of line LA3935 carries the genes encoding the prenyl transferase (*zFPS*) and terpene synthase (*ShSBS*) involved in the formation of α-santalene, α-bergamotene and β-bergamotene (Sallaud et al. 2009). Thus, to further confirm the activity of these sesquiterpenes towards *M. euphorbiae*, we utilized the introgression line LA3935, the parents *S. lycopersicum* LA4024 and *S. habrochaites* LA1777, as well as three additional introgression lines from this collection, LA3934, LA3936, and LA3937, that contain *S. habrochaites* introgressions on chromosome 4 overlapping with the respective introgression in line LA3935.

To characterize the production and emission of glandular trichome derived terpenes for these tomato lines we performed leaf dip extractions as well as headspace collections from leaves, respectively, and analyzed the resulting samples by GC-MS. The *S. lycopersicum* accession LA4024 produced and emitted the expected mixture of monoterpenes including α-pinene, δ-2-carene, α-phellandrene, α-terpinene and β-phellandrene, as well as the sesquiterpenes β-caryophyllene, α-humulene, and δ-elemene (Table S2.3 and S2.4). Similar profiles were observed for the introgression lines LA3934, LA3936, and LA3937 suggesting that their *S. habrochaites* introgressions on chromosome 4 had no effect on the terpene production compared to the parent LA4024. The leaf dip extracts and headspace collections of *S. habrochaites* LA1777, like those of the chemotype 4 accessions LA1775 and LA1779, are characterized by γ-elemene, δ-elemene and β-elemene (indicative of the *in planta* formation of germacrene B, C and A, respectively) as well as α-santalene, α-bergamotene and β-bergamotene (Table S2.3 and S2.4). The introgression line LA3935 was found to produce and emit α-santalene, α-bergamotene and β-bergamotene (Table S3 and S4) as had been shown previously (van der Hoeven et al. 2000; Sallaud et al. 2009).

When these parental and introgression lines were used to perform non-choice assays with *M. euphorbiae* no significant differences were observed for the longevity and fecundity of aphid

apterae arrested on the leaf surface of *S. lycopersicum* LA4024 and the introgression lines LA3934, LA3936, and LA3937 (Figure 2.5A). In contrast, longevity and fecundity of *M. euphorbiae* on *S. habrochaites* accession LA1777 were significantly reduced (22.0% of the longevity and 2.0% of the fecundity of aphids on LA4024) (Figure 2.5A) similar to the results obtained with the accessions LA1775 and LA1779 (Figure 2.1). Both parameters were also significantly affected for *M. euphorbiae* kept on leaves of the introgression line LA3935 (Figure 2.5A) with a 18.9% reduction in longevity and a 32.4% reduction in the number of *M. euphorbiae* offspring compared to the LA4024 background, thus suggesting that the observed effects on the *M. euphorbiae* performance are indeed due to the produced α-santalene, α-bergamotene and β-bergamotene.

Leaf dip extracts from the parental and introgression lines were used to perform feeding assays to further verify if α-santalene, α-bergamotene and β-bergamotene also affect the feeding behavior of *M. euphorbiae*. Addition of leaf dip extracts from the introgression lines LA3934, LA3936, and LA3937 to the feeding diet did not affect *M. euphorbiae* apterae as their survivorship, gel saliva investment and honeydew production (Figure S2.2) were not significantly different compared to the performance of aphids feeding on a diet containing extracts from *S. lycopersicum* LA4024. In contrast, the performance of aphids was affected when leaf dip extracts from the *S. habrochaites* accession LA1777 as well as the introgression line LA3935 were added to feeding diets. The survivorship, gel saliva investment, and honeydew production of aphids (Figure 2.5B, C, and D, respectively) on diets with extracts from LA1777 and LA3935 were significantly reduced compared to aphids feeding on diets containing *S. lycopersicum* LA4024 extracts, thus further confirming that α-santalene, α-bergamotene and β-bergamotene have an effect on *M. euphorbiae* feeding.

To further confirm the potential of α-santalene, α-bergamotene and β-bergamotene to affect the choice behavior of *M. euphorbiae* we performed olfactometer assays using the *S. lycopersicum* LA4024 and *S. habrochaites* LA1777 parental lines, as well as the different introgression lines. When given the choice between pure air and the odors of tomato leaves (Figure S2.3A) between 61.9% and 67.4% of *M. euphorbiae* alatae orientated towards the odors of the parental line LA4024 and the introgression lines LA3934, LA3936 and LA3937, thus further confirming the previously observed (Figure 2.3A) attraction of *M. euphorbiae* towards

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the *S. lycopersicum* background. In contrast, only 34.3% and 41.0% of the aphids orientated towards the odors of the parental line LA1777 and the introgression line LA3935, respectively, suggesting that the emitted α-santalene, α-bergamotene and β-bergamotene have repellent activity. This was also supported by the observation that only 32.5% and 33.3% of *M. euphorbiae* alatae oriented towards the parental line LA1777 and the introgression line LA3935, respectively, when these santalene/bergamotene producing lines were paired with *S. lycopersicum* LA4024 (Figure S2.3B), while no aphid preference was detected in pairs between LA4024 and the introgression lines LA3934, LA3936 and LA3937. To further test the repellent activity of α-santalene, α-bergamotene and β-bergamotene *M. euphorbiae* alatae were given the choice between the odors of *S. lycopersicum* LA4024 leaves alone, and LA4024 leaves to which different amounts (100 and 300 μL) of leaf dip extracts from the parental *S. habrochaites* accession LA1777 and the introgression line LA3935 had been added on filter papers. In both cases the addition of leaf dip extracts reduced the attraction of aphids to LA4024 in a dose-dependent manner (Figure 2.5E). Significantly less aphids, 30.6% and 26.3% respectively, oriented towards LA4024 when 100 μL ($\chi^2 = 5.44$, $P = 0.020$) and 300 μL ($\chi^2 = 8.53$, $P = 0.004$) of the LA1777 leaf dip extract were added. Likewise, a significant lower number of aphids (33.3%, $\chi^2 = 4.00$, $P = 0.046$) oriented towards LA4024 upon addition of 300 μL of the LA3935 leaf dip extract, while the addition of 100 μL of LA3935 extract had a weaker effect with only 40.0% of aphids orienting towards the mixed odors. In summary, these results suggest that α-santalene, α-bergamotene and β-bergamotene have repellent activity and thus affect the choice behavior and host plant preference of *M. euphorbiae*.

2.4 Discussion

It is well documented that wild tomato species such as *S. habrochaites* and *S. pennellii* Correll possess trichome-based resistance against various insect pests (Simmons and Gurr 2005). While glandular trichomes of these wild tomato species are known to produce a number of different secondary metabolites involved in the defense against pests, it has been shown that in particular some of their terpenes act repellent and/or toxic against pests (Bleeker et al. 2009; 2011a; Carter et al. 1989a, b; Frelichowski and Juvik 2001). Analysis of terpene production in glandular trichomes of numerous *S. habrochaites* accessions revealed the presence of different chemotypes that are characterized by the dominant accumulation of distinct

sesquiterpenes (Gonzales-Vigil et al. 2012). Thus, we hypothesized that wild *S. habrochaites* accessions could also be a good source for the identification of defensive volatile terpene traits with activity against *M. euphorbiae*.

Among the different *S. habrochaites* chemotypes tested in this study we found that chemotype 4 and 5, characterized by the production of α-santalene/α-bergamotene/β-bergamotene and β-caryophyllene/α-humulene, respectively, consistently and negatively affected *M. euphorbiae*. Remarkably, the identified sesquiterpenes appear to affect multiple aspects of the aphid feeding behavior. When the aphids were exposed to these two groups of sesquiterpenes, either on the leaf surface (Figure 2.1) or in feeding experiments (Figure 2.2), their life span and the number of offspring were significantly reduced. These effects might be at least in part due to a reduced feeding frequency and intensity as indicated by the lower gel saliva investment and production of honeydew (Figure 2.2B and C). In addition, the two groups of sesquiterpenes displayed repellent activity towards *M. euphorbiae* (Figure 2.3) thus interfering with their host plant choice. Further assays with pure β-caryophyllene and α-humulene (Figure 2.4), and an α-santalene/α-bergamotene/β-bergamotene producing tomato introgression line (Figure 2.5) confirmed that the observed effects were indeed due to these sesquiterpenes and not to other secondary metabolites produced in glandular trichomes of *S. habrochaites*.

The formation of β-caryophyllene, which is often produced together with α-humulene by terpene synthases like the *S. lycopersicum* and *S. habrochaites* TPS12 (Bleeker et al. 2011b; Schilmiller et al. 2010), is found in many plants upon herbivory suggesting a role in the defense against pests. In some plants β-caryophyllene contributes to direct defense by affecting the development and survival of herbivores (Langenheim 1994), like in *Hymenaea courbaril* where it significantly affects the mortality of the beet armyworm (*S. exigua*). In addition, β-caryophyllene and α-humulene formed in tree species including *Hymenaea* appear to have repellent activity against leaf-cutting ants (Hubbell et al. 1983; Messer et al. 1990). In other plants, such as corn and rice, β-caryophyllene and α-humulene are involved in indirect defense by attracting parasitic wasps to oviposit on larvae or eggs of herbivores (Cheng et al. 2007; Köllner et al. 2008) or entomopathogenic nematodes to attack coleopteran larvae feeding on roots (Rasmann et al. 2005). In contrast to β-caryophyllene and α-humulene, very little has been reported about potential effects on herbivores caused by the blend of sesquiterpenes,

including α-santalene, α- and β-bergamotene, produced by the *S. habrochaites* 'Santalene and Bergamotene Synthase' (SBS) (Sallaud et al. 2009). Frelichowski and Juvik (2001) observed that sesquiterpene containing extracts from the α-santalene/α-bergamotene/β-bergamotene producing *S. habrochaites* accession LA1777 significantly reduced the developmental rates and survival of larvae of the tomato fruitworm (*H. zea*) and beet armyworm (*S. exigua*).

While these previous studies have primarily described the activity of β-caryophyllene/α-humulene and α-santalene/α-bergamotene/β-bergamotene against biting-chewing herbivores, our studies demonstrate for the first time the adverse effects of these two groups of sesquiterpenes on the performance, feeding and behavior of *M. euphorbiae*, a piercing-sucking herbivore. In a recent metabolic engineering study (Yin and Wong 2019) overexpression of the Santalene Synthase (SaSSy) from sandalwood (*Santalum album*) in tobacco plants resulted in the production of a blend of primarily α-santalene and β-santalene, as well as smaller amounts of α-bergamotene. When these transgenic plants were used for choice assays the tobacco lines producing santalenes and bergamotene were found to be more attractive to the green peach aphid (*Myzus persicae*). In contrast, when one *Mentha × piperita* sesquiterpene synthase was expressed in transgenic *Arabidopsis thaliana* the produced (*E*)-β-farnesene, an alarm pheromone in aphids, had repellent activity against *M. persicae* (Beale et al. 2006). Likewise, endogenous (*E*)-β-farnesene produced in glandular trichomes of the wild potato *Solanum berthaultii* repelled *M. persicae* (Gibson and Pickett 1983). Another example for the activity of terpenes against a piercing-sucking insect was found in *S. pennellii* and *S. habrochaites*. The sesquiterpenes 7-epizingiberene and *R*-curcumene produced in the glandular trichomes of some accessions of these wild tomato species were observed to have repellent activity towards whiteflies (*Bemisia tabaci*) and to significantly reduce their survival (Bleeker et al. 2009; 2011a). In contrast, in our analyses 7-epizingiberene and *R*-curcumene producing *S. habrochaites* accessions (LA1691 and LA2650) appeared to have no major effect on the performance, feeding and choice behavior of *M. euphorbiae* (Figure 2.1, S2.1, and 2.3). Indeed 7-epizingiberene and santalene/bergamotene producing *S. habrochaites* accessions originate from separate geographic regions in Peru and Ecuador characterized by differences in temperature, precipitation and elevation (Gonzales-Vigil et al. 2012) which suggests that their distinct terpene profiles are adaptations to environments with specific herbivore challenges.

Remarkably, β-caryophyllene and α-humulene, which our analyses showed to be highly effective in modifying the performance, feeding and choice behavior of *M. euphorbiae*, is also produced in the glandular trichomes of cultivated tomato *S. lycopersicum*, although at significantly lower levels compared to the *S. habrochaites* accessions of chemotype 5 (Table S2.1 and S2.2). Since β-caryophyllene and α-humulene seem to be effective even at lower concentrations as indicated by our analysis with pure sesquiterpenes (Figure 2.4), a similar though weaker effect on *M. euphorbiae* would be expected from the β-caryophyllene and α-humulene produced in *S. lycopersicum*. Thus, it was surprising to find that *M. euphorbiae* were attracted by *S. lycopersicum* (Figure 2.3), which suggested that some of the other volatile compounds produced by *S. lycopersicum* leaves besides β-caryophyllene and α-humulene were responsible for the attraction of *M. euphorbiae*. The identification and characterization of these attractive volatile compounds emitted from *S. lycopersicum* will require further investigation. At the same time the observed attraction of *M. euphorbiae* highlights the need to improve the defense of cultivated tomato against these pests. A previous study (Goffreda et al. 1988) as well as our analysis (Figure 2.3C) demonstrated that already the simple transfer of glandular trichome extracts from leaves of *S. pennellii* and *S. habrochaites* accessions, respectively, to *S. lycopersicum* could reduce attraction and feeding of *M. euphorbiae*. For the stable introduction of defensive terpene traits originating from *S. habrochaites* into *S. lycopersicum* two alternative strategies can be utilized: (i) the introgression of such traits into the *S. lycopersicum* background via interspecific crosses between cultivated and respective wild tomato accessions, or (ii) the genetic engineering of respective terpene biosynthetic pathways identified in wild tomato accessions into *S. lycopersicum*. In this study we have tested the introgression line LA3935 that was previously shown to carry a *S. habrochaites* introgression including the prenyl transferase (*zFPS*) and terpene synthase (*ShSBS*) genes involved in the formation of α-santalene, α-bergamotene and β-bergamotene (Sallaud et al. 2009; van der Hoeven et al. 2000). We found that introduction of α-santalene, α-bergamotene and β-bergamotene formation in the line LA3935 indeed affected the performance, feeding and choice behavior of *M. euphorbiae* (Figure 2.5 and S2.3). However, compared to the parental *S. habrochaites* accession L1777 the santalene/bergamotene formation (Table S2.3, S2.4) and in consequence the effects on *M. euphorbiae* (Figure 2.5 and S2.3) were significantly lower in the introgression line LA3935. Likewise, the 7-epizingiberene formation in F2 lines derived from the cross between a

respective *S. habrochaites* accession and *S. lycopersicum* was found to be significantly lower than in the parental *S. habrochaites* line (Bleeker et al. 2012). These obvious differences in the production of sesquiterpenes between introgression and parental *S. habrochaites* lines are likely due to the higher expression level of MVA and MEP pathway genes in *S. habrochaites* compared to *S. lycopersicum* (Besser et al. 2009), which suggests that a larger pool of precursors for sesquiterpene formation is available in glandular trichomes of *S. habrochaites*. The formation of 7-epizingiberene in *S. lycopersicum* was also achieved by genetic engineering via overexpression of *Z,Z-farnesyl diphosphate synthase* (*zFPS*) and *7-epizingiberene synthase* (*ShZIS*) from *S. habrochaites* under the control of glandular trichome specific promoters (Bleeker et al. 2012). Although the 7-epizingiberene levels measured in these transgenic tomato lines were lower than in *S. habrochaites*, these still negatively affected the performance and behavior of whiteflies and spider mites.

2.5 Conclusions

Glandular trichomes of cultivated and wild tomato produce a number of secondary metabolites including mono- and sesquiterpenes that contribute to host plant resistance against pests. By screening a collection of *S. habrochaites* accessions that produce different sets of sesquiterpenes we have identified two chemotypes that affect *M. euphorbiae*. Additional assays performed with pure terpene compounds and a tomato introgression line confirmed that β-caryophyllene/α-humulene and α-santalene/α-bergamotene/β-bergamotene are responsible for the observed effects on performance, feeding and choice behavior of *M. euphorbiae*. In *S. lycopersicum* only small amounts of β-caryophyllene/α-humulene are produced, while α-santalene/α-bergamotene/β-bergamotene are absent. Since respective biosynthetic genes have been identified in *S. habrochaites*, production of these sesquiterpenes with activity against *M. euphorbiae* now can be introduced or enhanced in *S. lycopersicum* via targeted breeding and/or metabolic engineering approaches. Further research will also be required to unravel the mode of action of the effective sesquiterpenes identified here and to characterize their specific targets in aphids.

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2.7 Tables and figures
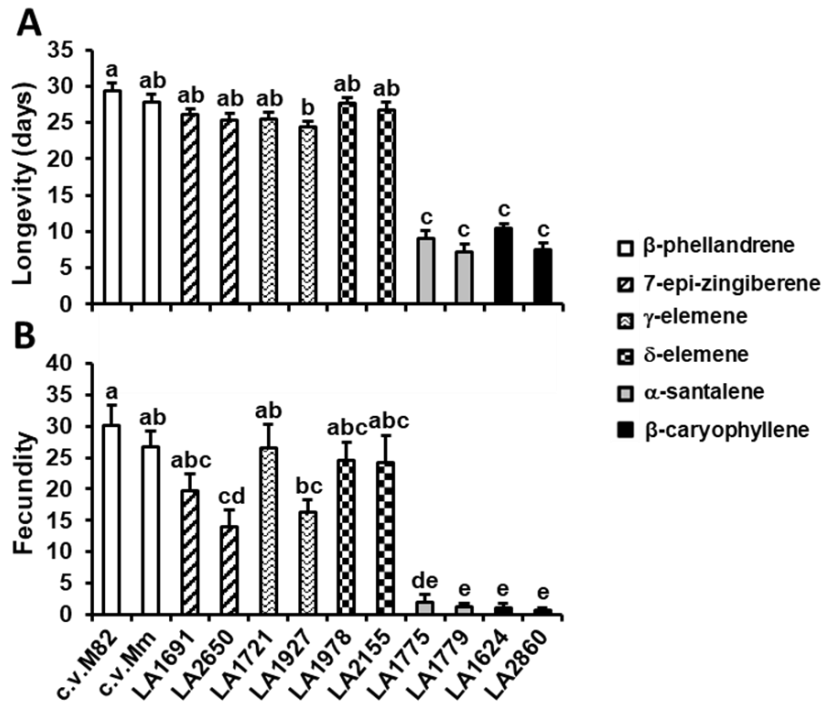
Figure 2.1 Longevity (A) and fecundity (B) of *M. euphorbiae* apterae arrested on the leaf surface of two *S. lycopersicum* cultivars and different *S. habrochaites* accessions. *S. habrochaites* accessions represent five chemotypes characterized by the production of different sesquiterpenes in their glandular trichomes. Values for longevity and fecundity are presented as mean ± SE. Different letters indicate that logarithmic values were significantly different (Tukey's HSD test, $\alpha = 0.05$).
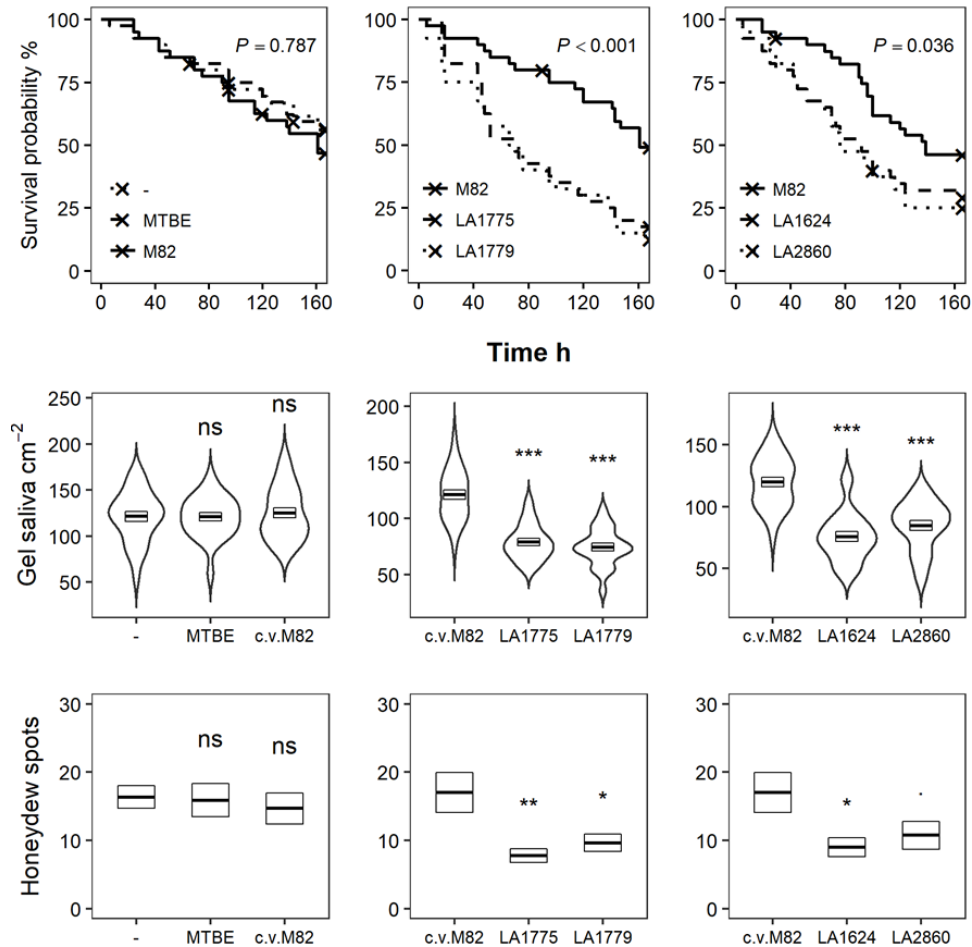
Figure 2.2 Feeding performance of *M. euphorbiae* apterae on artificial diets containing leaf dip extracts of *S. lycopersicum* c.v. M82 and different *S. habrochaites* accessions (MTBE solvent control). (**A**) Kaplan-Meier estimates of survivorship and analysis of log-rank test ($\alpha = 0.05$). (**B**) Box and violin plots represent mean $\pm$ SE of gel saliva density (cm^{-2}) and the probability density, respectively. (**C**) Number of honeydew drops accumulated in the feeding chambers. Asterisks in (B) and (C) represent significant differences between diets with leaf dip extracts and control based on Dunnett's test ($\cdot$, $P < 0.08$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
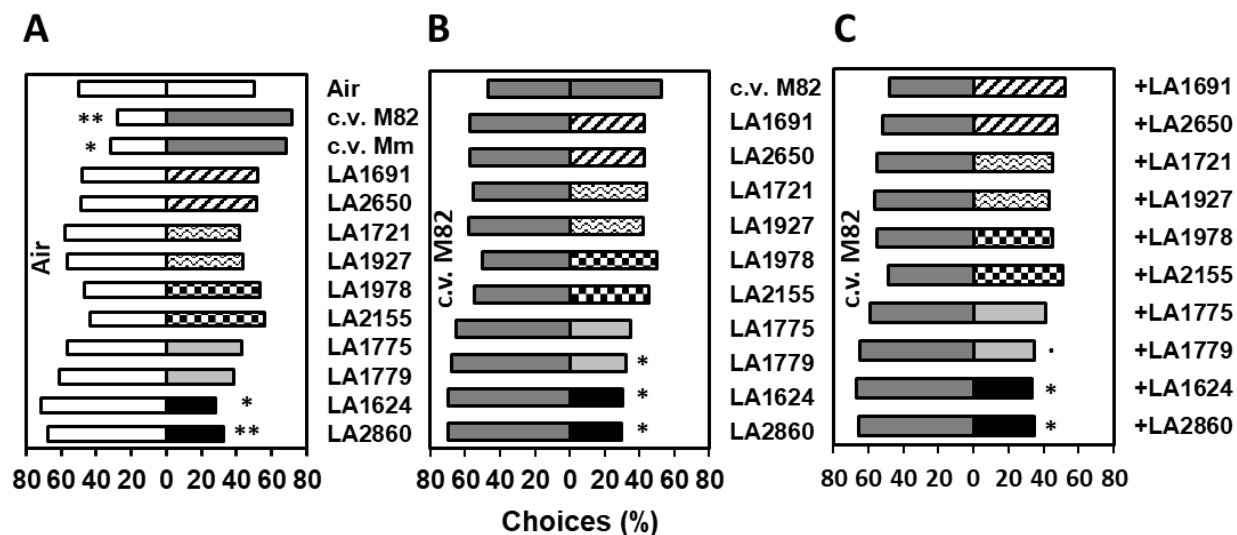
Figure 2.3 Choice behavior of *M. euphorbiae* alatae in an open Y-track olfactometer. (**A**) Choice of aphids between air and odors from leaves of different *S. lycopersicum* cultivars or *S. habrochaites* accessions. (**B**) Choice of aphids between odors from leaves of c.v. M82 and different *S. habrochaites* accessions. (**C**) Choice of aphids between odors from leaves of c.v. M82 leaves alone (left bars) and from leaves of c.v. M82 leaves with added *S. habrochaites* leaf dip extracts (right bars). Asterisks following each pair of bars indicates significant differences according to Chi-square goodness of fit ($\cdot$, $P < 0.07$; *, $P < 0.05$; **, $P < 0.01$).
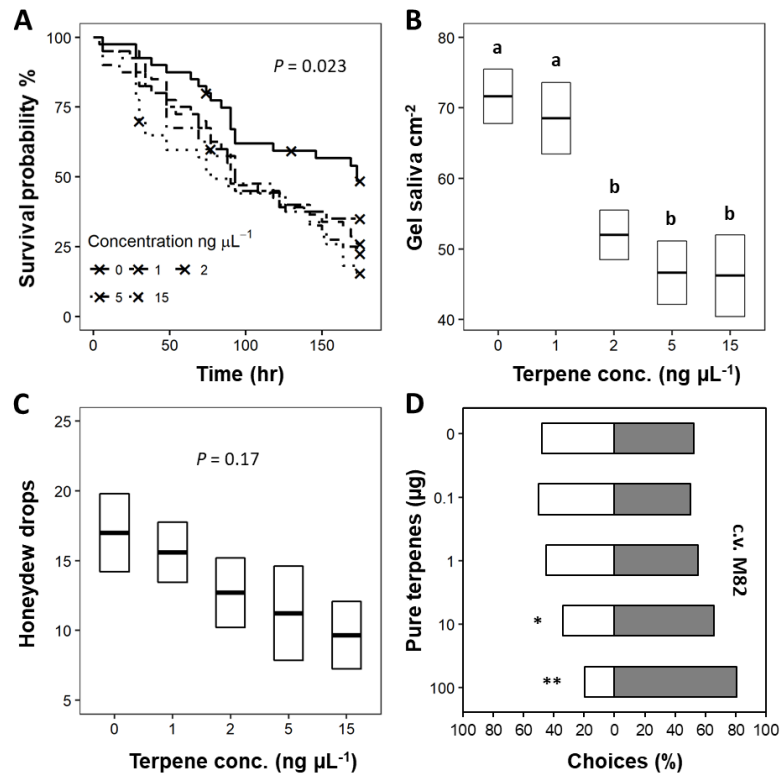
Figure 2.4 Effect of pure β-caryophyllene and α-humulene on *M. euphorbiae* feeding performance (A-C) and choice behavior (D). Different amounts of a mix of pure β-caryophyllene and α-humulene (3:1 ratio) were added to the feeding diet. The data in (**A**), (**B**), and (**C**) show the comparison of Kaplan-Meier survival curves (log-rank test, $\alpha = 0.05$), gel saliva density (Tukey's HSD, $\alpha = 0.05$), and number of honeydew drops (ANOVA, $\alpha = 0.05$), respectively, upon addition of different amounts of pure sesquiterpenes to the artificial diet. The data in (**D**) represent the behavioral responses of aphid alatae to odors from leaves of c.v. M82 alone (right bars) or from leaves of c.v. M82 leaves in combination with different amounts of a mix of pure β-caryophyllene and α-humulene (left bars) (Chi-square goodness of fit; *, $P < 0.05$; **, $P < 0.01$).
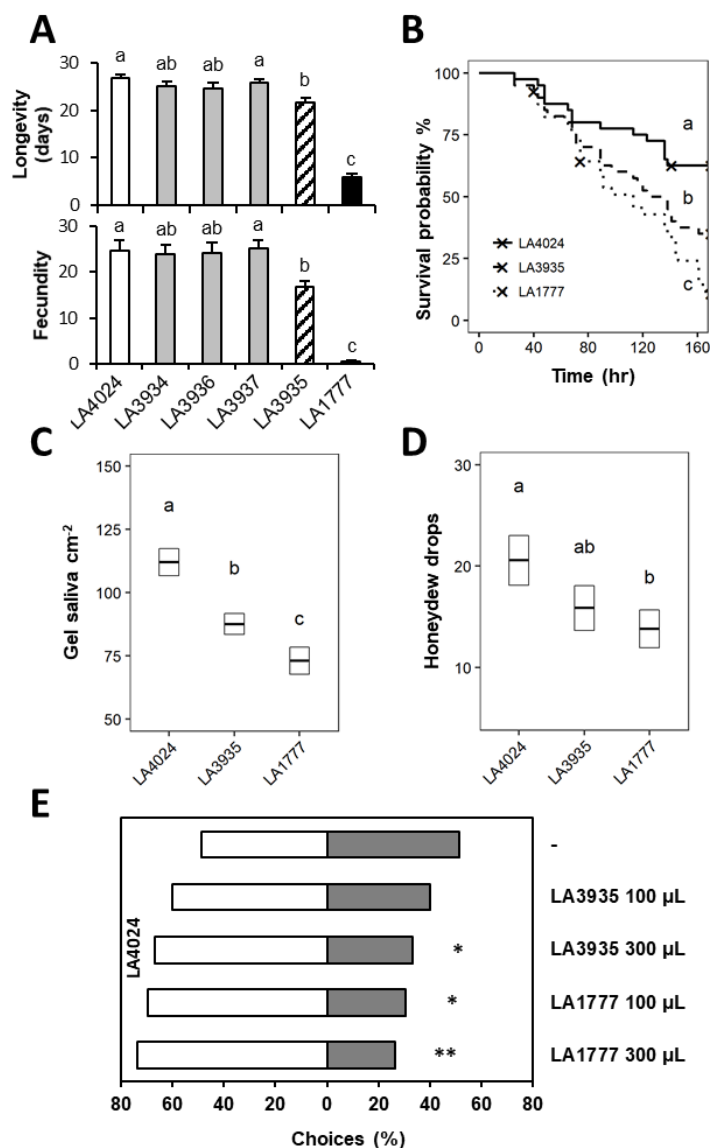
Figure 2.5 α-Santalene and α-bergamotene producing tomato introgression line affects performance, feeding and choice behavior of *M. euphorbiae*. (**A**) Performance of *M. euphorbiae* apterae arrested on the leaf surface of *S. lycopersicum* LA4024, *S. habrochaites* LA1777, and the introgression lines LA3935, LA3934, LA3936, and LA3937. Values for longevity and fecundity are presented as mean ± SE and compared by Tukey's HSD test ($\alpha = 0.05$). (**B**) Kaplan-Meier estimates of survivorship of *M. euphorbiae* apterae feeding on artificial diets containing leaf dip extracts of *S. lycopersicum* LA4024, *S. habrochaites* LA1777, and the introgression line LA3935 (log-rank test, $\alpha = 0.05$). (**C**) and (**D**) Box plots represent means ± SE of gel saliva density (cm^{-2}) and number of honeydew drops, respectively. Tukey's HSD tests ($\alpha = 0.05$) are used for post-hoc analysis. (**E**) Choice of *M. euphorbiae* alatae between odors from LA4024 leaves alone (left bars) and from LA4024 leaves with added leaf dip extracts (100 and 300 μL) from the introgression line LA3935 or *S. habrochaites* LA1777 (right bars) (Chi-square goodness of fit; *, $P < 0.05$; **, $P < 0.01$).

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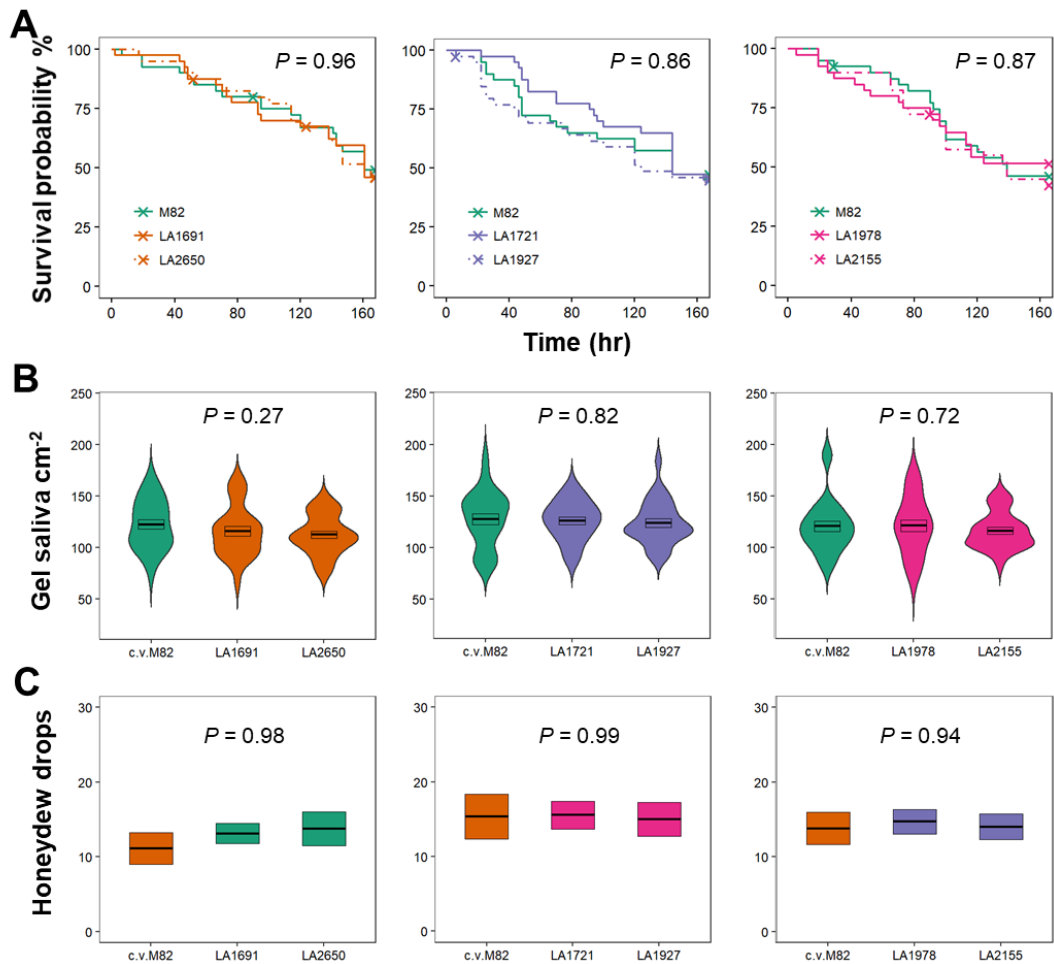
Figure S2.1 Feeding performance of *M. euphorbiae* apterae on artificial diets containing leaf dip extracts of *S. lycopersicum* c.v. M82 and *S. habrochaites* accessions representing the chemotypes 1, 2, and 3. (**A**) Kaplan-Meier estimates of aphid apterae survivorship on diets containing leaf-dip extracts. Survival curves are compared by log-rank test ($\alpha = 0.05$). (**B**) Box and violin plots represent mean ± SE of gel saliva density (cm^{-2}) (ANOVA, $\alpha = 0.05$) and the probability density, respectively. (C) Number of honeydew drops accumulated in the feeding chambers. Crossbars represent means ± SE (ANOVA, $\alpha = 0.05$).
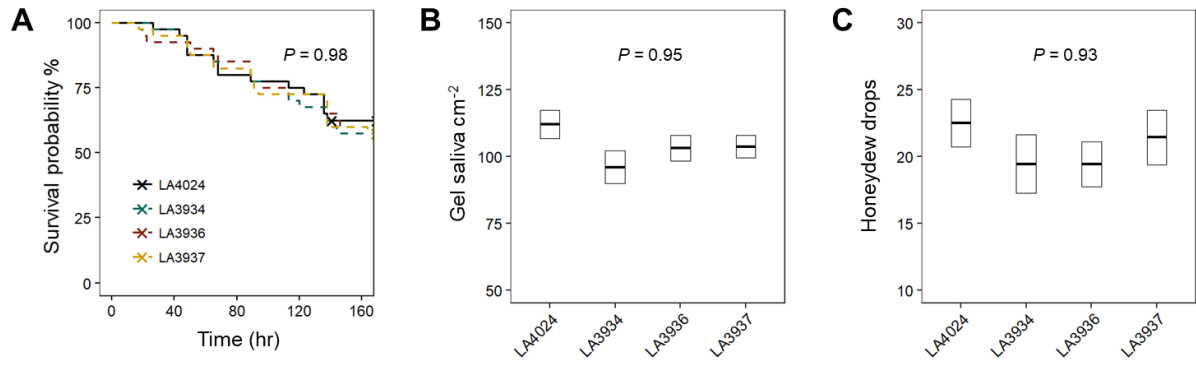
Figure S2.2 Feeding performance of *M. euphorbiae* apterae on artificial diets containing leaf-dip extracts from *S. lycopersicum* LA4024 and the introgression lines LA3934, LA3936, and LA3937. (**A**) Kaplan-Meier estimates of aphid apterae survivorship on diets. Survival curves are compared by log-rank test ($\alpha = 0.05$). Box plots represent means ± SE of gel saliva density (cm^{-2}) (**B**) and number of honeydew drops accumulated in the feeding chambers (**C**). Values are compared by ANOVA ($\alpha = 0.05$).
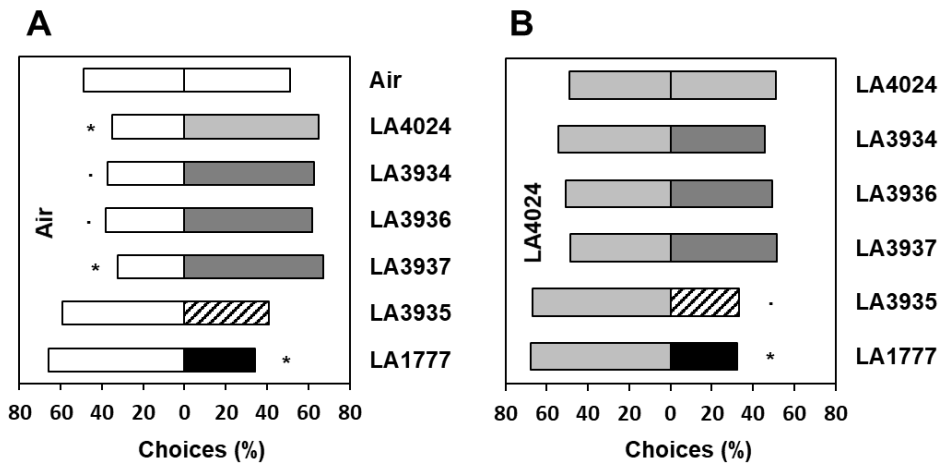
Figure S2.3 Choice behavior of *M. euphorbiae* alatae in an open Y-track olfactometer. (**A**) Choice of aphids between air (left bars), and odors from leaves of *S. lycopersicum* LA4024, *S. habrochaites* LA1777, and the introgression lines LA3935, LA3934, LA3936, and LA3937 (right bars). (**B**) Choice of aphids between odors from leaves of *S. lycopersicum* LA4024 (left bars), and *S. habrochaites* LA1777 or different introgression lines (right bars). Asterisks following each pair of bars indicate significant differences according to Chi-square goodness of fit ($\cdot$, $P < 0.1$; *, $P < 0.05$).

Table S2.1 Accumulated terpenes in leaf glandular trichomes of different tomato accessions. Terpene compounds were extracted from different *S. lycopersicum* and *S. habrochaites* accessions by briefly dipping leaves in methyl *tert*-butyl ether. The resulting extracts were analyzed by GC/MS. Values are nmol g FW^{-1} (means ± SE, *n* = 3).

Terpene compounds	c.v. M82	c.v. MM	LA1691	LA2650	LA1721	LA1927	LA1978	LA2155	LA1775	LA1779	LA1624	LA2860
α-pinene	0.32 ±0.02	0.38 ±0.02	-	-	-	-	-	-	-	-	-	-
δ-2-carene	1.71 ±0.13	4.61 ±0.71	-	-	-	-	-	-	-	-	-	-
α-phellandrene	0.25 ±0.06	0.19 ±0.02	-	-	-	-	-	-	-	-	-	-
β-phellandrene	9.70 ±0.63	36.39 ±3.67	-	-	-	-	-	-	-	-	-	-
β-myrcene	-	0.13 ±0.01	-	-	-	-	-	-	-	-	-	-
δ-elemene	0.37 ±0.04	-	-	-	7.82 ±0.64	14.6 ±0.89	62.46 ±3.04	36.32 ±1.28	19.60 ±0.33	5.86 ±0.55	-	-
β-caryophyllene	2.85 ±0.12	1.58 ±0.57	-	-	-	-	-	-	-	-	26.89 ±1.63	40.93 ±2.95
α-humulene	0.53 ±0.02	0.28 ±0.07	-	-	-	-	-	-	-	-	8.41 ±0.81	14.38 ±0.75
7-epi-zingiberene	-	-	33.56 ±3.97	94.23 ±12.12	-	5.22 ±0.33	-	15.87 ±1.43	-	-	-	-
R-curcumene	-	-	3.65 ±0.52	-	-	-	-	-	-	-	-	-
γ-elemene	-	-	-	-	135.34 ±10.08	157.39 ±16.62	4.96 ±0.19	2.42 ±0.10	160.09 ±5.02	93.08 ±0.78	-	-
β-elemene	-	-	-	-	-	-	-	-	8.06 ±0.29	11.58 ±0.84	-	-
α-bergamotene	-	-	-	-	-	-	-	-	17.63 ±1.21	20.82 ±1.39	-	-
α-santalene	-	-	-	-	-	-	-	-	15.89 ±0.32	46.34 ±5.06	-	-
β-bergamotene	-	-	-	-	-	-	-	-	33.87 ±3.26	41.51 ±11.67	-	-
(Z)-α-farnesene	-	-	-	-	-	-	-	-	6.34 ±0.41	2.39 ±0.37	-	-
(n)-trans-nerolidol	-	-	-	-	-	-	-	-	-	-	6.66 ±0.49	3.2 ±0.09
Z-α-trans-bergamotol	-	-	-	-	-	-	-	-	-	-	5.35 ±0.45	4.4 ±1.16
trans-z-bisabolene epoxide	-	-	-	-	-	-	-	-	-	-	-	4.02 ±0.13

Table S2.2 Terpenes emitted by leaves of different tomato accessions. Terpene compounds emitted from leaves of different *S. lycopersicum* and *S. habrochaites* accessions were collected by closed-loop stripping and analyzed by GC/MS. Values are pmol g FW^{-1} h^{-1} (means ± SE, $n = 3$).

Terpene compounds	c.v. M82	c.v. MM	LA1691	LA2650	LA1721	LA1927	LA1978	LA2155	LA1775	LA1779	LA1624	LA2860
α-pinene	53.70 ±4.62	29.16 ±3.65	-	-	-	-	-	-	-	-	-	-
δ-2-carene	90.03 ±30.65	97.25 ±11.51	-	-	-	-	-	-	-	-	-	-
α-phellandrene	8.15 ±2.64	20.38 ±3.21	-	-	-	-	-	-	-	-	-	-
β-phellandrene	978.53 ±221.50	1262.56 ±116.70	-	-	-	-	-	-	-	-	-	-
α-terpinene	5.12 ±3.28	19.85 ±1.89	-	-	-	-	-	-	-	-	-	-
δ-elemene	11.69 ±4.59	5.65 ±0.43	-	-	104.25 ±27.19	258.35 ±47.43	691.47 ±101.09	477.99 ±88.15	62.66 ±15.72	177.44 ±56.67	-	-
β-caryophyllene	64.39 ±5.42	61.21 ±11.34	-	-	-	-	-	-	-	-	1018.53 ±98.94	1495.12 ±298.56
α-humulene	46.81 ±9.49	29.86 ±2.15	-	-	-	-	-	-	-	-	518.48 ±34.43	1087.76 ±205.28
7-epi-zingiberene	-	-	454.08 ±136.24	1152.25 ±361.82	-	-	-	193.86 ±84.22	-	-	-	-
R-curcumene	-	-	54.94 ±13.88	27.29 ±4.39	-	-	-	-	-	-	-	-
γ-elemene	-	-	-	-	1847.40 ±102.43	2969.05 ±596.95	69.98 ±24.58	68.5 ±12.76	2040.73 ±218.17	1738.31 ±407.63	-	-
β-elemene	-	-	-	-	-	-	-	-	100.13 ±34.26	42.77 ±13.18	-	-
α-bergamotene	-	-	-	-	-	-	-	-	542.97 ±148.82	496.30 ±120.41	-	-
α-santalene	-	-	-	-	-	-	-	-	874.96 ±78.84	739.37 ±140.69	-	-
β-bergamotene	-	-	-	-	-	-	-	-	1251.31 ±85.26	883.83 ±102.72	-	-
(Z)-α-farnesene	-	-	-	-	-	-	-	-	55.27 ±21.68	53.29 ±16.02	-	-
Z-α-trans-bergamotol	-	-	-	-	-	-	-	-	-	-	20.48 ±4.90	190.82 ±49.93

Table S2.3 Accumulated terpenes in leaf glandular trichomes of tomato introgression lines. Terpene compounds were extracted from the *S. lycopersicum* and *S. habrochaites* parental accessions (LA4024 and LA1777, respectively), and four introgression lines (LA3934, LA3935, LA3936, LA3937) by briefly dipping leaves in methyl *tert*-butyl ether. The resulting extracts were analyzed by GC/MS. Values are nmol g FW^{-1} (means ± SE, $n = 3$).

Terpene compounds	LA4024	LA1777	LA3935	LA3934	LA3936	LA3937
α-pinene	4.37±0.27	-	-	4.72±1.51	4.88±0.87	12.81±1.31
δ-2-carene	22.92±4.93	-	-	22.14±3.49	36.01±5.26	73.87±4.98
α-phellandrene	4.05±0.68	-	-	5.16±1.88	6.60±1.33	12.92±0.94
α-terpinene	0.91±0.23	-	-	1.28±0.65	1.70±0.44	3.24±0.35
β-phellandrene	77.45±4.37	-	-	77.03±14.84	94.92±9.59	175.49±9.87
δ-elemene	2.61±1.13	30.30 ±4.08	0.96±0.53	2.49±1.55	2.71±0.68	8.64±2.64
β-elemene	0.31±0.11	23.42±1.91	-	0.33±0.12	0.31±0.07	0.94±0.22
β-caryophyllene	2.09±0.24	-	-	3.53±0.43	2.70±0.22	1.38±0.10
α-humulene	0.76±0.14	-	-	1.46±0.40	1.26±0.15	0.30±0.03
α-bergamotene	-	31.37±1.69	7.00±0.64	-	-	-
α-santalene	-	46.28±1.90	12.61±0.90	-	-	-
β-bergamotene	-	26.82±2.83	11.75±1.34	-	-	-
γ-elemene	-	85.79±10.16	-	-	-	-
(Z)-α-farnesene	-	5.10±3.97	1.95±0.25	-	-	-
cedrene	-	4.73±1.08	-	-	-	-
α-bisabolene	-	-	1.13±0.11	-	-	-
α-sesquiphellandrene	-	-	1.87±0.41	-	-	-

Table S2.4 Terpenes emitted by leaves of tomato introgression lines. Terpene compounds emitted from leaves of the *S. lycopersicum* and *S. habrochaites* parental accessions (LA4024 and LA1777, respectively), and four introgression lines (LA3934, LA3935, LA3936, LA3937) were collected by closed-loop stripping and analyzed by GC/MS. Values are pmol g FW^{-1} h^{-1} (means $\pm$ SE, $n = 3$).

Terpene compounds	LA4024	LA1777	LA3935	LA3934	LA3936	LA3937
α-pinene	19.83±4.04	-	-	73.13±26.17	40.29±7.41	31.98±7.19
δ-2-carene	148.59±17.04	-	-	436.91±47.43	399.29±42.99	447.61±38.54
α-phellandrene	17.59±0.56	-	-	59.21±9.80	43.98±10.29	44.55±19.59
α-terpinene	-	-	-	20.15±5.14	13.61±2.71	-
β-phellandrene	834.04±73.47	-	-	889.87±86.30	1364.56±46.43	1629.70±151.69
δ-elemene	8.04±1.96	122.08±21.90	5.37±1.18	9.37±3.34	5.06±1.07	13.20±2.80
β-elemene	-	585.58±42.08	-	-	-	-
β-caryophyllene	99.26±14.55	-	-	124.92±17.32	78.40±7.32	48.86±5.27
α-humulene	18.88±2.92	-	-	39.92±14.46	27.90±4.69	12.79±2.01
α-bergamotene	-	570.06±55.42	222.40±33.09	-	-	-
α-santalene	-	1442.73±99.12	564.80±20.31	-	-	-
β-bergamotene	-	1317.38±121.72	469.55±37.43	-	-	-
γ-elemene	-	2056.00±179.62	-	-	-	-
(Z)-α-farnesene	-	64.29±9.49	33.33±4.09	-	-	-
cedrene	-	147.64±32.49	-	-	-	-
α-bisabolene	-	-	45.70±10.17	-	-	-
α-sesquiphellandrene	-	-	87.42±19.93	-	-	-

Chapter 3: Glandular trichome-derived mono- and sesquiterpenes of tomato have contrasting roles in the interaction with the potato aphid *Macrosiphum euphorbiae*

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Abstract: Secondary metabolites produced in glandular trichomes of tomato are involved in interactions with herbivores. In cultivated tomato (*Solanum lycopersicum*) glandular trichomes accumulate a blend of abundant monoterpenes and smaller amounts of a few sesquiterpenes. These mono- and sesquiterpenes are synthesized by three terpene synthases, TPS20 as well as TPS9 and TPS12, respectively. To study effects of these terpenes on performance and choice behavior of potato aphid (*Macrosiphum euphorbiae*), we utilized two tomato trichome mutants, *hairless* and *odorless-2*, that are differently affected in mono- and sesquiterpene production. Non-choice assays demonstrated that longevity and fecundity of *M. euphorbiae* were increased when kept on the trichome mutants. A principal component analysis of these aphid performance parameters and terpene production in the trichome mutants indicated that longevity and fecundity of *M. euphorbiae* were negatively correlated with production of the TPS12-derived sesquiterpenes β-caryophyllene and α-humulene. While we had previously shown that addition of pure β-caryophyllene/α-humulene to an artificial feeding diet affected *M. euphorbiae* apterae survivorship and feeding behavior, no such effects were observed here upon addition of a mixture of pure TPS20-derived monoterpenes. In olfactometer assays *M. euphorbiae* alatae displayed differential choice behaviors towards the *hairless* and *odorless-2* mutants suggesting a role of TPS20-derived monoterpenes in aphid attraction, which was further confirmed using a mixture of pure monoterpenes. Our analyses revealed contrasting roles of glandular trichome-derived terpenes in *S. lycopersicum*. While TPS12-derived sesquiterpenes contribute to host plant resistance against *M. euphorbiae*, TPS20-derived monoterpenes appear to be exploited as cue for host plant orientation by aphids.

Keywords: *Solanum lycopersicum*, tomato, *Macrosiphum euphorbiae*, potato aphid, glandular trichomes, terpenes

3.1 Introduction

Due to their sessile lifestyle plants face many challenges through interactions with their biotic environment and thus have evolved several distinct strategies to defend themselves against herbivores. Towards this goal plants produce a wide variety of secondary metabolites including volatile organic compounds that contribute directly to the defense against herbivores by acting as repellents or toxins, and indirectly by attracting natural enemies (Dudareva et al. 2013; Unsicker et al. 2009). Monoterpenes and sesquiterpenes are one of the largest groups of plant-derived volatile organic compounds (Dudareva et al. 2013). Volatile terpenes originate from two building blocks: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP are used by prenyl transferases to form larger prenyl diphosphate intermediates which serve as precursors for mono- and sesquiterpene formation. The diversity of terpenes found in plants is ultimately generated by terpene synthases (TPSs) which utilize one or several prenyl diphosphate substrates, and often have the ability to produce multiple terpene products from one prenyl diphosphate substrate (Degenhardt et al. 2009). To facilitate their role in the defense against herbivores terpenes are often produced in specific plant tissues like internal ducts and glandular trichomes (Gershenzon and Dudareva 2007). While in wild plants defensive traits such as volatile terpenes are likely under constant positive selection, it appears that they have been compromised in crop plants, because selective breeding has favored other agronomic traits (e.g. Köllner et al. 2008).

Glandular trichomes are found on the vegetative tissues of cultivated and wild tomato species. In cultivated tomato (*Solanum lycopersicum*) they produce a blend of abundantly present monoterpenes and only small amounts of a few sesquiterpenes (Schilmiller et al. 2009). The terpene synthase gene family in *S. lycopersicum* has been characterized in detail (Bleeker et al. 2011a; Falara et al. 2011) including the three TPSs involved in the exclusive formation of mono- and sesquiterpenes in glandular trichomes. While the *S. lycopersicum* TPS20 produces several monoterpenes, including α-pinene, δ-2-carene, α-phellandrene and β-phellandrene (Schilmiller et al. 2009), the sesquiterpene synthases TPS9 (Colby et al. 1998) and TPS12 (Schilmiller et al. 2010) are responsible for the formation of germacrene C and β-caryophyllene/α-humulene, respectively. The expression of the three terpene synthases, TPS20, TPS9 and TPS12, was found to be specific to glandular trichomes in *S. lycopersicum* (Schilmiller

et al. 2009; Bleeker et al. 2011a). These mono- and sesquiterpenes produced in glandular trichomes of *S. lycopersicum* were shown to affect biting-chewing herbivores such as Colorado potato beetle (*Leptinotarsa decemlineata*), tomato fruitworm (*Helicoverpa zea*), and tobacco hornworm (*Manduca sexta*) (Kang et al. 2010a, b; Tian et al. 2012; Gutensohn et al. 2014). In contrast, very little is known about how piercing-sucking herbivores such as whiteflies, spider mites and aphids are affected by glandular trichome-derived terpenes in cultivated tomato.

Piercing-sucking herbivores such as aphids represent a serious problem in the commercial tomato production. Although direct feeding by aphids generally does not cause severe damage, significant yield losses are caused indirectly through the transmission of viruses for which these herbivores serve as vectors (van Emden and Harrington 2007). In addition, the secretion of honeydew by aphids promotes the development of sooty mold on foliage and fruits. Aphid control strategies utilizing synthetic insecticides are becoming increasingly ineffective due to avoidance behavior and emerging resistance (Bass et al. 2014; Fray et al. 2014; Silva et al. 2012). Thus, the development of highly efficient and sustainable strategies for the control of aphids and, in consequence, virus transmission in tomato production is urgently required. One potential approach towards this goal could be to introduce new glandular trichome-derived terpene traits from wild into cultivated tomato. Candidates for introgression would be sesquiterpenes with activity against whiteflies and aphids that have recently been identified in *Solanum habrochaites* accessions (Bleeker et al. 2011b; Wang et al. 2020) and that are not found in *S. lycopersicum* (Gonzales-Vigil et al. 2012). However, at the same time, it is essential to obtain a detailed understanding about how the mono- and sesquiterpenes that are already produced in glandular trichomes of *S. lycopersicum* affect the performance and feeding behavior of aphids.

Here, we utilized two *S. lycopersicum* trichome mutants that are affected in mono- and sesquiterpene production to analyze the effect of these terpenes on performance, feeding and choice behavior of the potato aphid (*Macrosiphum euphorbiae*). This aphid is a major pest of cultivated tomato that can have an adverse economic impact and develop into a serious issue for greenhouse production (Tomescu and Negru 2003). *M. euphorbiae* also serves as a vector for over 40 non-persistent and five persistent viruses (Blackman and Eastop 2000). To study the potential effects of glandular trichome-derived terpenes in *S. lycopersicum* on *M. euphorbiae* we

utilized the two tomato trichome mutants, *hairless* and *odorless-2*, that had been shown earlier to be deficient in the production of distinct terpenes (Kang et al. 2010a, b). The *hairless* mutant (Kang et al. 2010b) is deficient in the TPS12-derived sesquiterpenes, however, it accumulates wild type levels of the TPS20-derived monoterpenes. In contrast, the *odorless-2* mutant (Kang et al. 2010a) accumulates only trace levels of both, mono- and sesquiterpenes, which allows to analyze the effect of each terpene group on the aphids. In addition, both trichome mutants are deficient in a number of trichome-derived flavonoids, while other defensive secondary metabolites are not affected (Kang et al. 2010a, b). The terpenes produced in glandular trichomes of *S. lycopersicum* can directly affect aphids when they move around on the leaf surface, thereby breaking glandular trichomes (Bergau et al. 2015; Tissier et al. 2017). To study such effects of the trichome-derived mono- and sesquiterpenes on the performance of *M. euphorbiae* we conducted a series of non-choice assays with the two trichome mutants and respective wild types. To verify if exposure to glandular trichome-derived terpenes can affect *M. euphorbiae*, we performed feeding assays on an artificial diet with a mixture of pure monoterpenes, similar to our recent assays with pure sesquiterpenes (Wang et al. 2020). In addition to affecting the performance and feeding of *M. euphorbiae* on the plant surface, the terpenes produced in glandular trichomes of *S. lycopersicum* are emitted into the surrounding atmosphere and could already influence the pre-landing orientation and host plant choice of the aphids. Thus, the potential of the glandular trichome-derived terpenes emitted from *S. lycopersicum* to affect the choice behavior of aphids was tested by olfactometer assays utilizing the two trichome mutants as well as a mixture of pure monoterpenes.

3.2 Methods and Materials

3.2.1 Plant material

Seeds of the tomato (*S. lycopersicum*) cultivars Ailsa Craig (LA2838A) and Castlemart (LA2400), as well as the *hairless* tomato trichome mutant (LA3556) were obtained from the C.M. Rick Tomato Genetics Resource Center, University of California Davis, CA, USA. Seeds of the *odorless-2* tomato trichome mutant (Kang et al. 2010a) were kindly provided by Dr. Gregg Howe (Michigan State University, MI, USA). All tomato plants used for leaf dip extractions, headspace collections, and aphid bioassays were grown from seeds in Sungro® soil mixture (Sun

Gro Horticulture, Agawam, MA, USA) under a 16-h photoperiod in standard greenhouse conditions (23–25 °C, 50–60% relative humidity) without pesticide application.

3.2.2 Aphid cultivation

The potato aphid, *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae), was collected from the West Virginia University Evansdale Greenhouse (Morgantown, WV, USA) and its species identification was confirmed through barcode sequencing. To avoid experience on tomato plants prior to any of the assays, the aphids were allowed to reproduce parthenogenetically on potted potato plants in an insect rearing room under a 16-h photoperiod at 20–22 °C. Winged *M. euphorbiae* (alates) used in this study were produced from a colony confined on potato leaves by applying Vaseline to the leaf petioles.

3.2.3 Extraction, collection, and analysis of terpenes from tomato leaves

To extract terpenes from leaf glandular trichomes of *S. lycopersicum*, fresh leaves of 6- to 8-week-old plants were dipped and gently shaken for 30 s in 30 ml of methyl *tert*-butyl ether (MTBE). The extracts were concentrated under a gentle stream of nitrogen gas and centrifuged. For the analysis of terpene profiles, extracts were transferred into GC vials and supplemented with 3.33 μg of naphthalene as an internal standard. Terpenes emitted from tomato leaves were collected using a closed-loop stripping method (Dudareva et al. 2005). Headspace collections from detached tomato leaves supplemented with 20% (*w/v*) sucrose were performed for 24 h using Porapak-Q traps (Volatile Collection Trap (VCT) LLC, Gainesville, FL, USA). After the collection traps were immediately eluted with dichloromethane and 3.33 μg of naphthalene was added to the eluate as an internal standard.

Samples from leaf extractions and headspace collections were analyzed by combined gas chromatography/mass spectrometry (GC/MS) using a TRACE 1310 gas chromatograph system linked to a TSQ 8000 Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Two μl of each sample were injected, volatilized at 220 °C and then separated on a TraceGOLD TG-5MS GC column (30 m length, 0.25 mm I.D., 0.25 μm film; Thermo Fisher Scientific, Pittsburgh, PA, USA). The initial column temperature was held at 40 °C for 3 min and then increased to 120 °C at 5 °C/min, 10 °C/min to 180 °C and 20 °C/min to 300 °C which was maintained for 2 min. The helium carrier gas flow was 1.3 ml/min. All samples from leaf dip

extractions and headspace collections were analyzed by combined GC/MS using the total ion chromatogram (TIC) mode. Individual terpene compounds were identified using the Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific) by comparing their mass spectra with those deposited in the NIST/EPA/NIH Mass Spectral Library (NIST11) (National Institute of Standards and Technology NIST, Scientific Instrument Services, Inc., NJ, USA; https://chemdata.nist.gov/mass-spc/ms-search/). In addition, the identity of compounds was confirmed by comparison of retention times and mass spectra with authentic terpene standards including β-phellandrene (P294560, Toronto Research Chemicals Inc., Toronto, Canada), as well as α-pinene (147524), δ-2-carene (232386), β-caryophyllene (22075) and α-humulene (53675) (all purchased from Sigma-Aldrich, St. Louis, MO, USA). Representative mono- and sesquiterpene standards were also used to determine average response factors for both compound classes, which were used in combination with the internal standard (naphthalene) for the quantification of the analyzed compounds.

3.2.4 Aphid non-choice assays on wild type and mutant tomato

The performance of wingless *M. euphorbiae* (apterae) on the *hairless* and *odorless-2* mutants as well as on their wild type, the Ailsa Craig and Castlemart cultivars, respectively, was determined under greenhouse conditions as described previously (Eichele-Nelson et al. 2018). At the beginning of the assay two wingless individuals (F_0) reared on potato plants were placed on the surface of a young leaf (2nd or 3rd fully expanded leaf) of a 6-week old tomato plant and subsequently enclosed in a clip cage (BioQuip Products, Rancho Dominguez, CA, USA) that was attached to the leaf. Three tomato plants each with two clip cages attached (in total 6 clip cages) were used for each non-choice assay. After four days all aphids except three F_1 neonate nymphs (considered as 1 day old) were removed from each cage. Over the course of the experiment F_2 nymphs and exuviae were removed daily from the clip cages. The longevity of F_1 aphids and their fecundity represented by the number of F_2 nymphs in each cage were recorded.

3.2.5 Principal component analysis

The object-variable data matrix used for the principal component analysis (PCA) consisted of data that were collected from the four tomato accessions (*hairless*, Ailsa Craig, *odorless-2*, Castlemart) used for non-choice assays, with three replicates for each accession. The variables

included the total accumulation (nmol/g leaf FW) of the TPS20-derived monoterpenes (α-pinene, δ-2-carene, α-phellandrene, β-phellandrene), the TPS9-derived sesquiterpene (δ-elemene), and the TPS12-derived sesquiterpenes (β-caryophyllene, α-humulene), as well as the mean values of aphid longevity and fecundity. All variables had their scales equalized (standardization) prior to analysis to ensure that large values for some of the terpene concentrations would not bias the analysis. The first two dimensional spaces retaining most of the data variance were extracted for a biplot as an exploratory visualization of the data structure, focusing on the plant-plant and variable-variable relationships of interest. The PCA was conducted and the results were visualized by using "FactoMineR" and "factoextra" packages in R, respectively (version 3.4.3, R Development Core Team, www.R-project.org).

3.2.6 Aphid feeding assays

To determine the potential effects of TPS20-derived monoterpenes on the performance and feeding behavior of *M. euphorbiae* apterae, feeding assays on an artificial diet were performed as described previously by Mittler and Dadd (1963). Aliquots (100 μl) of an artificial diet were prepared by mixing 95 μl of a basal diet containing 20% (*w/v*) sucrose, 0.2% (w/v) neutral red, and 5 μl of a mixture of pure monoterpenes dissolved in MTBE. The monoterpene stock mixture was prepared by mixing the three pure compounds, β-phellandrene (Toronto Research Chemicals Inc., Toronto, Canada), δ-2-carene, and α-pinene (both Sigma-Aldrich, St. Louis, MO, USA), in a 1000:125:16 (*v/v/v*) ratio in MTBE according to their presence in the tomato leaf dip extracts (Table 1). Different amounts of the monoterpene stock mix were added to the artificial diets resulting in working concentrations of 0, 25, 50, 75, 100, 125 and 150 ng ul^{-1}. The solvent MTBE was allowed to evaporate from the diet for 5 min before each assay. Clear polystyrene plastic containers (31.75 mm diameter, 19.05 mm height; PSC Products, Beverly Hills, CA, USA) were used as feeding chambers. The inner side of each chamber cap was covered by an unstretched piece of Parafilm membrane and another layer of fully stretched Parafilm with 100 μl of the diet spread in between. Four aphid apterae (7–8 days old) were starved for 12 h and then introduced into a feeding chamber. This assay was replicated 10 times with a total of 40 apterae being used for each tested monoterpene concentration. The survival of the apterae was examined multiple times every day over the course of the experiment. Dead apterae and newly emerged offspring were removed immediately. In addition, immature aphids that had been

accidentally introduced and aphids that had been damaged during handling were removed and recorded. After 48 h, all feeding chambers with four apterae alive were assessed under a stereomicroscope (SZ-ST, Olympus, Tokyo, Japan) to quantify the number of feeding attempts, based on the sites with gel saliva produced, and honeydew droplets left behind by aphids on the Parafilm. Both gel saliva and honeydew droplets were stained by the neutral red included in the artificial diet. Three 0.25 cm^2 areas were randomly selected on the Parafilm for each feeding chamber and the number of punctures with stained gel saliva was determined. In addition, the number of honeydew droplets produced by aphids in each feeding chamber was counted.

3.2.7 Aphid olfactometer choice assays

An open Y-track system (Visser and Piron 1998) was utilized to study the choice behavior of *M. euphorbiae* alates. The vertical Y-track was constructed from an iron rod (diameter 2.5 mm, length 130 mm) and a Y-junction with a 50-mm vertical part and two 80-mm arms separated by 120 degrees. Each arm of the Y-junction was inserted by 6 cm into a separate glass tube from which a constant airflow (0.8 l/min) carrying odors from a source located in a separate Plexiglas container was driven by a 2-channel air delivery system (Analytical Research System Inc., Gainesville, FL, USA). The airflow was moistened and purified before reaching the enclosed odor source. The Y-track olfactometer was illuminated from the top by one halogen lamp.

M. euphorbiae alates were reared on potato plants, then collected from these potato plants, and starved for 12 h prior to each experiment. For each assay an aphid was introduced at the bottom of the rod, and a choice was considered complete when the aphid touched either end of the Y-junction. Each aphid was observed for no more than 3 min. At least 37 alates were tested for each pair of odor sources and the position of glass tubes relative to the Y-junction was switched after testing each set of 10 alates. All glassware and tubing were rinsed with 70% ethanol and dried after testing each pair of odor sources. The preferences of alates for specific *S. lycopersicum* accessions were tested by introducing 5.0 ± 0.2 g of fresh tomato leaves into either one or both of the Plexiglas containers of the 2-channel air delivery system. Leaf petioles were immersed into 20% (*w/v*) sucrose solution before introduction. Different amounts of the pure monoterpene mixture (see above) dissolved in MTBE were applied on round Whatman filter paper (8 cm diameter) to test their attractiveness to alates. The solvent was allowed to evaporate

2 min before the filter papers were transferred into the plexiglass containers of the 2-channel air delivery system.

3.2.8 Data analysis

All statistical analyses were conducted with R (version 3.4.3, R Development Core Team, www.R-project.org). The concentration of each terpene quantified in leaf dip extracts and headspace collections was summarized as mean ± SE. The content of individual terpenes in the tomato trichome mutants and the respective wild type cultivars were compared by Student's *t* test ($\alpha = 0.05$). Longevity and fecundity of wingless F_2 aphids in non-choice assay were also compared for each pair of tomato cultivar and trichome mutant to identify significant differences (Student's *t* test, $\alpha = 0.05$). In the feeding assays, the Kaplan-Meier survival curves estimated for four (0, 50, 100, and 150 ng μl^{-1}) of the seven monoterpene concentrations used for the assays were compared using nonparametric log-rank tests supported by "survival" package in R 3.4.3. Linear regressions were used to analyze the trend of gel saliva accumulation and the production of honeydew spots in response to the seven increasing concentrations of TPS20-derived monoterpenes. In choice assays, the observed frequencies of choices aphid alates made for odor sources were analyzed with Chi-Square test.

3.3 Results

3.3.1 Monoterpene and sesquiterpene production in the *hairless* and *odorless-2* trichome mutants

To verify the previous characterization of terpene profiles of the two tomato trichome mutants and respective wild type cultivars (Kang et al. 2010a, b) under our conditions, we prepared leaf dip extracts and analyzed these by combined gas chromatography/mass spectrometry (GC/MS). This analysis confirmed that the accumulation of monoterpenes and sesquiterpenes in glandular trichomes (Table 3.1) was indeed differently affected in the *hairless* and *odorless-2* mutants when compared to their wild type, Ailsa Craig and Castlemart, respectively. In the leaf dip extracts of the *hairless* mutant the concentrations of the TPS12-derived sesquiterpenes β-caryophyllene and α-humulene were reduced by 55.0% and 59.3%, respectively, compared to the Ailsa Craig cultivar (Table 3.1). In contrast, the concentrations of the TPS20-derived monoterpenes α-pinene, δ-2-carene, α-phellandrene, and β-

phellandrene, as well as the sesquiterpene δ-elemene were not significantly different in leaf dip extracts of the *hairless* mutant and Ailsa Craig. Although δ-elemene was detected upon analysis of leaf dip extracts, this is likely the result of a Cope rearrangement that occurs during sample injection into the hot (220 °C) GC injector port (Colby et al. 1998), thus suggesting that glandular trichomes of the analyzed tomato leaves accumulate the TPS9-derived sesquiterpene germacrene C. However, in leaf dip extracts of the *odorless-2* mutant both, the TPS20-derived monoterpenes as well as the TPS12- and TPS9-derived sesquiterpenes, were not or barely detected (Table 3.1) while they were present in extracts from the Castlemart cultivar.

In addition to analyzing the terpenes accumulated in glandular trichomes, we performed headspace collections from leaves to characterize the profile of terpenes emitted from the two tomato trichome mutants and respective wild types. The results of this analysis of emitted terpenes (Table S3.1) were consistent with those from our analysis of leaf dip extracts and further confirmed the observed differences in the terpene profile between the *hairless* and *odorless-2* mutants, and their respective wild type. The emission of the TPS12-derived sesquiterpenes β-caryophyllene and α-humulene from leaves of the *hairless* mutant was reduced by 52.4% and 49.5%, respectively, compared to Ailsa Craig (Table S3.1), while emission of the TPS20-derived monoterpenes and the TPS9-derived δ-elemene (germacrene C) were unchanged. In contrast, only trace amounts of the TPS20-derived monoterpenes as well as the TPS12- and TPS9-derived sesquiterpenes were emitted from the *odorless-2* mutant (Table S3.1).

3.3.2 glandular trichome-derived β-caryophyllene and α-humulene affect longevity and fecundity of aphids

We performed non-choice assays, utilizing both trichome mutants and respective wild types, as an initial approach to characterize the potential effects of the glandular trichome-derived monoterpenes and sesquiterpenes on *M. euphorbiae* when these are located on the leaf surface. The longevity of *M. euphorbiae* on the *odorless-2* mutant (26.9 ± 0.8 days), which is characterized by the almost complete lack of mono- and sesquiterpene production (Table 3.1), was significantly increased ($t = 2.45$, $P = 0.019$) compared to that on the Castlemart cultivar (24.1 ± 0.8 days) (Figure 3.1a). A slight but not significant increase ($t = 0.42$, $P = 0.68$) in longevity was also observed for *M. euphorbiae* on the *hairless* mutant (24.2 ± 0.9 days), which

shows only a reduced production of β-caryophyllene and α-humulene (Table 3.1), compared to the Ailsa Craig cultivar (23.7 ± 1.0 days) (Figure 3.1a). Similar trends as observed for the longevity of *M. euphorbiae* were also found for their fecundity (represented by the number of offspring) on the two trichome mutants. The number of *M. euphorbiae* offspring was significantly larger ($t = 2.27$, $P = 0.047$) on the *odorless-2* mutant (36.7 ± 4.1 nymphs) compared to that on Castlemart (25.3 ± 3.5 nymphs) (Figure 3.1b). A trend towards an increased number ($t = 1.00$, $P = 0.34$) of *M. euphorbiae* offspring was also found with the *hairless* mutant (23.8 ± 3.4 nymphs) compared to Ailsa Craig (19.5 ± 2.7 nymphs) (Figure 3.1b).

To better understand the total variance of the glandular trichome-derived monoterpene and sesquiterpene compounds of the trichome mutants and respective wild type obtained by leaf-dip extraction (Table 3.1), as well as of the aphid performance parameters, longevity and fecundity, observed by the non-choice assays (Figure 3.1a and b), a PCA was conducted. The total variance of the data matrix was explained by five dimensions. The eigenvalues of dimension 1 and 2 were 2.94 and 1.60, respectively, accounting for 58.8% and 32.0% (90.8% in summary) of the total variance (Figure 3.1c). Dimension 1 contributed towards the separation of the *odorless-2* trichome mutant and the Castlemart cultivar. On dimension 1 scores of Castlemart plants were close to the origin (mean score = 0.04), while those of *odorless-2* plants were strongly negative (mean score = −2.52). Dimension 2 separated the *hairless* mutant from Ailsa Craig. On dimension 2 scores of Ailsa Craig plants were negative (mean score = −1.38), while scores of *hairless* plants were highly positive (mean score = 1.92). Based on the angles of the arrows in the two-dimensional biplot (Figure 3.1c), the two aphid performance parameters, longevity (eigenvector coefficients: dim1 = −0.91, dim2 = 0.18) and fecundity (eigenvector coefficients: dim1 = −0.88, dim2 = 0.36), were highly positively correlated, both of which contributed greatly to the separation of the *odorless-2* mutant and the other tomato accessions in dimension 1 (longevity = 28.21%, fecundity = 26.71%). Neither the longevity nor fecundity variable established an obvious relationship with either the TPS9-derived sesquiterpene δ-elemene (germacrene C) (eigenvector coefficients: dim1 = 0.61, dim2 = 0.76) or the TPS20-derived monoterpenes (eigenvector coefficients: dim1 = 0.62, dim2 = 0.73). In contrast, a strong negative relationship was observed between the TPS12-derived sesquiterpenes (eigenvector coefficients: dim1 = 0.75, dim2 = −0.57) and both aphid performance parameters, longevity and fecundity (Figure 3.1c). Thus, the results of the non-choice assays and PCA suggested that the TPS12-

derived sesquiterpenes β-caryophyllene and α-humulene produced in the glandular trichomes of *S. lycopersicum* negatively affect the performance of *M. euphorbiae*.

3.3.3 TPS20-derived monoterpenes do not affect aphid performance and feeding behavior

While the accumulation of TPS12-derived sesquiterpenes negatively correlated with the aphid performance, no obvious relationship between the production of TPS20-derived monoterpenes and aphid performance was observed in the non-choice assays and PCA (Figure 3.1). To further test if these monoterpenes indeed lack the potential to affect the performance and feeding behavior of *M. euphorbiae*, we performed feeding assays. Towards this goal increasing amounts of a mixture of the three most abundant TPS20-derived monoterpenes (Table 3.1), β-phellandrene, δ-2-carene, and α-pinene were added to an artificial diet. When *M. euphorbiae* apterae were allowed to feed on the artificial diets containing the monoterpene mixture, their survivorship was not significantly different from that of aphids feeding on the artificial diet with only the solvent (MTBE) added (Figure 3.2a). Two additional parameters indicative of the aphid feeding behavior were analyzed: investment in the production of gel saliva, which normally is produced by aphids upon penetration of plant tissue and hardens forming a sheath around the inserted aphid stylet (Will et al. 2012), as well as the production of honeydew, which is secreted by aphids during feeding. However, neither the investment in gel saliva (Figure 3.2b) nor the honeydew production by the aphids (Figure 3.2c) was significantly influenced by increasing concentrations of the monoterpene mixture in the artificial diets, with the coefficient estimates for 'concentration' in both linear models not being significantly different from 0 (concentration estimate for gel saliva model: -0.0066 ± 0.0065, $t = -1.023$, $P = 0.308$; concentration estimate for honeydew production model: 0.017 ± 0.025, $t = -0.668$, $P = 0.507$). Thus, these results further confirmed that the TPS20-derived monoterpenes produced in glandular trichomes of *S. lycopersicum* do not affect the performance and feeding behavior of *M. euphorbiae*.

3.3.4 TPS20-derived monoterpenes emitted by *S. lycopersicum* are attractive for aphids

In order to test the behavioral response of *M. euphorbiae* alates towards the mono- and sesquiterpenes emitted by *S. lycopersicum* (Table S3.1) we performed choice assays using an open Y-track olfactometer. Initially aphids were given the choice between pure air and the odors

of tomato leaves (Figure 3.3a). A significantly different choice behavior of *M. euphorbiae* alates was observed with the *hairless* mutant resulting in 67.4% ($\chi^2 = 5.57$, $P = 0.02$) of the aphids orienting towards the leaf odors (Figure 3.3a), while only 58.1% ($\chi^2 = 1.14$, $P = 0.29$) of the aphids oriented towards the odors of Ailsa Craig. In contrast, a significant attraction of *M. euphorbiae* alates was observed for the Castlemart cultivar with 65.1% ($\chi^2 = 3.93$, $P = 0.05$) of the aphids orienting towards the leaf odors (Figure 3.3a), while only 56.8% ($\chi^2 = 0.68$, $P = 0.41$) of the aphids oriented towards the odors of the *odorless-2* mutant. The opposite trends in aphid attraction detected with the two trichome mutants became even more obvious in the second set of assays when *M. euphorbiae* alates were given the choice between the odor of one of the trichome mutants and its respective wild type (Figure 3.3a). When the *hairless* mutant was paired with Ailsa Craig 65.5% ($\chi^2 = 4.45$, $P = 0.03$) of the *M. euphorbiae* alates oriented towards the odors of the trichome mutant, indicating a repellent activity of the increased emission of β-caryophyllene and α-humulene by Ailsa Craig (Table S3.1). In contrast, when the *odorless-2* mutant was paired with Castlemart only 34.8% ($\chi^2 = 2.69$, $P = 0.10$) of the *M. euphorbiae* alates oriented towards the odors of the trichome mutant, suggesting that the monoterpenes emitted from Castlemart might act as attractant for the aphids.

To verify that the TPS20-derived monoterpenes produced in glandular trichomes of *S. lycopersicum* indeed affect the choice behavior of *M. euphorbiae*, we performed an additional set of olfactometer choice assays utilizing a mixture of the three pure monoterpenes, β-phellandrene, δ-2-carene, and α-pinene. When *M. euphorbiae* alates were given the choice between pure air and the odors of this monoterpene mixture, we observed an increased attraction of *M. euphorbiae* alates towards the monoterpene mixture in a dose-dependent manner (Figure 3.3b). Increasing numbers of aphids, 53.7%, 58.1%, and 65.2% respectively, oriented towards the TPS20-derived monoterpenes when 1 μg ($\chi^2 = 0.22$, $P = 0.64$), 10 μg ($\chi^2 = 1.14$, $P = 0.29$), and 100 μg ($\chi^2 = 4.26$, $P = 0.04$) of the pure terpene mixture were used. Only when higher amounts of the monoterpene mixture were used this trend reverted with only 59.1% and 47.6% of aphids orienting towards the monoterpene blend upon addition of 1000 μg ($\chi^2 = 1.45$, $P = 0.23$) and 10,000 μg ($\chi^2 = 0.10$, $P = 0.76$), respectively. Thus, these assays further confirmed that the TPS20-derived monoterpenes in *S. lycopersicum* indeed have the potential to positively affect the choice behavior and host plant preference of *M. euphorbiae*.

3.4 Discussion

This study provided evidence that the TPS12-derived sesquiterpenes and the TPS20-derived monoterpenes found in glandular trichomes of *S. lycopersicum* have contrasting roles in the interaction with *M. euphorbiae*. We utilized the *hairless* and *odorless-2* tomato trichome mutants, that previously have been shown to be differently affected in the production of these mono- and sesquiterpenes (Kang et al. 2010a, b). Our analysis of the emission and accumulation of terpenes (Table 3.1 and S3.1) confirmed that the formation of TPS12-derived sesquiterpenes is reduced in both trichome mutants, while only the *odorless-2* mutant is affected in the formation of TPS20-derived monoterpenes. In the non-choice and choice assays we found that the TPS12-derived sesquiterpenes β-caryophyllene and α-humulene contributed to host plant resistance against *M. euphorbiae* in cultivated tomato. Non-choice assays (Figure 3.1) demonstrated that longevity and fecundity of *M. euphorbiae* was increased when kept on the trichome mutants. A principal component analysis (Figure 3.1c) of these aphid performance parameters and terpene production in the trichome mutants indicated that longevity and fecundity of *M. euphorbiae* were negatively correlated with production of the TPS12-derived sesquiterpenes β-caryophyllene and α-humulene. In addition, the results of olfactometer choice assays showed that significantly more aphids oriented towards the leaf odors of the *hairless* mutant, which is only affected in the formation of TPS12-derived sesquiterpenes, than towards the Ailsa Craig cultivar (Figure 3.3), suggesting that these sesquiterpenes have repellent activity.

These results indicated a role of TPS12-derived sesquiterpenes in host plant resistance against *M. euphorbiae*, which is in line with the outcome of our recent study (Wang et al. 2020) demonstrating that aphid performance and behavior were negatively affected by several accessions of the wild tomato species *S. habrochaites* that produce β-caryophyllene and α-humulene. Aphids that were kept on the leaf surface of these *S. habrochaites* accessions, where they are exposed to at least ten-fold higher amounts of β-caryophyllene and α-humulene than in *S. lycopersicum*, showed a significantly reduced longevity and fecundity compared to those on *S. lycopersicum*. When *M. euphorbiae* were allowed to feed on an artificial diet to which leaf dip extracts from β-caryophyllene/α-humulene producing *S. habrochaites* accessions had been added (Wang et al. 2020), their survivorship as well as the production of gel saliva and

honeydew were significantly reduced. Thus, the effect on the performance of aphids when these are exposed to TPS12-derived sesquiterpenes, larger amounts on *S. habrochaites* leaves (Wang et al. 2020) and smaller amounts on *S. lycopersicum* leaves (Figure 3.1), might be at least in part due to a reduced feeding frequency and intensity as indicated by the lower gel saliva investment and production of honeydew (Wang et al. 2020). To verify that the observed effects are due to the TPS12-derived sesquiterpenes and not to other secondary metabolites produced in tomato glandular trichomes, additional feeding assays on artificial diets with a mixture of pure β-caryophyllene and α-humulene were performed (Wang et al. 2020). Indeed *M. euphorbiae* survivorship as well as investment of gel saliva and production of honeydew were reduced in a dose dependent manner by these two sesquiterpenes. In addition to affecting the aphid performance, the β-caryophyllene/α-humulene producing *S. habrochaites* accessions also displayed repellent activity towards *M. euphorbiae* in choice assays (Wang et al. 2020). Our choice assays performed with a mixture of pure β-caryophyllene and α-humulene compounds confirmed that the observed repellent effect was indeed due to these sesquiterpenes produced in tomato glandular trichomes. These results are in line with the increased attraction of aphids towards the *hairless* mutant (Figure 3.3), which only emits reduced amounts of these TPS12-derived sesquiterpenes (Table S3.1).

In summary, the results presented here, in combination with the results of the previous study (Wang et al. 2020), indicate that the TPS12-derived β-caryophyllene and α-humulene produced in glandular trichomes of *S. lycopersicum* negatively affect performance, feeding and choice behavior of *M. euphorbiae*. However, we cannot exclude that trichome-derived flavonoids might also contribute to the host plant resistance of *S. lycopersicum* against *M. euphorbiae*. While these are the first reports demonstrating that β-caryophyllene and α-humulene in tomato affect a piercing-sucking herbivore such as the potato aphid, herbivory was shown to induce the production of these sesquiterpenes in several plant species, suggesting that they also have a role in the defense against other pests. In some plants β-caryophyllene and α-humulene contribute to direct defense against herbivores by affecting their development and survival, or by acting as a repellent. For example, in *Hymenaea* tree species these sesquiterpenes increase the mortality of the beet armyworm (*Spodoptera exigua*) (Langenheim 1994) and repel leaf-cutting ants (Hubbell et al. 1983; Messer et al. 1990). In other plants, such as corn and rice, β-caryophyllene and α-

humulene are involved in indirect defense by attracting parasitoids and predators (Cheng et al. 2007; Köllner et al. 2008; Rasmann et al. 2005).

In contrast to the repellent activity of the TPS12-derived sesquiterpenes, the TPS20-derived monoterpenes produced in glandular trichomes of *S. lycopersicum* appear to be exploited as a cue for host plant orientation by aphids. Remarkably, in the olfactometer choice assays significantly less aphids oriented towards the leaf odors of the *odorless-2* mutant, affected in the formation of TPS20-derived monoterpenes, than towards the Castlemart cultivar (Figure 3.3a), suggesting a role of the TPS20-derived monoterpenes in aphid attraction. The potential of TPS20-derived monoterpenes to positively affect the choice behavior of *M. euphorbiae* was further confirmed using a mixture of pure monoterpenes (Figure 3.3b). In contrast, the addition of a mixture of pure TPS20-derived monoterpenes to an artificial feeding diet did not affect survivorship and feeding behavior of *M. euphorbiae* apterae (Figure 3.2). These results are in contrast to previous studies that had observed the effect of the *odorless-2* mutant and TPS20-derived monoterpenes on the performance and feeding of a number of other herbivores. The *odorless-2* mutant was highly susceptible to herbivory under field conditions. Both *L. decemlineata* and *M. sexta* larvae showed an increased gain of body mass when feeding on the mutant compared to control plants (Kang et al. 2010a). An increased gain of body mass was also found for *M. sexta* larvae, but not for *L. decemlineata*, when these herbivores were feeding on the *hairless* mutant which is only affected in the production of TPS12-derived sesquiterpenes (Kang et al. 2010b; Tian et al. 2012). Thus, it remains to be shown how much of the observed effect on the growth and performance of these biting-chewing herbivores is due to TPS20-derived monoterpenes or TPS12-derived sesquiterpenes, respectively. One of our earlier studies (Gutensohn et al. 2014) provided direct evidence for the role of TPS20-derived monoterpenes in the resistance against biting-chewing herbivores. When *H. zea* larvae were allowed to feed on tomato fruits that were engineered for the production of TPS20-derived monoterpenes their gain in body mass was significantly reduced compared to larvae feeding on control fruits lacking these monoterpenes. In conclusion, it appears that TPS20-derived monoterpenes in *S. lycopersicum* have distinct roles in the interaction with herbivores. While the TPS20-derived monoterpenes contribute to the defense of *S. lycopersicum* against some biting-chewing herbivores, the same monoterpenes are being exploited as a cue for host plant orientation by other pests such as *M. euphorbiae*.

3.5 Literature cited

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3.6 Figures and tables
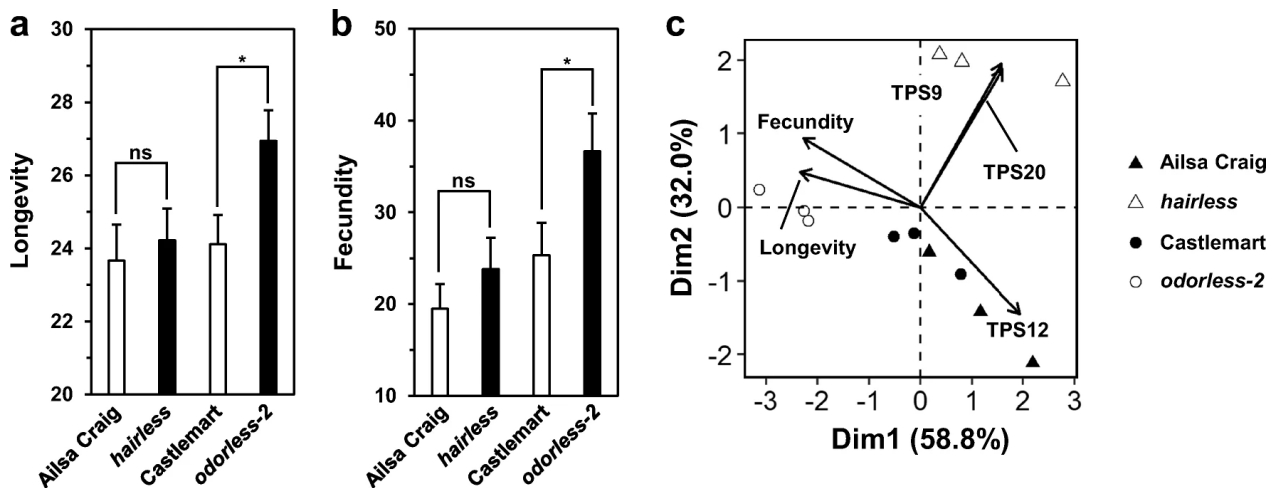
Figure 3.1 Longevity (a) and fecundity (b) of *M. euphorbiae* arrested on the leaf surface of the *hairless* and *odorless*-2 trichome mutants, and their respective wild type, Ailsa Craig and Castlemart. Values for longevity (in days) and fecundity (representing the number of offspring) are presented as means ± SE and asterisks indicate significant differences (*t*-test, $P < 0.05$). The relationship of both *M. euphorbiae* performance parameters with each terpene group found in *S. lycopersicum* glandular trichomes (TPS20-derived monoterpenes, TPS9-derived δ-elemene, and TPS12-derived sesquiterpenes) are shown in an object-variable biplot (**c**) derived from the principal component analysis of the terpene profiles and aphid performance for the four tomato lines
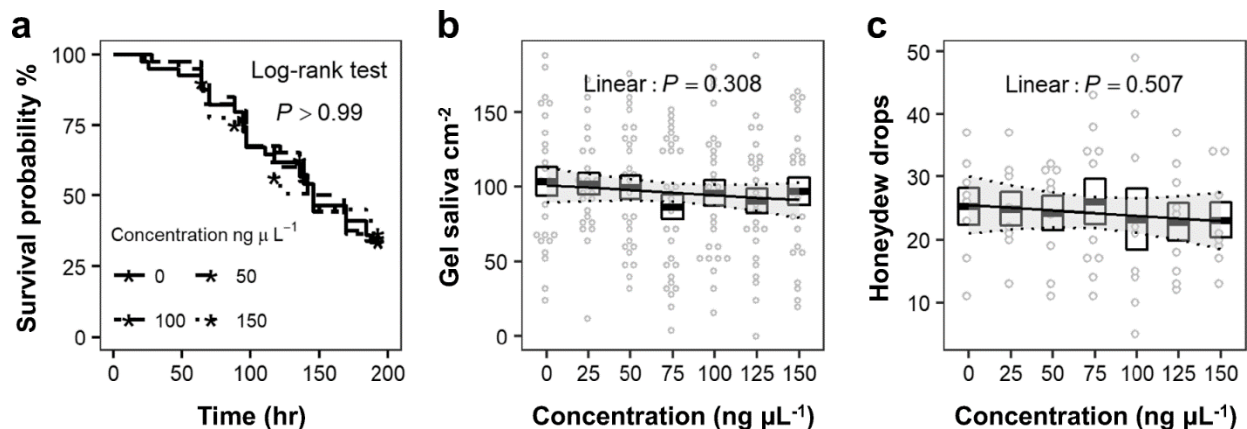
Figure 3.2 Feeding performance of *M. euphorbiae* apterae on artificial diets containing TPS20-derived monoterpenes (MTBE solvent control). Different amounts of a mix of pure β-phellandrene, δ-2-carene, and α-pinene were added to the feeding diet. (**a**) Survivorship of aphids was analyzed by Kaplan-Meier estimates followed by log-rank test ($\alpha = 0.05$). Box plots represent means ± SE of gel saliva density (cm^{-2}) (**b**) and the number of honeydew drops accumulated in the feeding chambers (**c**). Linear regressions ($\alpha = 0.05$) were used to analyze the relationship of gel saliva density and the number of honeydew drops with the concentration of monoterpenes. Shaded areas represent 95% CI for the linear regression estimation.
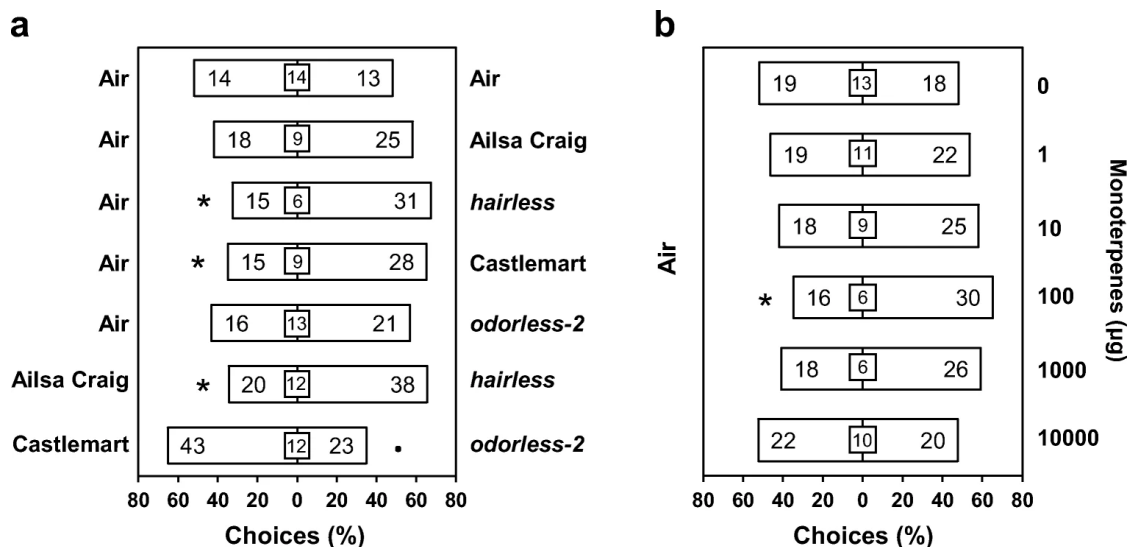
Figure 3.3 Choice behavior of *M. euphorbiae* alates in an open Y-track olfactometer. (**a**) Choice of aphids between air and odors from leaves of different *S. lycopersicum* lines, or between leaf odors of trichome mutants and respective wild type. (**b**) Choice of aphids between air and odors from different amounts of a mix of pure TPS20-derived monoterpenes added on filter paper. Numbers on the bars represent *M. euphorbiae* alates responsive to respective odors or unresponsive (center). Asterisks indicate significant differences in response frequencies according to Chi-square goodness of fit test (*, $P < 0.05$; ·, $P \leq 0.10$)

Table 3.1 Accumulation of glandular trichome derived terpenes in leaves of two *S. lycopersicum* trichome mutants and their respective wild type as determined by leaf dip extraction with methyl *tert*-butyl ether and subsequent analysis of extracts by GC/MS.

Terpene Group	Compound[a]	CAS	RI[b]	Terpene Content[c] (nmol · g FW^{-1})					
				Ailsa Craig	*hairless*	P[d]	Castlemart	*odorless-2*	P[d]
TPS20	α-pinene	80-56-8	937	0.42 ± 0.01	0.47 ± 0.15	ns	0.78 ± 0.1	-	*
	δ-2-carene	554-61-0	1001	3.39 ± 0.72	3.3 ± 0.83	ns	6.68 ± 1.02	-	*
	α-phellandrene	99-83-2	1005	-	-	ns	0.48 ± 0.08	-	*
	β-phellandrene	555-10-2	1031	19.81 ± 2.91	19.77 ± 2.77	ns	67.65 ± 3.35	0.01 ± 0.001	*
TPS9	δ-elemene	20307-84-0	1338	0.15 ± 0.04	0.13 ± 0.01	ns	1.06 ± 0.19	-	*
TPS12	β-caryophyllene	87-44-5	1419	2.29 ± 0.47	1.03 ± 0.18	*	0.82 ± 0.21	0.03 ± 0.01	*
	α-humulene	6753-98-6	1454	0.91 ± 0.07	0.37 ± 0.04	*	0.35 ± 0.08	0.02 ± 0.01	*

[a] Leaf dip extracts were analyzed by combined GC/MS using the total ion chromatogram mode. Mass spectra were scanned at a range of 30-500 (m/z) after electron ionization at 70 ev. Terpene compounds were identified by comparison of mass spectra with those deposited in the NIST/EPA/NIH MS library version 2.2 and with authentic terpene standards.
[b] Retention index median (semi-standard non-polar) according to NIST/EPA/NIH MS library version 2.2.
[c] Amounts of terpene compounds were quantified based on peak areas relative to the internal standard naphthalene using response factors determined for authentic terpene standards. Contents of terpene compounds are shown as nmol · g FW^{-1} (means ± SEM, $n = 3$)
[d] Contents of terpene compounds were compared between mutants and wild type by Student's *t*-test ($\alpha = 0.05$)

Table S3.1 Emission of glandular trichome derived terpenes from leaves of two *S. lycopersicum* trichome mutants and their respective wild type as determined by closed-loop stripping and subsequent analysis of collected terpenes by GC/MS.

Terpene Group	Compound[a]	CAS	RI[b]	Emitted Terpenes[c] (pmol · g FW^{-1} · hr^{-1})					
				Ailsa Craig	*hairless*	P[d]	Castlemart	*odorless-2*	P[d]
TPS20	α-pinene	80-56-8	937	49.32± 4.29	55.02 ± 7.22	ns	96.72 ± 15.41	0.65 ± 0.35	*
	δ-2-carene	554-61-0	1001	392.95 ± 54.01	384.04 ± 25.94	ns	793.57 ± 70.84	3.99 ± 0.7	*
	α-phellandrene	99-83-2	1005	2.32 ± 0.39	2.06 ± 0.6	ns	66.97 ± 10.96	-	*
	β-phellandrene	555-10-2	1031	2464.89 ± 287.41	2343.11 ± 203.79	ns	8499.35 ± 879.38	13.2 ± 4.23	*
TPS9	δ-elemene	20307-84-0	1338	24.29 ± 4.56	25.89 ± 3.43	ns	131.06 ± 16.94	-	*
TPS12	β-caryophyllene	87-44-5	1419	125.81 ± 17.31	59.84 ± 8.23	*	44.7 ± 3.55	4.14 ± 0.86	*
	α-humulene	6753-98-6	1454	47.97 ± 8.48	24.24 ± 2.18	*	21.97 ± 2.03	2.09 ± 0.66	*

[a] Emitted terpenes were analyzed by combined GC/MS using the total ion chromatogram mode. Mass spectra were scanned at a range of 30-500 (m/z) after electron ionization at 70 ev. Terpene compounds were identified by comparison of mass spectra with those deposited in the NIST/EPA/NIH MS library version 2.2 and with authentic terpene standards.
[b] Retention index median (semi-standard non-polar) according to NIST/EPA/NIH MS library version 2.2.
[c] Amounts of terpene compounds were quantified based on peak areas relative to the internal standard naphthalene using response factors determined for authentic terpene standards. Contents of terpene compounds are shown as pmol · g FW-1 · hr-1 (means ± SEM, n = 3).
[d] Contents of terpene compounds were compared between mutants and wild type by Student's t-test (α = 0.05).

Chapter 4: Epidermis-specific metabolic engineering of sesquiterpene formation in tomato affects the performance of potato aphid *Macrosiphum euphorbiae*

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Abstract: Tomato produces a number of terpenes in their glandular trichomes that contribute to host plant resistance against pests. While glandular trichomes of cultivated tomato *Solanum lycopersicum* primarily accumulate a blend of monoterpenes, those of the wild tomato species *Solanum habrochaites* produce various sesquiterpenes. Recently, we have identified two groups of sesquiterpenes in *S. habrochaites* accessions that negatively affect the performance and choice behavior of the potato aphid (*Macrosiphum euphorbiae*). Aphids are piercing-sucking herbivores that use their mouthpart to penetrate and probe plant tissues in order to ultimately access vascular tissue and ingest phloem sap. Because secondary metabolites produced in glandular trichomes can affect the initial steps of the aphid feeding behavior, introducing the formation of defensive terpenes into additional plant tissues *via* metabolic engineering has the potential to reduce tissue penetration by aphids and in consequence virus transmission. Here, we have developed two multicistronic expression constructs based on the two sesquiterpene traits with activity toward *M. euphorbiae* previously identified in *S. habrochaites*. Both constructs are composed of sequences encoding a prenyl transferase and a respective *S. habrochaites* terpene synthase, as well as enhanced green fluorescent protein as a visible marker. All three coding sequences were linked by short nucleotide sequences encoding the foot-and-mouth disease virus 2A self-processing oligopeptide which allows their co-expression under the control of one promoter. Transient expression of both constructs under the epidermis-specific *Arabidopsis CER5*-promoter in tomato leaves demonstrated that formation of the two sets of defensive sesquiterpenes, β-caryophyllene/α-humulene and (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene, can be introduced into new tissues in tomato. The epidermis-specific transgene expression and terpene formation were verified by fluorescence microscopy and tissue fractionation with subsequent analysis of terpene profiles, respectively. In addition, the longevity and fecundity of *M. euphorbiae* feeding on these engineered tomato leaves were significantly reduced, demonstrating the efficacy of this novel aphid control strategy.

Keywords: sesquiterpenes, metabolic engineering, multicistronic expression constructs, prenyl transferases, terpene synthases, epidermis, *Solanum lycopersicum*, potato aphid

4.1 Introduction

In nature, plants are an integral part of a complex system of antagonistic and mutualistic biotic interactions. Due to their sessile lifestyle, plants have adapted to the resulting challenges by evolving specific strategies for the defense against attacking as well as the attraction of beneficial organisms. Many of these strategies include the formation of secondary metabolites, such as volatile organic compounds (VOCs), that belong to three major categories: phenylpropanoids/benzenoids, fatty acid derivatives, and terpenes (Dudareva et al. 2013). Volatile mono- and sesquiterpenes synthesized by plants are known to contribute to the direct and indirect defense against phytophagous insects. While terpenes accumulated in plant tissues can be toxic to biting-chewing and piercing-sucking herbivores, their emission into the surrounding atmosphere can act repellent to these herbivores and attractive toward their natural enemies (Degenhardt et al. 2003; Gershenzon and Dudareva 2007; Unsicker et al. 2009). To facilitate their role in the antagonistic interactions, terpenes are often produced in specific plant tissues including internal ducts, extracellular cavities, and glandular trichomes (Gershenzon and Dudareva 2007; Zulak and Bohlmann 2010). Volatile terpenes, like all other terpenoids, are synthesized from the building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which in plants originate from two parallel pathways: the mevalonic acid (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Ashour et al. 2010; Hemmerlin et al. 2012). IPP and DMAPP are subsequently utilized by prenyl transferases to form larger *trans* and *cis* prenyl diphosphate intermediates. While geranyl diphosphate (GPP) and its *cis* isomer neryl diphosphate (NPP) are used for monoterpene synthesis, *trans-* and *cis-* farnesyl diphosphate (*E,E-* and *Z,Z-*FPP) serve as precursors for sesquiterpenes. The formation of terpenes is then catalyzed by terpene synthases (TPSs) which utilize one or several of the prenyl diphosphate substrates, and frequently have the ability to form multiple different terpene products from one prenyl diphosphate substrate (Degenhardt et al. 2009).

While defensive terpene traits are under positive selection pressure to ensure survival in wild plants, it appears that they have been compromised in cultivated crop plants since selective breeding has favored other agronomic traits (Köllner et al. 2008). Domestication and breeding have also resulted in the introduction of strong genetic bottlenecks in cultivated tomato (*Solanum lycopersicum*) which suffers from a higher susceptibility to various pests compared to wild

tomato species (Bai and Lindhout 2007). Although glandular trichomes are present on vegetative tissues of both cultivated and wild tomato species, these differ significantly in their profile of volatile terpenes. In *S. lycopersicum*, they primarily produce a blend of monoterpenes and only small amounts of a few sesquiterpenes (Schilmiller et al. 2009). In contrast, the systematic characterization of glandular trichome-derived terpenes in different accessions of the wild tomato species *Solanum habrochaites* (previously *Lycopersicon hirsutum*), for example, revealed several chemotypes characterized by the dominant formation of distinct blends of sesquiterpenes (Gonzales-Vigil et al. 2012), of which most are not present in *S. lycopersicum*. Numerous studies have shown that glandular trichome-derived terpenes found in *S. lycopersicum* as well as the *S. habrochaites* accessions have repellent and toxic activity against different biting-chewing herbivores including Colorado potato beetle (*Leptinotarsa decemlineata*), tobacco hornworm (*Manduca sexta*), tomato fruitworm (*Helicoverpa zea*), and beet armyworm (*Spodoptera exigua*; Carter et al. 1989a,b; Frelichowski and Juvik 2001; Kang et al. 2010a,b; Tian et al. 2012; Gutensohn et al. 2014). In contrast, relatively little is known about the potential effects of glandular trichome-derived terpenes in cultivated and wild tomato on piercing-sucking herbivores, such as whiteflies, spider mites, and aphids. The sesquiterpene 7-*epi*-zingiberene and some of its derivatives produced in the glandular trichomes of several *S. habrochaites* accessions were shown to have repellent and/or toxic activity against silverleaf whiteflies (*Bemisia tabaci*) and two spotted-spider mites (*Tetranychus urticae*) (Bleeker et al. 2011a, 2012; Dawood and Snyder 2020; Zabel et al. 2021). Utilizing a collection of *S. habrochaites* accessions with different glandular trichome-derived terpenes, we have recently identified two groups of sesquiterpenes that affect the potato aphid (*Macrosiphum euphorbiae*) (Wang et al. 2020). *S. habrochaites* accessions producing β-caryophyllene and α-humulene, or (−)-*endo*-α-bergamotene, (+)-α-santalene, and (+)-*endo*-β-bergamotene, respectively, not only reduced the aphid longevity and fecundity significantly, but also had repellent activity against *M. euphorbiae*. Remarkably, by utilizing two tomato trichome mutants, *hairless* and *odorless-2*, that are differently affected in mono- and sesquiterpene production (Kang et al. 2010a,b), we demonstrated that the relatively small amounts of β-caryophyllene and α-humulene in glandular trichomes of cultivated tomato still have some effect on the performance of *M. euphorbiae* (Wang et al. 2021). However, the same analysis also suggested a role of the highly

abundant TPS20-derived monoterpenes in the attraction of aphids to cultivated tomato, which was further confirmed using a mixture of pure monoterpenes (Wang et al. 2021).

Macrosiphum euphorbiae is an important agricultural pest that causes economic losses to horticultural crop production including tomato (Tomescu and Negru 2003). Damage is caused not only by the direct feeding of *M. euphorbiae*, but even more by the transmission of multiple non-persistent and persistent viruses for which these aphids serve as vectors (Blackman and Eastop 2000; van Emden and Harrington 2007). This results in reduced crop yield and quality, and often plant death even at low levels of aphid infestation (Lange and Bronson 1981). Current aphid control strategies utilizing synthetic insecticides are increasingly inefficient due to emerging resistances and avoidance behavior (Silva et al. 2012; Bass et al. 2014; Fray et al. 2014). Thus, introducing the production of the two groups of sesquiterpenes that we have identified in the *S. habrochaites* accessions and that affected choice behavior and performance of *M. euphorbiae* (Wang et al. 2020) into cultivated tomato represents a promising approach toward developing a sustainable aphid control strategy. One possible way for the introduction of these defensive terpene traits is the classical genetic approach by crossing *S. lycopersicum* with a respective *S. habrochaites* accession, followed by several backcrosses into the cultivated tomato background to create an introgression line carrying the terpene trait from *S. habrochaites*. Indeed, we have used a near isogenic line containing a small *S. habrochaites* introgression on chromosome 8 (van der Hoeven et al. 2000) that carries the genes encoding the respective prenyl transferase and terpene synthase, and was found to produce (−)-*endo*-α-bergamotene, (+)-α-santalene and (+)-*endo*-β-bergamotene (Sallaud et al. 2009). Our assays performed with this line confirmed that introgression of (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene formation into the cultivated tomato background had successfully transferred this defensive trait affecting performance and choice behavior of *M. euphorbiae* (Wang et al. 2020). However, compared to the parental *S. habrochaites* accession the amounts of (−)-*endo*-α-bergamotene, (+)-α-santalene, and (+)-*endo*-β-bergamotene, and in consequence, the effects on *M. euphorbiae* were significantly lower in the near isogenic line (Wang et al. 2020) highlighting a potential limitation of this classical genetic approach. The observed difference in sesquiterpene production is likely due to a higher expression level of MVA and MEP pathway genes (Besser et al. 2009) and a larger storage capacity in the internal cavity of the glandular trichomes (Therezan et al. 2021) in the parental *S. habrochaites* accession compared to the

introgression line. The described classical genetic approach limits the introduction of defensive terpene traits into cultivated tomato to the tissue(s) where the respective biosynthetic genes are expressed in wild tomato, for example, glandular trichomes. In contrast, metabolic engineering represents an efficient approach to introduce a respective biosynthetic pathway into a plant tissue and/or species naturally devoid of a terpene compound of interest. Multiple examples for successful metabolic engineering of terpenes in different plant species have been reported (summarized in Lange and Ahkami 2013; Vickers et al. 2014) with three types of genes being used individually or in combination: MVA/MEP pathway, prenyl transferase, and TPS genes. Two of our previous metabolic engineering studies in tomato (Gutensohn et al. 2013, 2014) demonstrated that co-expression of prenyl transferases and respective TPSs utilizing the produced prenyl diphosphates resulted in the formation of significant amounts of the expected terpenes. To avoid potential negative effects on plant growth and performance recent engineering strategies utilized specific promoters to restrict the expression of terpene biosynthetic genes to particular plant tissues or organs (Lewinsohn et al. 2001; Davidovich-Rikanati et al. 2007, 2008; Morris et al. 2011; Bleeker et al. 2012; Borghi and Xie 2016).

Aphids as piercing-sucking herbivores use their stylet, a specialized mouthpart, to ultimately access vascular tissue and ingest phloem sap. However, to achieve sustained phloem sap ingestion, the feeding behavior of aphids progresses through several stages (Powell et al. 2006): (i) pre-alighting behavior, (ii) initial plant contact and assessment of surface cues before stylet insertion, (iii) probing of the plant epidermis, (iv) stylet pathway activity, (v) sieve element puncture and salivation, and (vi) phloem acceptance and ingestion. Blocking or hindering these feeding behaviors could lead to the development of a novel aphid control strategy. Terpenes produced in glandular trichomes of tomato are likely only affecting the initial steps of the aphid feeding behavior. However, introducing the formation of defensive terpenes into additional plant tissues *via* metabolic engineering could have the potential to reduce or even eliminate plant tissue penetration by aphids and in consequence virus transmission. In this study, we have developed two multicistronic expression constructs based on the two sesquiterpene traits previously identified in *S. habrochaites* (Wang et al. 2020) that are composed of the coding sequences for prenyl transferases and respective *S. habrochaites* terpene synthases, as well as enhanced green fluorescent protein as a visible marker. Transient expression of both constructs under the epidermis-specific *CER5*-promoter in tomato leaves demonstrated that formation of the

two sets of defensive sesquiterpenes, β-caryophyllene/α-humulene and (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene, can be introduced into new tissues in tomato. The epidermis-specific transgene expression and terpene formation were verified by fluorescence microscopy and tissue fractionation with subsequent analysis of terpene profiles, respectively. In addition, the longevity and fecundity of *M. euphorbiae* that fed on these engineered tomato leaves were significantly reduced, demonstrating the efficacy of this novel aphid control strategy.

4.2 Materials and methods

4.2.1 Plant material

Seeds of the tomato (*S. lycopersicum*) trichome mutant *odorless-2* (Kang et al. 2010a) were kindly provided by Dr. Gregg Howe (Michigan State University, MI, United States). Tomato plants used for *Agrobacterium* leaf infiltration and all subsequent analyses were grown from seeds in Sungro® soil mixture (Sun Gro Horticulture, Agawam, MA, United States) in multi-trays (288 cells, 5 ml per cell), and seedlings were transplanted into 4-inch square pots. Plants were grown under a 16-h photoperiod in a climate-controlled growth room (23–25°C, 50–60% relative humidity) without pesticide application.

4.2.2 Cloning and plasmid construction

For the cloning of the two multicistronic gene constructs under the control of a tissue-specific promoter, the binary vector pMCS:GW was used (obtained from the Arabidopsis Biological Resource Center, stock # CD3-1933) that contains a multiple cloning site upstream of the Gateway cassette (Michniewicz et al. 2015). To obtain the promoter region of the *AtCER5* gene (At1g51500; Pighin et al. 2004), a 2,614 base pair fragment was amplified by PCR from *Arabidopsis thaliana* genomic DNA using Taq polymerase (GenScript, Piscataway, NJ, United States) and a pair of oligonucleotides each carrying a specific restriction site (*AtCER5*-fwd-*Eco*RI: 5'-CGGAATTCTTTAGTTTGCTTG AGTTCTCATG-3'; *AtCER5*-rev-*Sal*I: 5'-GCGTCGACTGTTTT TGTTTGATCTTGAAAAAGATC-3'). The resulting PCR product was cloned into the vector pMD20 (Takara Bio, United States) and sequenced to verify its correct amplification. The *AtCER5* promoter fragment was subsequently excised from the pMD20 vector

by *Eco*RI and *Sal*I digestion, and then ligated between the *Eco*RI and *Xho*I sites of the pMCS:GW vector.

The two multicistronic gene constructs were obtained by gene synthesis (Twist Bioscience, San Francisco, CA, United States). These gene constructs were flanked by attL-sequences and inserted into the Gateway Entry vector pTwist ENTR (Twist Bioscience, San Francisco, CA, United States). Both of these multicistronic constructs contained three consecutive coding regions encoding a prenyltransferase, a terpene synthase, and enhanced green fluorescent protein, respectively. Stop codons of the prenyl transferase and terpene synthase coding sequences were removed and instead 60-bp wild type F2A sequences (5'- CAGCTGTTGAATTTTGACCTT CTTAAGCTTGCGGGAGACGTCGAGTCCAACCCTGGG CCC-3'; Ryan et al. 1991; Kenneth et al. 2012) inserted such that the three coding regions were linked into one ORF. In addition, the 5'-UTR sequence of the *A. thaliana Farnesyl Diposphate Synthase 2 AtFPPS2* gene (At5g47770) was fused upstream of the start codon of the prenyl transferase coding sequence in both gene constructs.

Each of the two multicistronic gene constructs was transferred from the pTwist ENTR vector into the pMCS:GW vector carrying the *AtCER5* promoter fragment by performing a standard LR recombination reaction. One μl of the destination vector (150 ng/μl) and 0.5 μl of the entry vector (30 ng/μl) were mixed with 6.5 μl TE buffer and 2 μl LR Clonase II Plus enzyme mix (Invitrogen, Thermo Fisher Scientific), and incubated at 25°C for 1 h. After the addition of 1 μl of proteinase K (Invitrogen, Thermo Fisher Scientific), reactions were incubated at 37°C for 10 min. Aliquots of each LR reaction were transformed into competent *Escherichia coli* DH5α cells (Thermo Fisher Scientific) and colonies with recombinant plasmids selected on LB agar plates with kanamycin (100 μg/ml).

4.2.3 Agrobacterium leaf infiltration

The resulting binary pMCS:GW vectors carrying the multicistronic gene constructs under the control of the *AtCER5* promoter were introduced into *Agrobacterium tumefaciens* (strain GV3101), and those were subsequently used for transient transformation *via* infiltration of tomato leaves as described previously (Norkunas et al. 2018). Selected *Agrobacterium* clones were grown in LB medium containing kanamycin (100 μg/ml), rifampicin (25 μg/ml), and gentamycin (50 μg/ml) to an OD_{600} of 0.8–1.0. Bacterial cultures were harvested by

centrifugation and resuspended in infiltration buffer [10 mm MES-KOH (pH 5.7), 10 mm $MgCl_2$ and 200 μm acetosyringone]. *Agrobacterium* suspensions were adjusted to an OD_{600} of 0.6–0.8 and further incubated at room temperature for 2 h prior to leaf infiltration. *Agrobacterium*-mediated transient transformation was subsequently conducted on the second and older true leaves of 4-week-old *odorless-2* tomato plants. The *Agrobacterium* suspension was injected into leaves from the abaxial side using a 5 ml plastic syringe (without hypodermic needle) thus infiltrating the intercellular spaces of the entire leaves. After the infiltration plants were covered by a humidity dome and continued to grow under a 16-h photoperiod at 23–25°C until further analysis.

4.2.4 RNA extraction and RT-PCR analysis

Six days after the *Agrobacterium* infiltration total RNA was isolated from infiltrated tomato leaves as previously described (Eggermont et al. 1996). For the RT-PCR analyses, total RNA was pretreated with RNase-free DNase (New England Biolabs, Ipswich, MA, United States) and cDNA was synthesized using reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA, United States). To evaluate the expression of both multicistronic gene constructs, the cDNA was subsequently used for PCR utilizing three primer pairs specific for: *AtFPPS2* (*AtFPPS*-fwd: 5'-CGGATCTGAAATCAACCTTCCTCGAC-3'; *AtFPPS*-rev: 5'-CAATGCCTTAACTACCAACCAGGAGC-3'), *ShzFPPS* (*ShzFPPS*-fwd: 5' -CAAATTCACCTCTGACAGTGTCTGC-3'; *ShzFPPS*-rev: 5'-GTGTGTCCACCAAAACGTCTATGCC-3'), and *eGFP* (*eGFP*-fwd: 5'-CGACGTAAACGGCCACAAGTT CA-3'; *eGFP*-rev: 5'- ACTTGTACAGCTCGTCCATGCC-3'). The PCR conditions were as follows: 94°C for 5 min for one cycle, followed by 35 cycles of 95°C for 60 s, 57°C for 60 s and 72°C for 60 s, and a final extension at 72°C for 10 min. The amplification products were separated by agarose gel electrophoresis, stained with GelRed® (Biotium, United States), and analyzed using the ChemiDoc Gel Imaging System and Image Lab 5.1 software (Bio-Rad, Hercules, CA, United States).

4.2.5 Fluorescence microscopy

Four to five days after *Agrobacterium* infiltration tomato leaves were collected, and cross- and surface sections were prepared. Leaf cross- and surface sections were analyzed with a Zeiss Axio Imager M1 compound microscope (Zeiss, Oberkochen, Germany) equipped with an X-Cite

series 120Q fluorescent illuminator. Fluorescence microscopy was performed using a 485/20 excitation filter in combination with a green filter cube, and a 545/25 excitation filter in combination with a red filter cube for the analysis of GFP fluorescence and chlorophyll autofluorescence, respectively. All images were captured using a monochrome AxioCam MRm camera mounted on the Imager M1 microscope and further processed with the AxioVision software. Composite images from GFP and chlorophyll fluorescence microscopy were overlaid using the ImageJ software (National Institutes of Health).[1]

4.2.6 Extraction of terpenes from whole tomato leaves

Tomato leaves were collected at different time points (0, 3, 6, 9, 12, and 15 days) after the *Agrobacterium* infiltration. Leaves were photographed, and their surface areas were determined using the ImageJ software (National Institutes of Health, see footnote 1). Leaves were stirred in 50 ml of methyl *tert*-butyl ether (MTBE) for 20 min to extract terpenes as described previously (Gutensohn et al. 2013). After removing the leaves, extracts were concentrated under a gentle stream of nitrogen gas to a volume of 200 µl and centrifuged for further purification. For the analysis of terpene profiles, extracts were transferred into GC vials and supplemented with 3.33 µg of naphthalene as an internal standard.

4.2.7 Extraction of terpenes from isolated trichomes, epidermis, vasculature, and mesophyll from tomato leaves

To further investigate the accumulation of terpenes in different leaf tissues, glandular trichomes, epidermis, vasculature, and mesophyll were isolated from tomato leaves 15 days after *Agrobacterium* infiltration. Type VI glandular trichomes were collected from tomato leaves as described previously (Schilmiller et al. 2009) using a stretched Pasteur pipette under a stereomicroscope (SZ-ST, Olympus, Tokyo, Japan). A total of 200 trichomes from each leaf sample were accumulated into 10 ml of MTBE for the extraction of terpenes. For the isolation of the other leaf tissues, a protocol was adapted from previous studies (Endo et al. 2016; Svozil et al. 2016). Entire tomato leaflets were sandwiched between two layers of clear scotch tape, and the abaxial leaf epidermis was peeled by gently pulling off one tape. The tape with the attached abaxial leaf epidermis was immediately transferred into 10 ml MTBE for terpene extraction. The other layer of tape with the remainder of the leaf was transferred into a 50 ml tube containing 15 ml of enzyme solution [1.00% (w/v) cellulase R-10, 0.25% (w/v) macerozyme R-10, 0.4 M

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mannitol, 8 mm CaCl2, and 5 mm MES-KOH, pH 5.7]. After a 10 min incubation, the leaf vasculature was isolated by using a dissecting needle and transferred into 10 ml MTBE. The remaining leaf tissue was further digested for an additional 15 min for removing mesophyll cells into the enzyme solution. Subsequently, the tape with the attached adaxial leaf epidermis was transferred into the vial which already contained the tape with the abaxial leaf epidermis. The mesophyll cells isolated by the enzyme treatment were further purified using a 30 μm cell strainer (pluriSelect, Leipzig, Germany), centrifuged at 1,000 g for 5 min, and resuspended into 10 ml MTBE. All isolated leaf tissue fractions were allowed to shake in MTBE at 100 rpm for 30 min for terpene extraction. All extracts were concentrated under a gentle stream of nitrogen gas to a volume of 200 μl and centrifuged for further purification. Concentrated and purified extracts were transferred into GC vials and supplemented with 3.33 μg of naphthalene as an internal standard.

4.2.8 Gas chromatography–mass spectrometry analysis

All extracts from entire tomato leaves and leaf tissue fractions were analyzed by combined gas chromatography–mass spectrometry (GC–MS) using a TRACE 1310 gas chromatograph system linked to a TSQ 8000 Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States). Two μl of each sample was injected under a spitless mode, volatilized at 220°C, and then separated on a TraceGOLD TG-5MS GC column (30 m length, 0.25 mm I.D., and 0.25 μm film; Thermo Fisher Scientific, Pittsburgh, PA, United States). The initial column temperature was held at 40°C for 3 min and then ramped at 5°C/min to 120°C, 10°C/min to 180°C, and 20°C/min to 300°C which was maintained for 2 min. The helium carrier gas flow was 1.3 ml/min. All samples were analyzed using the total ion chromatogram (TIC) mode. Individual terpene compounds were identified by comparing their mass spectra (15–300 m/z) with those deposited in the NIST/EPA/NIH Mass Spectral Library (NIST11; National Institute of Standards and Technology NIST, Scientific Instrument Services, Inc., NJ, United States),[2] as well as those reported previously (Sallaud et al. 2009). Terpenes identified in the leaf and leaf tissue extracts were quantified using a previously determined average response factor for sesquiterpenes (Wang et al. 2020) in combination with the internal naphthalene standard.

4.2.9 Aphid culture

A potato aphid (*M. euphorbiae*) colony was established from apterae collected in the WVU Evansdale Greenhouse (Morgantown, WV, United States). To avoid experience on tomato plants prior to the non-choice assays on *odorless-2* plants, aphids were allowed to reproduce parthenogenetically on potted potato plants in an insect rearing room under a 16-h photoperiod at 20–22°C. The aphid species was confirmed through barcode sequencing.

4.2.10 Aphid non-choice assays

The performance (longevity and fecundity) of *M. euphorbiae* apterae on agroinfiltrated leaves of *odorless-2* tomato plants was determined in a climate-controlled growth room (23–25°C, 50–60% relative humidity, and 16-h photoperiod) as described previously (Eichele-Nelson et al. 2018; Wang et al. 2020). Before the assay apterae (F_0) reared on potato plants were introduced on the leaf surface of *odorless-2* plants. Four days after the introduction three neonate F_1 nymphs (considered as 1 day old) were carefully transferred to the surface of a young *odorless-2* leaf (second or third fully expanded leaf) which had been infiltrated with *Agrobacterium* 2 days earlier, and subsequently enclosed in a clip cage (BioQuip Products, Rancho Dominguez, CA, United States) that was attached to the leaf. Four tomato plants each with three clip cages attached (in total 12 clip cages) were used for each of the treatments (two expression constructs and one control). Over the course of the experiment F_2 nymphs and exuviae were removed daily from the clip cages. The longevity of F_1 nymphs and their fecundity represented by the number of F_2 nymphs in each cage were recorded. The longevity and fecundity of aphids were analyzed by one-way ANOVA followed by multiple comparisons using Tukey's HSD ($\alpha = 0.05$).

4.3 Results

4.3.1 Design of two multicistronic expression constructs for epidermis-specific engineering of sesquiterpene formation

To engineer formation of the two groups of sesquiterpenes with activity against *M. euphorbiae* that we have identified in *S. habrochaites* (Wang et al. 2020) into the epidermis of *S. lycopersicum*, we have designed two multicistronic expression constructs (Figure 4.1) in the binary vector pMCS:GW (Michniewicz et al. 2015). Both constructs were put under the control

of the promoter of the *AtCER5* gene (At1g51500; Figure 4.1), encoding a plasma membrane localized ABC transporter involved in the export of cuticular wax, that was shown to direct epidermis-specific expression (Pighin et al. 2004). To achieve high levels of terpene formation, we opted to co-express each of the selected *S. habrochaites* terpene synthases together with a respective prenyl transferase which assures sufficient availability of the required prenyl diphosphate substrate in the correct subcellular compartment. Despite the significant difference in β-caryophyllene/α-humulene formation between cultivated and wild tomato accessions (Wang et al. 2020), in both tomato species these sesquiterpenes are produced by Terpene Synthase 12 (TPS12) utilizing *E,E*-FPP as substrate (Schilmiller et al. 2010; Bleeker et al. 2011b). Thus, for the design of the respective expression construct (Figure 4.1), the coding sequence for the *S. habrochaites Terpene Synthase 12* (*ShTPS12*, GenBank accession JN402389; Bleeker et al. 2011b) was paired with that for the *A. thaliana Farnesyl Diphosphate Synthase 2* (*AtFPPS2*, At4g17190; Keim et al. 2012). The terpene synthase responsible for the formation of (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene in some *S. habrochaites* accessions, Santalene and Bergamotene Synthase (ShSBS), accepts *Z,Z*-FPP as substrate which is synthesized by *cis*-Farnesyl Diphosphate Synthase (ShzFPPS; Sallaud et al. 2009). In contrast to AtFPPS2 and ShTPS12, which are localized in the cytosol, ShzFPPS and ShSBS both carry N-terminal transit peptides that target them toward plastids. Thus, for the design of the second expression construct (Figure 4.1), the coding sequences of *ShSBS* (GenBank accession FJ194970) and *ShzFPPS* (GenBank accession FJ194969) (Sallaud et al. 2009) were paired. To obtain coordinated and stable expression of the multiple transgenes under the control of the *AtCER5* promoter, the open reading frames encoding the terpene synthase and prenyl transferase in both expression constructs (Figure 4.1) were linked by a short 60 bp nucleotide sequence encoding the foot-and-mouth disease virus 2A oligopeptide (F2A; Ryan et al. 1991; Kenneth et al. 2012). This F2A sequence represents a self-processing peptide that *via* a ribosome skipping mechanism during the translation process leads to the separation between the upstream polypeptide ending with the C-terminal 2A sequence and the next translation product downstream (Ryan and Flint 1997; Donnelly et al. 2001). As third part, the coding region of *enhanced green fluorescent protein* (*eGFP*) was added to both multicistronic expression constructs (Figure 4.1) and was likewise linked by an F2A sequence to the 3' end of the terpene synthases, *ShTPS12* and *ShSBS*, respectively. In summary, both constructs (Figure 4.1), the pC5-

FTG construct (*AtCER5P-AtFPPS-ShTPS12-eGFP*) and the pC5-zFSG construct (*AtCER5P-ShzFPPS-ShSBS-eGFP*), will result in the formation of three separate proteins upon expression *in planta*: a prenyl transferase and terpene synthase pair catalyzing the synthesis of the desired sesquiterpenes, as well as eGFP that will serve as a visual marker of the epidermis-specific expression.

4.3.2 Transient expression of the multicistronic constructs in tomato leaves

We tested the function of the newly designed multicistronic expression constructs by infiltrating leaves of cultivated tomato with *Agrobacterium* carrying the pMCS binary vector with the inserted pC5-FTG and pC5-zFSG constructs (Figure 4.1), respectively, as well as the empty pMCS vector as a negative control. For these transient transformation assays, we used the tomato trichome mutant *odorless-2* (Kang et al. 2010a) since it is deficient in the formation of TPS20-derived monoterpenes and TPS12-derived sesquiterpenes that are naturally found in *S. lycopersicum* trichomes and thus could interfere with the analysis of the engineered sesquiterpenes. To determine the expression of both multicistronic constructs upon transient transformation of the tomato leaves reverse transcription-PCR (RT-PCR) analyses were performed using primer pairs (Figure 4.1) specific for the first coding region (*AtFPPS* and *ShzFPPS*) and the third coding region (*eGFP*) in the pC5-FTG and pC5-zFSG constructs, respectively. An *AtFPPS* specific 788 base pair cDNA fragment could be amplified from leaves infiltrated with *Agrobacterium* carrying the pC5-FTG construct (Figure 4.2A). However, this *AtFPPS* fragment was not detected with untransformed *odorless-2* control leaves or leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector and the pC5-zFSG construct, respectively. In contrast, a *ShzFPPS*-specific 794 base pair cDNA fragment was only amplified from leaves infiltrated with *Agrobacterium* carrying the pC5-zFSG construct (Figure 4.2B) but was absent from the *odorless-2* and empty vector controls as well as leaves infiltrated with *Agrobacterium* carrying the pC5-FTG construct. The *eGFP*-specific 656 base pair cDNA fragment could be amplified from leaves infiltrated with *Agrobacterium* carrying either the pC5-FTG or pC5-zFSG construct (Figure 4.2C) but was not found with the *odorless-2* and empty vector controls. The fact that transcripts of the prenyl transferases and *eGFP* representing the first and last coding region in both constructs were detected by RT-PCR upon the transient

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transformation of tomato leaves (Figure 4.2) suggests that the entire multicistronic constructs were expressed under the control of the *AtCER5* promoter.

4.3.3 Epidermis-specific expression of the multicistronic constructs

While the RT-PCR analysis (Figure 4.2) in general demonstrated expression of the multicistronic constructs in tomato leaves upon transient transformation, the tissue specificity of their expression under the control of the *AtCER5* promoter remained to be shown. Toward this goal, we performed confocal fluorescence microscopy of tomato leaves that had been infiltrated with *Agrobacterium* carrying the empty pMCS vector, pC5-FTG construct, and pC5-zFSG construct, respectively, to determine the tissue-specific accumulation of eGFP which is encoded in both multicistronic constructs. When cross-sections of tomato leaves were analyzed, GFP fluorescence was exclusively detected in both epidermal layers of leaves transiently transformed with the pC5-FTG and pC5-zFSG constructs (Figure 4.3A), while no respective fluorescence was observed with the empty vector control. Moreover, the GFP fluorescence did not overlap with the chlorophyll fluorescence detected in the chloroplast containing parenchyma cells of the leaf cross-sections (Figure 4.3A). In addition, we analyzed surface sections of the transiently transformed tomato leaves by fluorescence microscopy to further verify the expression of *eGFP* in epidermis cells. Upon the transient expression of the pC5-FTG and pC5-zFSG constructs in tomato leaves, GFP fluorescence could be observed in the cytosolic rim of the epidermal pavement cells (Figure 4.3B). The results of these fluorescence microscopy analyses not only provide further evidence that the entire pC5-FTG and pC5-zFSG constructs including *eGFP* are expressed, but also indicate that their expression under the *AtCER5* promoter is indeed restricted to the epidermis of transformed tomato leaves.

4.3.4 Sesquiterpene formation in the epidermis of tomato leaves transiently expressing the pC5-FTG and pC5-zFSG constructs

To determine if the transient expression of the multicistronic constructs, both encoding pairs of prenyl transferases and terpene synthases, in the leaf epidermis, resulted in the formation of the expected sesquiterpenes, tomato leaves were extracted with methyl *tert*-butyl ether (MTBE) 15 days after *Agrobacterium* infiltration. The subsequent analysis of the leaf extracts by combined gas chromatography–mass spectrometry (GC–MS) demonstrated that leaves of the *odorless-2* tomato mutant expressing the pC5-FTG construct had accumulated the expected

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ShTPS12 products β-caryophyllene and α-humulene (Figure 4.4A; Figure S4.1). A similar analysis of leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector found no β-caryophyllene and α-humulene accumulation (Figure 4.4C) which is in line with the previous characterization of the *odorless-2* tomato mutant (Kang et al. 2010a; Wang et al. 2021) showing the absence of these two sesquiterpenes in this trichome mutant. In contrast, the analysis of tomato leaves expressing the pC5-zFSG construct revealed a different profile of accumulated terpenes (Figure 4.4B; Figure S4.2) including (−)-*endo*-α-bergamotene, (+)-α-santalene, (−)-*exo*-α-bergamotene, (−)-*epi*-β-santalene, and (+)-*endo*-β-bergamotene. These five sesquiterpenes have been observed previously in *in vitro* enzyme assays as well as in a transgenic tobacco line as products of ShSBS when Z,Z-FPP was provided as substrate by ShzFPPS (Sallaud et al. 2009).

The quantitative analysis of the terpene accumulation in tomato leaves expressing the pC5-FTG and pC5-zFSG constructs showed that the ShTPS12- and ShSBS-derived sesquiterpene products, respectively, could be detected for the first time 6 days after the *Agrobacterium* infiltration (Figures 4.4D,E; Table S4.1). Subsequently, the amounts of the sesquiterpene products in the tomato leaves continued to increase until 12 days after the *Agrobacterium* infiltration and appeared to remain constant afterward (Figures 4.4D,E; Table S4.1). Remarkably, the total amount of sesquiterpenes produced after 15 days in leaves expressing the plastid localized ShzFPPS and ShSBS were 3.1-fold higher than in leaves expressing the cytosolic AtFPPS and ShTPS12 (Figures 4.4D,E; Table S4.1).

To further verify the tissue specificity of the novel metabolic engineering approach described here, we studied the accumulation of sesquiterpenes in different tissues of tomato leaves transiently expressing the pC5-FTG and pC5-zFSG constructs. Fifteen days after *Agrobacterium* infiltration tomato leaves were separated into epidermis, mesophyll and vasculature fractions which were subsequently extracted with MTBE and analyzed for their terpene content by GC–MS. The ShTPS12-derived sesquiterpenes β-caryophyllene and α-humulene were found in the epidermis and mesophyll fractions of tomato leaves expressing the pC5-FTG construct (Table 4.1), while they were absent in the vasculature. Likewise, three of the ShSBS-derived sesquiterpenes, (−)-*endo*-α-bergamotene, (+)-α-santalene, and (+)-*endo*-β-bergamotene, were found in the epidermis fraction of leaves expressing the pC5-zFSG construct

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(Table 4.1), while only (+)-α-santalene and (+)-*endo*-β-bergamotene were detected in the mesophyll fraction and no ShSBS-derived sesquiterpenes were present in the vasculature of these leaves. In addition to the epidermis, mesophyll, and vasculature fractions, we also analyzed the terpene content of glandular trichomes collected from tomato leaves expressing the pC5-FTG and pC5-zFSG constructs, however, did not observe any accumulation of ShTPS12- and ShSBS-derived sesquiterpenes, respectively (Table 4.1). In summary, these analyses revealed that the vast majority of the ShTPS12- and ShSBS-derived sesquiterpenes, 92.99 and 92.24%, respectively, accumulate in the epidermis (Table 4.1) of the transiently transformed tomato leaves, thus indicating that expression of the pC5-FTG and pC5-zFSG constructs under the control of the *AtCER5* promoter indeed results in the epidermis-specific production of the engineered sesquiterpenes.

4.3.5 Engineered sesquiterpene formation in the epidermis affects the longevity and fecundity of aphids

As a first approach to characterize the potential of the sesquiterpene formation engineered into the leaf epidermis to affect the potato aphid (*M. euphorbiae*), we performed non-choice assays utilizing tomato leaves that transiently express the pC5-FTG and pC5-zFSG constructs. Newly emerged *M. euphorbiae* nymphs were reared in clip cages on the surface of tomato leaves that previously have been infiltrated with *Agrobacterium* carrying the pC5-FTG and pC5-zFSG constructs or the empty pMCS vector control, and their longevity and fecundity (represented by the number of offspring) were determined. Compared to the non-infiltrated *odorless-2* control, infiltration of leaves with *Agrobacterium* carrying the empty pMCS vector did not significantly affect longevity ($t = 0.201$, $p = 0.997$) or fecundity ($t = 0.368$, $p = 0.983$) of *M. euphorbiae*. In contrast, the longevity of *M. euphorbiae* on tomato leaves expressing the pC5-FTG construct (20.39 ± 0.55 days), characterized by β-caryophyllene and α-humulene production in their epidermis (Figure 4.4; Table 4.1), was significantly decreased ($t = 5.420$, $p = 0.001$) compared to that on leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector (22.78 ± 0.53 days; Figure 4.5A). The longevity of *M. euphorbiae* (Figure 4.5A) on tomato leaves expressing the pC5-zFSG construct (18.33 ± 0.65 days), which accumulated the ShSBS-derived sesquiterpenes in their epidermis (Figure 4.4; Table 4.1), was also significantly decreased ($t = 2.678$, $p = 0.048$) compared to that on leaves infiltrated with the empty pMCS

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vector control, and even further decreased compared to that on leaves expressing the pC5-FTG construct ($t = 2.742$, $p = 0.035$). Similar effects as observed for the longevity were also found for the fecundity of *M. euphorbiae* (Figure 4.5B) on tomato leaves expressing the pC5-FTG and pC5-zFSG constructs, while their fecundity on leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector was not significantly affected ($t = 0.368$, $p = 0.983$). The number of *M. euphorbiae* offspring was significantly reduced ($t = 3.160$, $p = 0.015$) on leaves expressing the pC5-zFSG construct (17.83 ± 2.01 nymphs) compared to leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector (25.33 ± 1.94 nymphs; Figure 4.5B). A similar trend toward a reduced number of offspring (20.83 ± 1.86 nymphs) was observed with *M. euphorbiae* on leaves expressing the pC5-FTG construct (Figure 4.5B), although their fecundity was not significantly different to that of aphids on leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector ($t = 1.679$, $p = 0.347$) or the pC5-zFSG construct ($t = 1.481$, $p = 0.457$).

4.4 Discussion

It is well known that wild tomato species, such as *S. habrochaites*, have glandular trichome-derived resistance traits against numerous pests (Simmons and Gurr 2005). In particular, some terpenes produced in the glandular trichomes of wild tomato accessions have been shown to act repellent and/or toxic against pests (Carter et al. 1989a,b; Frelichowski and Juvik 2001; Bleeker et al. 2009, 2011a). In our previous study (Wang et al. 2020), we have identified two groups of *S. habrochaites* accessions producing β-caryophyllene/α-humulene and (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene, respectively, that significantly reduced the longevity and fecundity of *M. euphorbiae*, and also had repellent activity against the aphids. Thus, introducing these defensive sesquiterpene traits identified in *S. habrochaites* into cultivated tomato represents a logical step toward developing a novel aphid control strategy. One avenue toward achieving this goal is the classical genetic approach by crossing *S. lycopersicum* and respective *S. habrochaites* accessions, followed by backcrosses into the cultivated tomato background to obtain an introgression line carrying the *S. habrochaites* sesquiterpene trait. A near isogenic tomato line with a small *S. habrochaites* introgression carrying the *ShzFPPS* and *ShSBS* genes was previously isolated (van der Hoeven et al. 2000) and found to produce (−)-*endo*-α-bergamotene, (+)-α-santalene, and (+)-*endo*-β-bergamotene (Sallaud et al. 2009). While our assays demonstrated that introgression of

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the (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene formation into the cultivated tomato background indeed affected the performance and choice behavior of *M. euphorbiae*, it became obvious that the sesquiterpene levels and the resulting effects on *M. euphorbiae* were significantly lower in the introgression line (Wang et al. 2020). In contrast to the limitations of the genetic approach, metabolic engineering has been shown to offer an efficient approach to introduce the biosynthesis of terpene compounds of interest into plants (Lange and Ahkami 2013; Vickers et al. 2014) that in addition can be steered toward specific tissues through the choice of respective promoters. To engineer high levels of terpene formation in many cases multiple biosynthetic genes have to be introduced into the host plant including MVA/MEP pathway, prenyl transferase, and terpene synthase genes. However, the introduction of multiple individual transgenes and their combination in one plant line through subsequent crosses is a time-consuming process. In addition, the stacking of several transgenes that are all expressed under the identical type of promoter bears the risk of gene silencing. In contrast, the utilization of the viral self-processing 2A sequences circumvents these problems and allows the co-expression of multiple genes under the control of a single promoter (de Felipe et al. 2006).

Here, we designed two multicistronic expression constructs, each composed of the coding sequences for a prenyl transferase, a respective *S. habrochaites* terpene synthase, and enhanced green fluorescent protein linked by short nucleotide sequences encoding the foot-and-mouth disease virus 2A self-processing oligopeptide (Figure 4.1). Both constructs are under the control of the *AtCER5* promoter that directs epidermis-specific gene expression (Pighin et al. 2004). Infiltration of tomato leaves with *Agrobacterium* carrying the pC5-FTG and pC5-zFSG constructs resulted in the transient expression of all three genes included in each expression construct. The RT-PCR analyses (Figure 4.2) demonstrated the expression of the first coding region, *AtFPPS* and *ShzFPPS*, respectively, and the third coding region, *eGFP*, from each of the two multicistronic constructs. Moreover, the expression of *eGFP* was further verified through fluorescence microscopy that detected GFP fluorescence in the epidermis (Figure 4.3). The formation of the expected sesquiterpenes, β-caryophyllene and α-humulene (Figure 4.4A), and (−)-*endo*-α-bergamotene, (+)-α-santalene, (−)-*exo*-α-bergamotene, (−)-*epi*-β-santalene, and (+)-*endo*-β-bergamotene (Figure 4.4B), upon leaf infiltration with *Agrobacterium* carrying the pC5-FTG and pC5-zFSG construct, respectively, provided further evidence for the expression of the prenyl transferases and terpene synthases included in these constructs. Our observation that the

total sesquiterpene amounts produced in leaves expressing the plastid localized ShzFPPS and ShSBS were higher than in leaves expressing the cytosolic AtFPPS and ShTPS12 (Table S4.1) is in line with earlier studies showing that the plastidic MEP pathway is often metabolically more active than the cytosolic MVA pathway (Ashour et al. 2010; Hemmerlin et al. 2012). A similar co-expression system based on viral 2A sequences has previously been used to engineer the formation of a precursor of artemisinin, a plant derived sesquiterpene lactone highly effective in the treatment of malaria, into tobacco leaves (van Herpen et al. 2010). Transient expression of a multicistronic construct, containing the reading frames for amorpha-4,11-diene synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and farnesyl diphosphate synthase linked by 2A sequences, in *Nicotiana benthamiana* leaves resulted in the formation of the artemisinin precursor amorpha-4,11-diene. Moreover, a viral 2A sequence system has been used for the co-expression of the carotenoid biosynthetic genes encoding phytoene synthase and carotene desaturase in rice endosperm to obtain an improved version of the β-carotene producing Golden Rice (Ha et al. 2010; Jeong et al. 2017). Another study (Møldrup et al. 2012) utilized the viral 2A sequence co-expression system to engineer the six-step benzylglucosinolate pathway from *A. thaliana* into *Nicotiana tabacum*, thus converting the resulting tobacco lines into a trap crop for the pest diamondback moth (*Plutella xylostella*).

In vegetative parts of plants, the formation of terpenes is often restricted to specific tissues, such as glandular trichomes on the leaf surface (Gershenzon and Dudareva 2007; Zulak and Bohlmann 2010). Therefore, the goal of this study was to test if the formation of terpenes with activity against aphids could be engineered into new vegetative tissues, specifically the epidermis, where terpenes are naturally not found. The fluorescence microscopy analyses (Figure 4.3) of tomato leaves infiltrated with *Agrobacterium* carrying the pC5-FTG and pC5-zFSG constructs detected GFP fluorescence exclusively in both epidermal layers, thus confirming the tissue specificity of the expression under the control of the *AtCER5* promoter. Moreover, the analysis of the terpene content (Table 4.1) in different tissue fractions of the tomato leaves transiently expressing the multicistronic constructs revealed that the vast majority of the ShTPS12- and ShSBS-derived sesquiterpenes is indeed produced in the epidermis. These results suggest that sufficient pools of IPP and DMAPP are available in the cytosol and plastids of these epidermis cells that can serve as substrates for the cytosolic AtFPPS2 and ShTPS12, and the plastid localized ShzFPPS and ShSBS, respectively. Although the minor amounts of

sesquiterpenes (Table 4.1) found in the mesophyll fractions of leaves expressing the pC5-FTG and pC5-zFSG constructs are likely the consequence of contamination by epidermis cells, we cannot exclude that there might be a symplastic transport of some sesquiterpenes produced in the epidermis cells toward neighboring mesophyll cells. While to the best of our knowledge this is the first report on the metabolic engineering of terpene formation in the epidermis of leaves, there are examples of natural terpene formation in epidermis cells. The flowers of *Clarkia breweri* are strongly scented and one of the major volatile compounds emitted is the monoterpene *S*-linalool. *In situ* localization studies of the *S*-linalool synthase transcripts demonstrated that this terpene synthase is mainly expressed in the epidermal cell layers of the *C. breweri* flower petals (Dudareva et al. 1996). Likewise, both epidermal layers of the petals in rose (*Rosa* x *hybrida*) flowers were found to produce, accumulate, and emit a number of monoterpenes including geraniol, citronellol, and nerol (Bergougnoux et al. 2007).

Remarkably, the formation of the ShTPS12- and ShSBS-derived sesquiterpenes in the epidermis of tomato leaves expressing the pC5-FTG and pC5-zFSG constructs significantly affected the longevity and fecundity of *M. euphorbiae* (Figure 4.5). Recently, we observed similar effects on the longevity and fecundity of *M. euphorbiae* when the aphid performance was tested on the leaf surface of *S. habrochaites* accessions producing β-caryophyllene/α-humulene and (−)-*endo*-α-bergamotene/(+)-α-santalene/(+)-*endo*-β-bergamotene in their glandular trichomes (Wang et al. 2020). The fact that the effect on the performance of *M. euphorbiae* was less severe on the engineered leaves with the epidermis-specific sesquiterpene formation (Figure 4.5) compared to that of the glandular trichome-derived sesquiterpenes in *S. habrochaites* accessions (Wang et al. 2020) could be due to a difference in the amounts of sesquiterpenes produced. On the other hand, the reduced longevity and fecundity of *M. euphorbiae* observed in this study are clearly due to the sesquiterpene formation engineered into the leaf epidermis, since we have used the *odorless-2* mutant that is deficient in the formation of the glandular trichome-derived terpenes normally found in tomato leaves (Kang et al. 2010a; Wang et al. 2021). This result of our study is in line with previous studies which have revealed that the host plant selection by aphids is not only affected by glandular trichomes, but also by factors located in the epidermis including epicuticular lipids, cell wall barriers, and the presence or absence of certain metabolites that serve as gustatory cues upon probing (Alvarez et al. 2006; Schwarzkopf et al. 2013).

4.5 Conclusion

In this study, we have taken a novel approach toward developing a sustainable aphid control strategy that specifically considers the feeding behavior of these piercing-sucking pests. By utilizing the viral 2A sequence system, we co-expressed two pairs of prenyl transferases and *S. habrochaites* terpene synthases under the control of the epidermis-specific *AtCER5* promoter. This metabolic engineering approach resulted not only in the exclusive accumulation of the desired sesquiterpenes in the epidermis of tomato leaves, but also significantly affected the aphid performance. Thus, the metabolic engineering of sesquiterpenes into the leaf epidermis introduced an additional layer of defense against aphids, besides the glandular trichome-derived terpenes naturally present in cultivated and wild tomato species that in particular affect the aphid choice behavior. Future metabolic engineering approaches could now also test the effects of sesquiterpene formation in other tissues relevant to aphid feeding, such as the mesophyll and phloem, by expressing the newly designed multicistronic constructs under the control of respective tissue-specific promoters. While the outcome of our transient engineering study and the aphid bioassays highlighted the potential and efficacy of the tissue-specific metabolic engineering approach described here, further detailed characterization of respective stable transgenic tomato lines and aphids feeding on them, including electrical penetration graph analysis, will be required to verify which specific stages of the aphid feeding behavior are affected by the sesquiterpene formation in the epidermis and other tissues.

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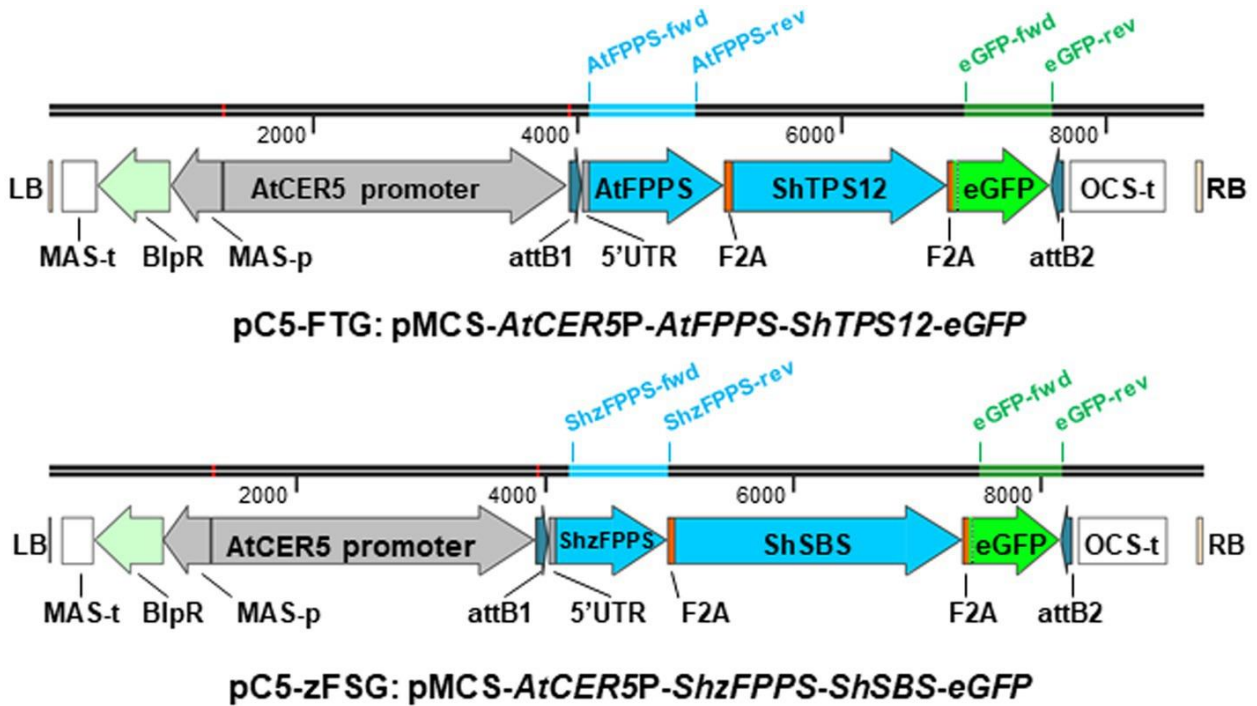
Figure 4.1 Schematic representation of multicistronic expression constructs for epidermis-specific engineering of sesquiterpene formation. Two expression constructs were designed within the T-DNA, indicated by the left (LB) and right (RB) borders, of the binary pMCS vector. Both synthetic expression constructs are put under the control of an *AtCER5* promoter sequence and inserted between the gateway attachments sites (attB1 and attB2). Each of the multicistronic expression constructs contains the coding sequences for three proteins: a prenyl transferase (AtFPPS or ShzFPPS), a terpene synthase (ShTPS12 or ShSBS), and enhanced green fluorescent protein (eGFP). The three individual coding sequences within both multicistronic expression constructs are linked by a short nucleotide sequence encoding the self-processing foot-and-mouth disease virus 2A oligopeptide (F2A). Other elements located within the T-DNA are the octopine synthase terminator (OCS-t), mannopine synthase promoter (MAS-p) and terminator (MAS-t) and phosphinothricin acetyltransferase (BlpR). The bars above the pC5-FTG and pC5-zFSG constructs indicate their size (in base pairs) and the location of the three primer pairs used for RT-PCR analysis of *AtFPPS*, *ShzFPPS*, and *eGFP* expression.
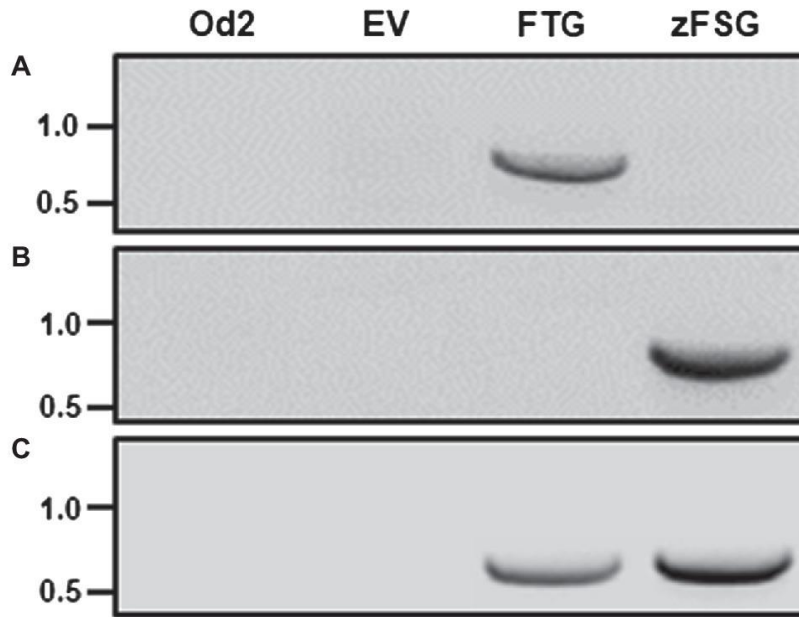
Figure 4.2 Transient expression of the multicistronic constructs in tomato leaves. Leaves of the tomato *odorless-2* mutant were infiltrated with *Agrobacterium* carrying the pC5-FTG construct, the pC5-zFSG construct, or the empty pMCS vector (EV). Transcript levels in *Agrobacterium* infiltrated leaves and *odorless-2* control (Od2) leaves were analyzed by RT-PCR utilizing *AtFPPS* (**A**), *ShzFPPS* (**B**), and *eGFP* (**C**) specific primer pairs (see **Figure 4.1** for location). The amplification products indicating *AtFPPS* (788 bp), *ShzFPPS* (794 bp), and *eGFP* (656 bp) expression were separated by agarose gel electrophoresis (size marker in kb indicated with each panel).
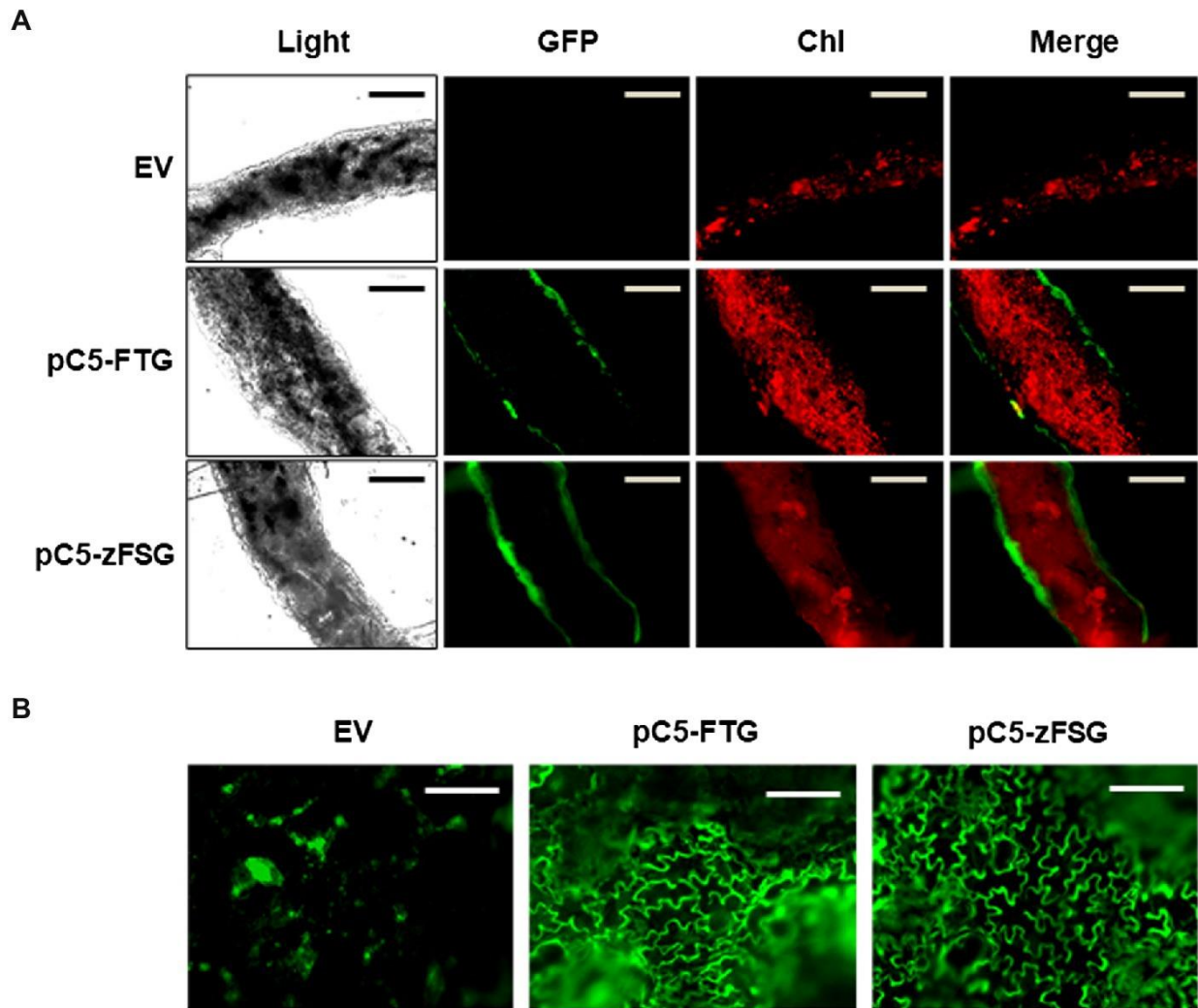
Figure 4.3 Tissue-specific expression of the multicistronic constructs. Cross sections **(A)** and surface sections **(B)** of tomato leaves infiltrated with *Agrobacterium* carrying the pC5-FTG construct, the pC5-zFSG construct, or the empty pMCS vector (EV) were analyzed by light and confocal laser scanning microscopy. Panels show fluorescence of green fluorescent protein (GFP) and chlorophyll autofluorescence (Chl). Scale bars represent 100 μm.
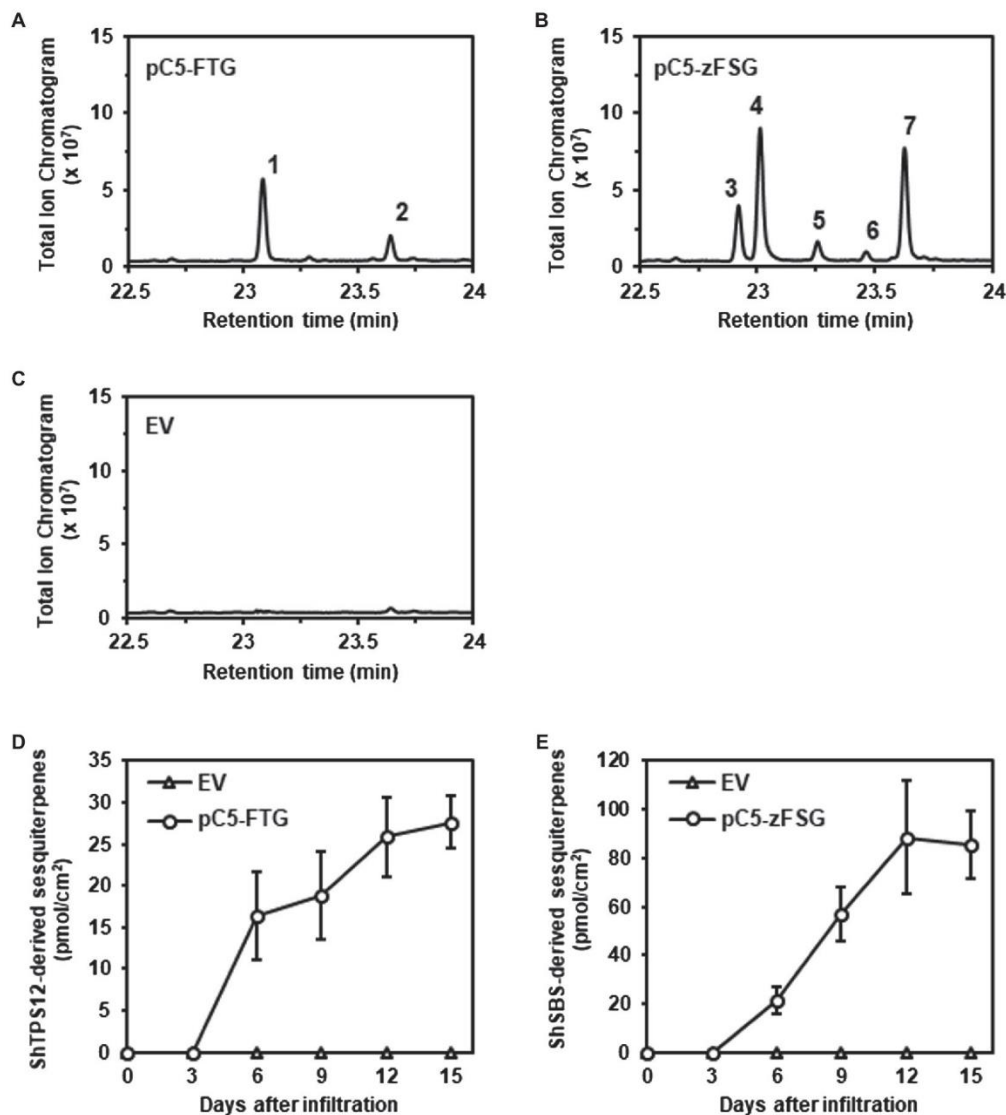
Figure 4.4 Accumulation of ShTPS12- and ShSBS-derived sesquiterpenes in tomato leaves expressing the multicistronic constructs. Terpenes were extracted from tomato leaves infiltrated with *Agrobacterium* carrying the pC5-FTG construct (**A**), the pC5-zFSG construct (**B**), or the empty pMCS vector (EV) (**C**), and were analyzed by GC-MS (total ion chromatograms are shown). ShTPS12-derived sesquiterpenes: 1, β-caryophyllene; 2, α-humulene. ShSBS-derived sesquiterpenes: 3, (-)-*endo*-α-bergamotene; 4, (+)-α-santalene; 5, (-)-*exo*-α-bergamotene; 6, (-)-*epi*-β-santalene; 7, (+)-*endo*-β-bergamotene. The total amounts (pmol/cm^2 leaf area) of ShTPS12-derived (**D**) and ShSBS-derived (**E**) sesquiterpenes were determined in tomato leaves at different time points after the *Agrobacterium* infiltration. Data are means ± SEM (n = 3).
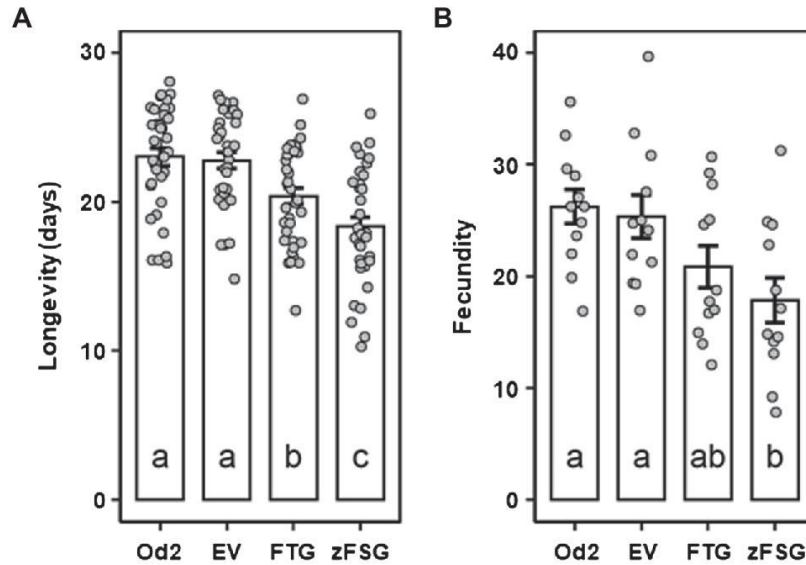
Figure 4.5 Longevity and fecundity of potato aphids on tomato leaves expressing the multicistronic constructs. Longevity **(A)** and fecundity **(B)** of *M. euphorbiae* on leaves of the *odorless-2* tomato mutant (Od2), and leaves infiltrated with *Agrobacterium* carrying the empty pMCS vector (EV), the pC5-FTG construct, or the pC5-zFSG construct. Newly emerged aphid nymphs were arrested onto tomato leaves two days after *Agrobacterium* infiltration. Values for longevity (n = 36) and fecundity (n = 12) are presented as means ± SEM. Values of different leaf samples were compared by ANOVA and Tukey's HSD test, and different letters indicate significant differences ($P < 0.05$).
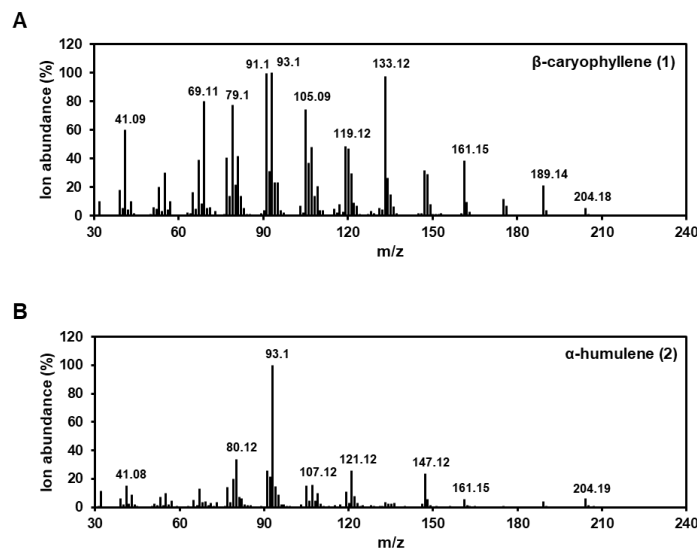
Figure S4.1 Mass spectra of ShTPS12-derived sesquiterpenes. Mass spectra of β-caryophyllene **(A)** and α-humulene **(B)** corresponding to peaks 1 and 2 (**Figure 4.4A**), respectively, are shown. Terpenes were extracted from tomato leaves infiltrated with *Agrobacterium* carrying the pC5-FTG construct, and were analyzed by GC-MS. Mass spectra were scanned at a range of 30-500 (m/z) after electron ionization at 70eV.

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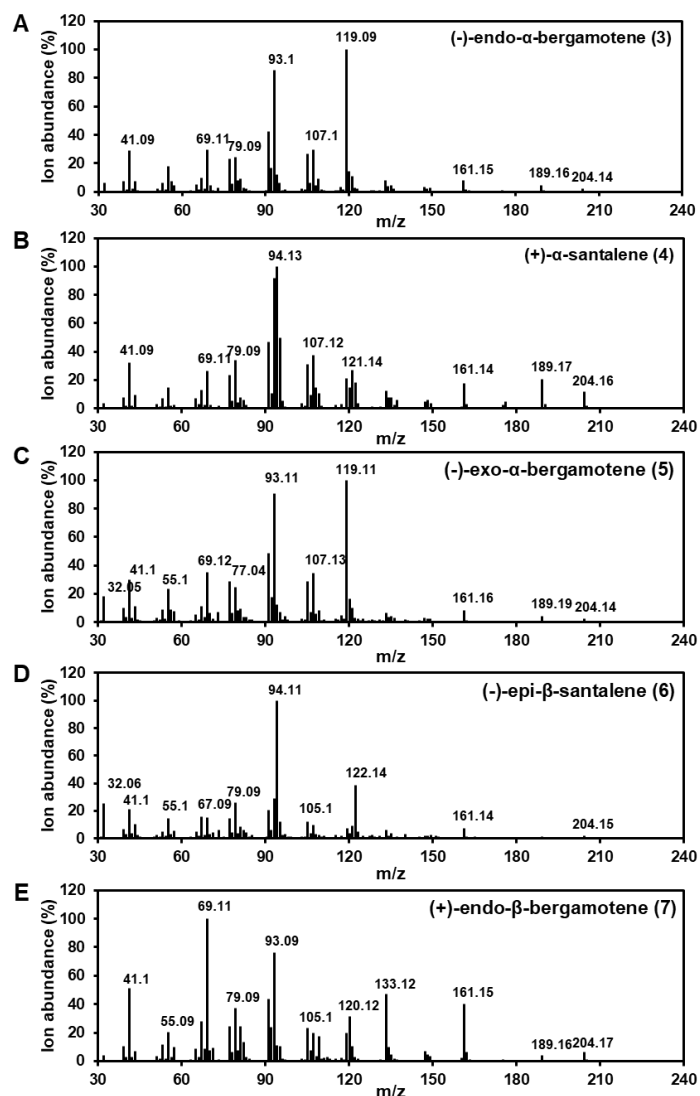
Figure S4.2 Mass spectra of ShSBS-derived sesquiterpenes. Mass spectra of (-)-*endo*-α-bergamotene (**A**), (+)-α-santalene (**B**), (-)-*exo*-α-bergamotene (**C**), (-)-*epi*-β-santalene (**D**), and (+)-*endo*-β-bergamotene (**E**) corresponding to peaks 3 - 7 (**Figure 4.4B**), respectively, are shown. Terpenes were extracted from tomato leaves infiltrated with *Agrobacterium* carrying the pC5-zFSG construct, and were analyzed by GC-MS. Mass spectra were scanned at a range of 30-500 (m/z) after electron ionization at 70eV.

Chapter 5: *Agrobacterium*-mediated tomato transformation for trichome- and companion cell-specific production of defensive sesquiterpenes; a lab report

5.1 Introduction

Aphids are typical piercing-sucking herbivores which utilize the specialized mouthpart (stylet) to ingest phloem sap, and during the process the stylet pathway involves intensive intra- and extracellular activities in different leaf tissues. After landing on a plant, aphid stylet carries out several events before sustained phloem sap ingestion, including probing of plant epidermis, mesophyll activity, sieve element puncture, and phloem acceptance (Powell et al. 2006). These stages are essential in determining how plants interact with aphids, and thus modulating them is of significance to reducing plant tissue penetration and has the potential to prevent virus transmission to plants. In previous chapters, it was shown that two sesquiterpene mixtures catalyzed respectively by *ShTPS12* and *ShSBS* had the ability to suppress aphid feeding and salivation when they were tested in artificial diets. Their constitutive production in glandular trichomes of *Solanum habrochaites* was likely to affect the initial step of the aphid infestation while longevity and fecundity of wingless aphids introduced to the wild tomato accessions were negatively affected (Wang et al. 2020). In addition, the epidermis is the tissue proven to contribute to aphid resistance. Transient expression of two constructs under the epidermis-specific *Arabidopsis thaliana CER5*-promoter (*AtCER5*p) in tomato leaves demonstrated the accumulation of *ShTPS12*- and *ShSBS*-derived sesquiterpenes specifically in epidermal cells, and the leaves infiltrated with *Agrobacterium* harboring the constructs also reduced both longevity and fecundity of aphids (Wang et al. 2021).

Phloem companion cells form an additional layer that is known to develop multiple mechanisms to defend against aphid infestation (Klingler et al. 2005; Tjallingii 2006; Lü et al. 2011). Factors contributing to phloem-based defense mechanisms include sieve-specific phloem proteins, polysaccharides and other secondary metabolites (Tjallingii 2006; Lü et al. 2011; Fu et al. 2014), which are usually active in affecting the feeding activities and performance of aphid species. For example, the cotton aphid, *Aphis gossypii* (Glover), introduced on a *Cucumis melo*

line AR showed prolonged salivation after sieve element puncture and reduced phloem sap ingestion (Klingler et al. 1998). The green peach aphid *Myzus persicae* (Sülzer) spent more time actively feeding on the sieve elements of a *Arabidopsis pad4* (*phytoalexin deficient4*) mutant than from wild-type plants, and less time feeding on transgenic plants in which *PAD4* is ectopically expressed (Pegadaraju et al. 2007). The *Arabidopsis* PAD4 protein distributes between cytoplasm and nucleus of phloem sieve element or companion cells. Therefore, it is conceivable that the accumulation of the *ShTPS12*- and *ShSBS*-derived sesquiterpenes in companion cells might form another layer abating feeding activities and therefore reducing aphid performance.

The purpose of this chapter is to investigate whether the stable expression of the genes responsible for producing the two sesquiterpene mixtures in glandular trichome cells, epidermis cells, and companion cells of cultivated tomato plants, respectively, affect the interaction with the potato aphid. Toward this goal, recombinant binary vectors were cloned using the cloning strategy employed in the previous chapter, which involves the two multicistronic expression constructs based on the two sesquiterpene traits (Wang et al. 2021). The expression of genes was controlled respectively by a trichome-specific *TPS9* promoter in *Solanum lycopersicum* (*SlTPS9*p) and a companion cell-specific *SUC2* promoter in *A. thaliana* (*AtSUC2*p). The trichome-specific promoter *SlTPS*9 was shown to drive *sYFP* expression specifically in type VI glandular trichomes (Kortbeek et al. 2016), whereas the companion cell-specific promoter *AtSUC2*p was known to control the expression of sucrose carrier *AtSUC2* of *A. thaliana*, where it catalyzes the uptake of sucrose from the apoplast into companion cells (Stadler and Sauer 2019). The expression cassettes cloned in the binary vectors were transformed into tomato explants via *Agrobacterium*-based plant transformation. Additionally, the resistances to kanamycin and phosphinothricin (glufosinate) were both used as selection makers for evaluating transformation efficiency.

5.2 Material and method

5.2.1 Amplification of promoter sequences from genomic DNA

The promoter sequences of *AtSUC2* (At1g22710) and *SlTPS9* (JN408289) (named hereafter *AtSUC2*p and *SlTPS9*p) were amplified by Taq DNA Polymerase using two pairs of

oligonucleotides both having restriction sites incorporated: *AtSUC2*p-fwd (5'-CGGAATTCGCAAAATAGCACACCATTTATG-3'; *Eco*RI site underlined), *AtSUC2*p-rev (5'-CGCTCGAGTTTGACAAACCAAGAAAGTAAG-3'; *Xho*I site underlined), *SlTPS9*p-fwd (5'-GCGAATTCGAGCCAAAATGCCTTATTGAGGT-3'; *Eco*RI site underlined) and *SlTPS9*p-rev (5'-CGCTCGAGTGCTTGTTTGTTCTAAGGTTTGC-3'; *Xho*I site underlined). Genomic DNA from *A. thaliana* and *S. lycopercisum* c.v. M82 were used as templates for the polymerase chain reactions.

5.2.2 Preparation of gateway destination vectors by TA- and restriction-cloning

The two promoter sequences amplified by PCR were ligated into a pGEM-T vector by TA-cloning. The recombinant plasmids were transformed into DH5α *Escherichia coli* competent cells by a traditional heat-shock method and then plated out on LB/Amp/IPTG/X-gal media for blue-white screening. To get the promoter fragments with sticky cloning sites, the recombinant pGEM-T plasmids were digested with the restriction enzymes recognizing the cut sites incorporated in the primers. On the other hand, the pMCS:GW vector (CD3-1933) (Michniewicz et al. 2015) with a *BlpR* gene encoding a phosphinothricin acetyltransferase in expression cassette was also digested with compatible restriction enzymes recognizing the cutting sites in its multiple cloning sites. The pMCS:GW backbone after digestion was ligated with the digested promoter fragments to produce Gateway Destination vectors named pS2:GW and pT9:GW, respectively.

5.2.3 Tri-cistronic 2A constructs and Gateway Cloning

Two *attB*-flanked tri-cistronic fragments having genes producing respectively mixtures of caryophyllene/humulene and santalene/bergamotene were obtained by commercial DNA synthesis (Twist Bioscience ®, San Francisco CA). The fragments were incorporated in the pTwist ENTR vector and were used as a Gateway Entry clone. Each of the tri-cistronic constructs included sequences from three genes, encoding respectively a prenyl transferase (*AtFPPS or ShzFPPS*), a terpene synthase (*ShTPS12 or ShSBS*), and the enhanced green fluorescent protein (*eGFP*). The 5'-UTR of *AtFPS1* gene (At5g47770) from *A. thaliana* was fused before the start codon of the prenyl transferase for translational regulation. The stop codons of prenyl transferase and terpene synthase were removed and a nucleotide sequence (F2A) encoding a "2A peptide" (Yu et al. 2012) was used to link the three genes into one ORF.

A standard LR recombination reaction between each Gateway Destination vector and pTwist ENTR vector was conducted using LR Clonase (Invitrogen, Thermo Fisher Scientific), following the manufacturer's protocol. After LR reactions, the four subsequent recombinant plasmids (Figure 5.1), i.e., pS2-*BlpR-AtFPPS-ShTPS12-eGFP* (pS2-B-FTG), pS2-*BlpR-ShzFPPS-ShSBS-eGFP* (pS2-B-zFSG), pT9-*BlpR-AtFPPS-ShTPS12-eGFP* (pT9-B-FTG), and pT9-*BlpR-ShzFPPS-ShSBS-eGFP* (pT9-B-zFSG) were transformed into an *Agrobacterium tumefaciens* strain GV3101::pMP90 via a traditional freeze-thaw method.

5.2.4 Introducing a kanamycin resistance gene into recombinant plasmids via In-Fusion Cloning

To linearize the recombinant plasmids, pS2-FTG and pT9-FTG were digested by the *Sac*I restriction enzyme, while pS2-zFSG and pT9-zFSG were digested by *Sac*I and *Eco*RI, respectively. On the other hand, a 1418bp region located in the expression cassette of a binary pK7WGF2 vector (Karimi et al. 2002), which flanks a *NOS* promoter, the coding region of an aminoglycoside phosphotransferase (*NeoR*/*KanR*), and a *NOS* terminator, was amplified by one forward and two reverse primers, i.e., IF-KAN-fwd (5'-CGCCGAATTAATTCGATTATCAGCTTGCATGCCGGT-3'), IF-KAN-rev1 (5'-AAATTATCAGATCCGCCGGGTACCGCGAATTATCA-3') and IF-KAN-rev2 (5'-GCAAACTAAAGAATTCCGGGTACCGCGAATTATCA-3'), producing two types of fragments (inserts) differ in their end sequences (underlined in primers) homologous to the plasmids with two ways of linearization. The linearized plasmids and corresponding inserts were combined In-Fusion enzyme mix (Takara Bio Inc., Japan) and incubate for 15 min. The recombinant plasmids with the introduced kanamycin resistance gene (Figure 5.2), i.e., pS2-*BlpR-KanR-AtFPPS-ShTPS12-eGFP* (pS2-K-FTG), pS2-*KanR-ShzFPPS-ShSBS-eGFP* (pS2-K-zFSG), pT9-*BlpR-KanR-AtFPPS-ShTPS12-eGFP* (pT9-K-FTG), and pT9-*KanR-ShzFPPS-ShSBS-eGFP* (pT9-K-zFSG), were further cloned in DH5α competent cells and transformed into the *A. tumefaciens* strain GV3101::pMP90.

5.2.5 *Agrobacterium*-mediated plant transformation

A cultivated tomato line (c.v. MP-1), known for its superior transformation competence, was used for *Agrobacterium*-mediated transformation, using a protocol adapted from previous studies (Pino et al. 2010; Kortbeek et al. 2016). The protocol was divided into seven steps: seed

sterilization and germination, *Agrobacterium* preparation, explanting, co-cultivation, shoot induction and elongation, root induction, and acclimation. Hormone solutions of BAP (6-benzylaminopurine) and NAA (1-naphthaleneacetic acid) were respectively prepared by mixing the chemicals with drops of 1 M HCl and 1 M KOH, and further dissolved in water to 5 mM (BAP) and 0.4 mM (NAA). Acetosyringone (AS) stock solution was prepared in 70% ethanol to 100 mM. Before use, all the hormone stock solutions were filter-sterilized (0.2 μm).

5.2.5.1 Seed sterilization and germination

Seeds of c.v. MP-1 were sterilized by shaking for 10 min in 70% ethanol and 15 min in commercial bleach (dilute with sterile water to get 3% NaClO) and they were rinsed a few times with sterilized water. The seeds were transferred to flasks (20 seeds per flask) containing 30 mL autoclaved germination medium (GM) which includes 2.5 g/L Murashige and Skoog medium-including Gamborg B5 vitamins (MS + Vit B5), 15 g/L sucrose and 6 g/L agar (pH = 5.8). For cotyledon germination, the seeds were allowed to grow at 25°C in dark for 4 days and under a 16L:8D photoperiod for another 4 days.

5.2.5.2 *Agrobacterium* preparation and cotyledon explanting

The *A. tumefaciens* strain GV3101::pMP90 containing the plasmids obtained from Gateway-Cloning and In-Fusion Cloning was grown initially on YEB *Agrobacterium* growth medium containing 100 mg/L kanamycin, 50 mg/L rifampicin and 50 mg/L gentamicin. A single colony with each plasmid was propagated sequentially in 5 mL and 50 mL of liquid YEB media at 28°C for 24h at 200 rpm, both containing the same antibiotics. The culture was harvested by centrifuging at 2000-3000 g for 15 min and resuspended in suspension medium (SM) (4.5 g/L MS + Vit B5, 30 g/L sucrose) to an OD_{600} of 0.3-0.35. Before cocultivation, acetosyringone (AS) stock solution was added to the suspension to a concentration of 100 μM.

Cotyledons isolated from seedlings were cut transversally into 2-3 pieces (explants) with distal and proximal tips removed. The explants were directly placed with the abaxial side down into a Petri dish (90 × 15 mm) containing a solid Root Induction Medium (RIM) (4.5 g/L MS + Vit B5, 30 g/L sucrose, 6 g/L agar, 0.4 μM NAA and 100 μM AS). Each dish plate had 20 explant pieces and 6 dishes were used for each type of suspension. The plates were incubated for 1 day before co-cultivation.

5.2.5.3 Co-cultivation

By using a micropipette, two droplets of the prepared *Agrobacterium* suspension were applied to cover completely the surface and border of each explant. After 10 minutes of incubation at room temperature, excessive suspension from explants was removed by sterile pipette, and filter papers were used for brief dehydration. The explants were incubated in dark at 25°C for two days.

5.2.5.4 Shoot induction, elongation, and plantlet acclimatization

The explants were transferred to plates that contain Shoot Inducing Medium (SIM) (4.5 g/L MS + Vit B5, 30 g/L sucrose, 6 g/L agar, 5 µM BAP). The SIM plates also contained 100 mg/L kanamycin alone or its combination with 100 mg /L glufosinate (phosphinothricin), respectively for explants cocultured with In-Fusion and Gateway Cloning plasmids. The plates were incubated under a 16h photoperiod at 25 °C for ~3 weeks to develop 2-4 mm shoots. For shoot elongation, well-developed shoots were isolated and transferred to flasks with 30 mL of media, composed of 4.5 g/L MS + Vit B5, 30 g/L sucrose, and 6 g/L agar, as well as the corresponding antibiotics. After two weeks, plantlets were gently removed and grown in soil-containing 4-inch pots under greenhouse conditions.

5.2.6 Testing the transient expression of the *NeoR/KanR* cassette by leaf-infiltration

To verify the feasibility of the cloning strategies for plant transformation, another recombinant plasmid, i.e., pC5-K-zFSG vector (Figure 5.4A), was designed from pC5-zFSG (see the previous chapter), using the same In-Fusion cloning method by which pS2-K-zFSG and pS2-K-zFSG were created. The same agroinfiltration method, as shown in the previous chapter, was used to infiltrate 4-week-old c.v. MP1 tomato leaves with *Agrobacterium* carrying pC5-zFSG. Six days after infiltration, total RNA from different leaves was isolated, pretreated with RNase-free DNase (New England Biolabs, Ipswich, MA, United States), and then used to synthesize cDNA using reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA, United States). The expression of the *NeoR/KanR* and *eGFP* was evaluated by PCR utilizing two primer pairs, i.e., *KanR*-fwd (5'- GAAGAACTCGTCAAGAAGGCGATAGAAG-3'), *KanR*-rev (5'-GATGGATTGCACGCAGGTTCTC-3'), *eGFP*-fwd (5'-CGACGTAAACGGCCACAAGTTCA-3'), and *eGFP*-rev (5'-ACTTGTACAGCTCGTCCATGCC-3'). The PCR conditions were as

follows: 96°C for 5 min for one cycle, followed by 40 cycles of 95°C for 60 s, 57°C for 1.0 min and 72°C for 60 s, and a final extension at 72°C for 10 min. The amplification products were separated by agarose gel electrophoresis, stained with GelRed® (Biotium, United States), and analyzed using the ChemiDoc Gel Imaging System and Image Lab 5.1 software (Bio-Rad, Hercules, CA, United States).

5.3 Results and Discussion

The explants co-incubated with the eight recombinant plasmids were not able to regenerate during the shoot induction procedure. Instead, all the necrotic explants exhibit a symptom resembling those inhibited by herbicide (Figure 5.3). Thus, it was hypothesized that the selection gene (*BlpR* or *NeoR*/*KanR*) in each plasmid used for plant transformation was not efficiently expressed, and thus the development of explants were inhibited by the selection chemicals introduced in SIM plates. To test this, *Agrobacterium* carrying another vector pC5-K-zFSG (Figure 5.4A) was used to infiltrate tomato leaves, and transient expression of the *NeoR*/*KanR* and *eGFP* was analyzed by RT-PCR. The results indicated that *eGFP* was successfully expressed in each leaf infiltrated (Figure 5.4C), which is consistent with its expression in pC5-FTG and pC5-zFSG as shown in the previous chapter. In contrast, none of the infiltrated leaves have the expression of *NeoR*/*KanR* detected (Figure 5.4B), supporting the hypothesis that kanamycin eliminates the explants in SIM plates which were not able to produce aminoglycoside phosphotransferase to compensate for the damage by kanamycin.

On the other hand, explants were also eliminated in SIM plates containing 10 mg/L glufosinate, although they were co-cultured by *Agrobacterium* carrying *BlpR*-containing plasmids (Figure 5.3). From previous studies it can be included that plants with the transformed *BlpR* vary tremendously in resistance to glufosinate, and the optimized concentration of glufosinate used for selection range from 0.1-185 mg/L depending on plant species (Zhang et al. 1999; Brukhin et al. 2000; Sarria et al. 2000; Zeng et al. 2004; Nakamura et al. 2010; Khuong et al. 2013; Fartyal et al. 2018). In addition, the selection test for soybean explants introduced in selective SIM plates showed a narrow concentration range of glufosinate in killing response (Hada et al. 2016). This might be caused by a too high concentration of glufosinate that I used in SIM plates. Therefore, future studies need to test more concentrations to refine the selection.

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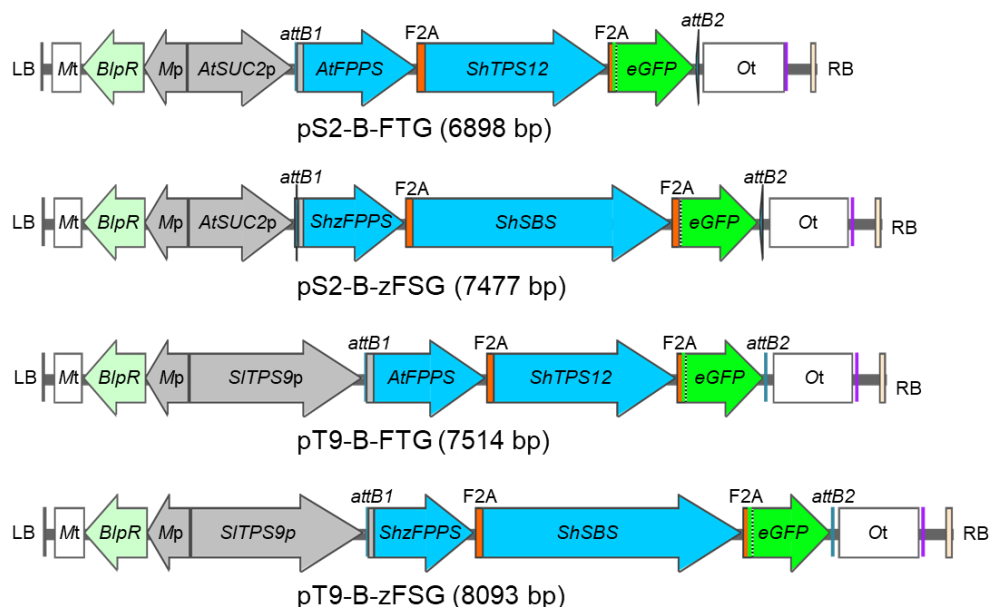
Figure 5.1 Expression cassettes of the recombinant plasmids from Gateway Cloning. The T-DNA regions, indicated by the left (LB) and right (RB) borders, are from the binary pMCS vector. The constructs are put under the control of *AtSUC2* or *SlTPS9* promoter sequence (*AtSUC2*p or *SlTPS9*p) inserted between the gateway attachments sites (*attB1* and *attB2*). Each construct contains three coding sequences downstream the promoter sequence, including a prenyl transferase (*AtFPPS* or *ShzFPPS*), a terpene synthase (*ShTPS12* or *ShSBS*), and enhanced green fluorescent protein (*eGFP*). The three protein-coding sequences are linked by a short nucleotide sequence encoding the self-processing foot-and-mouth disease virus 2A oligopeptide (F2A). Other elements include the mannopine synthase promoter (*M*p) and terminator (*M*t), the octopine synthase terminator (*O*t), and the coding sequence for phosphinothricin acetyltransferase (*BlpR*).
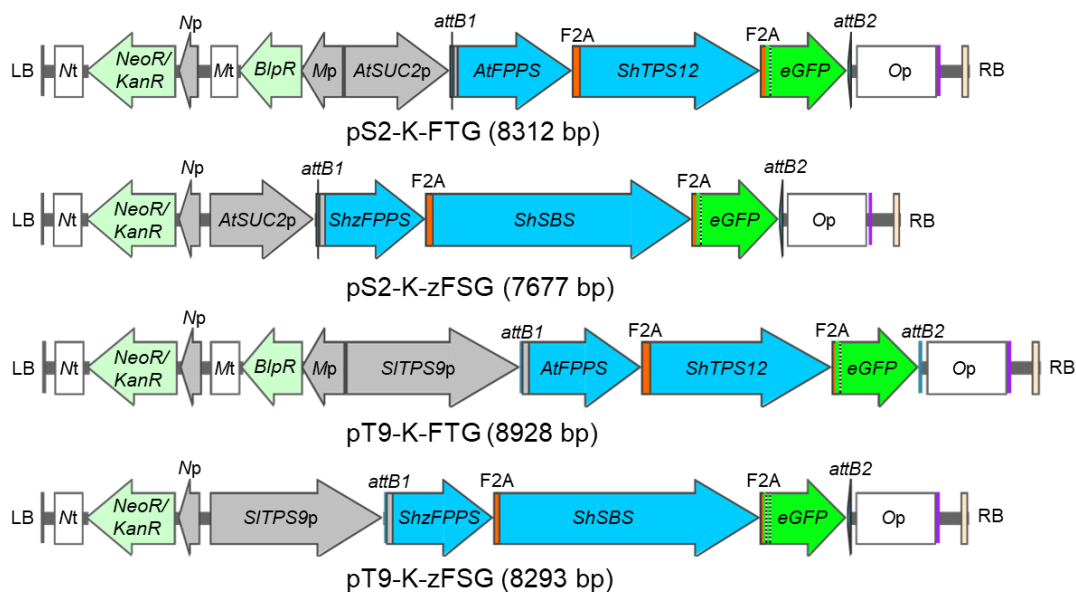
Figure 5.2 Expression cassettes of the recombinant plasmids from In-Fusion Cloning. The expression cassettes of pS2-K-FTG and pT9-K-FTG are originated from pS2-B-FTG and pS2-B-zFTG by fusing a nopaline synthase promoter (*N*p) and terminator (*N*t) and a coding sequence for aminoglycoside phosphotransferase (*NeoR*/*KanR*). The same sequences are used to replace the mannopine synthase promoter (*M*p) and terminator (*M*t), the octopine synthase terminator (*O*t), and the coding sequence for phosphinothricin acetyltransferase (*BlpR*) in pS2-B-zFSG and pT9-B-zFSG, to generate pS2-K-zFSG and pT9-K-zFSG.
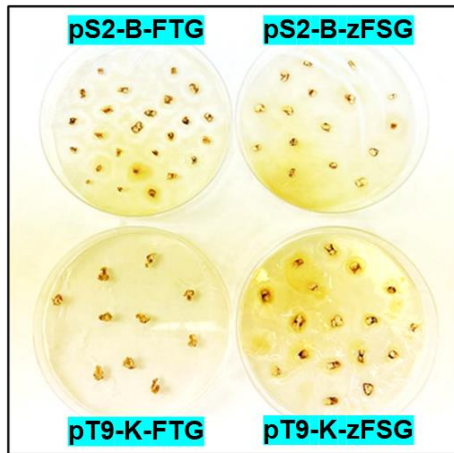
Figure 5.3 Explants unable to regenerate in vitro in Shoot Inducing Medium. The two plates at the top contain 100 mg/L kanamycin and 10 mg /L glufosinate, while the bottom two contain 100 mg/L kanamycin only.
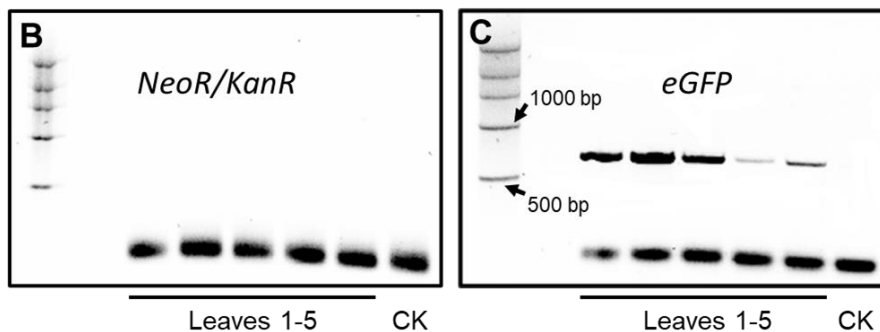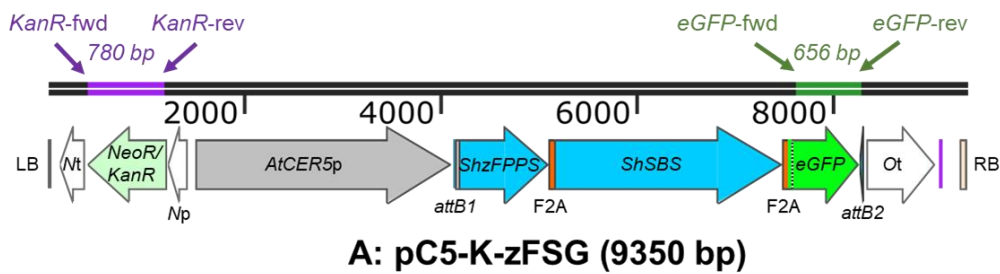
Figure 5.4 Transient expression of genes in c.v. MP1 tomato leaves agroinfiltrated by *Agrobacterium* **harboring pC5-K-zFSG.** The bar above the expression cassette of pC5-K-zFSG (**A**) indicate its size (in base pairs) and the location of the two primer pairs used for RT-PCR analysis of *NeoR/KanR* and *eGFP* expression, respectively. The amplified products for *NeoR/KanR* (**B**; 780 bp) and *eGFP* (**C**; 656 bp) were separated by agarose gel electrophoresis. CK, negative control.

Chapter 6: Summary and further perspective

6.1 Summary

This project was dedicated to developing new efficient, sustainable, and environmentally friendly approaches to control the potato aphid, a major piercing-sucking herbivore that heavily infests domesticated tomato *Solanum lycopersicum* in the greenhouse. The first task of the project was to explore potential defensive traits localized in glandular trichomes of wild tomato *Solanum habrochaites* accessions, which were known to produce a great variety of secondary metabolites that affect a broad category of different herbivores (Kang et al. 2010a, 2010b; Tian et al. 2012; Gutensohn et al. 2014). The second purpose was to improve the resistance of cultivated tomato lines by engineering the production of the defensive traits from wild tomato in multiple tissues of cultivated tomato along the stylet pathway of the potato aphid.

For the first task, a collection of *Solanum habrochaites* (wild tomato) accessions which were known to produce different sesquiterpenes being major compounds in their type VI glandular trichomes (Gonzales-Vigil et al. 2012), as well as their leaf surface extracts, were prepared. The wild tomato accessions were separated into 5 groups (type I-V) based on their major sesquiterpene compounds, and they were all different from the two cultivated tomato lines, *i.e.*, c.v. M82 and c.v. Moneymaker, which produce a mixture of TPS20-derived monoterpenes as major compounds (Wang et al. 2020).

1) Defensive traits against the potato aphid were identified from two groups of the wild tomato accessions, *i.e.*, LA1775 and LA1779 of group IV and LA1624 and LA2860 of group V. The accessions from the two groups produce respectively isomeric mixtures of santalene/bergamotene and caryophyllene/humulene as the major compounds in glandular trichomes. These accessions reduced the longevity and fecundity of wingless aphids, while their surface leaf surface extracts affected the feeding performance of wingless aphids, indicated by the affected survivorship, gel saliva and honeydew production. The choice behaviors of winged aphids were also influenced by the odors emitted from the leaves and extracts of the accessions.

2) The resistance trait in LA1624 and LA2860 was confirmed to be the ShTPS12-derived sesquiterpene mixture β-caryophyllene and α-humulene. Applying the mixture in a ratio produced in glandular trichome lowered the parameters of feeding and choice behaviors of the potato aphid. The resistance trait in LA1775 and LA1779 was indirectly determined to be the ShSBS-derived santalene/bergamotene sesquiterpenes, rather than γ-elemene, the most abundant compound produced in glandular trichome. The introgression line LA3935, which originated from LA4024 and a wild tomato LA1777, had the ShSBS-derived sesquiterpenes produced predominately in glandular trichome, while it affected feeding and choice behaviors of the potato aphid.

The result from the first task demonstrated that the cultivated tomato lines susceptible to the potato aphid contain large amounts of TPS20-derived monoterpenes and tiny amounts of TPS12-derived sesquiterpenes, while large amounts of such sesquiterpenes were also found in the accessions of group V. This furthermore led to the assumption that terpene chemistry in glandular trichome is the primary factor determining the plant-aphid interaction. To test this assumption, two cultivated tomato lines, Ailsa Craig and Castlemart, their respective trichome mutant, *hairless* and *odorless-2*, as well as all their leaf surface extracts were prepared. The *hairless* mutant was known to produce fewer amounts of TPS12-derived sesquiterpenes compared to the Ailsa Craig background, whereas all terpene compounds were nearly absent in the *odorless-2* mutant (Kang et al. 2010a, 2010b).

1) The wingless aphid performed better on the trichome mutants compared to their respective background lines with higher parametric values of longevity and fecundity. A principal component analysis (PCA) demonstrated that aphid performance parameters negatively correlated with the production of the TPS12-derived sesquiterpenes, but not obviously correlated with that of the TPS20-derived monoterpenes.

2) The relationship between aphid performance and the TPS-20 monoterpenes was further validated in feeding experiments. The survivorship, gel saliva and honeydew production of wingless aphids were not significantly affected by the monoterpene mixture when they were mixed in artificial diets with increasing concentrations.

3) The TPS-20 derived monoterpenes played otherwise a positive role on the choice behaviors of winged aphids. The *hairless* mutant was highly attractive to the winged

aphids, while the *odorless-2* did not. Aphids were also attracted significantly to the monoterpene mixtures emitted in a certain concentration.

4) The result indicated that the terpene chemistry in glandular trichomes of cultivated tomato lines contributed mainly to their susceptibility to the potato aphid. While a large concentration of TPS20-derived monoterpenes produced in cultivated lines is used by the potato aphid as the orientation cue, the small amounts of TPS12-derived sesquiterpenes are insufficient to be resistant against the aphid.

For the second task of the project, multiple binary vectors were designed and cloned to engineer the production of the two sesquiterpene mixtures respectively in the epidermal cell, companion cell, and glandular trichome of cultivated tomato leaf. Two multicistronic expression constructs were synthesized, aiming to produce a prenyl transferase (AtFPPS or ShzFPPS), a terpene synthase (ShTPS12 or ShSBS), and an enhanced green fluorescent protein (eGFP). The two constructs were put under the control of respectively an epidermis-specific *AtCER5* promoter, a companion cell-specific *AtSUC2* promoter, and a glandular trichome-specific *SlTPS9* promoter (Truernit and Sauer 1995; Pighin et al. 2004; Kortbeek et al. 2016).

1) The two binary vectors with genes controlled by the *AtCER5* promoter (pC5-FTG and pC5-zFSG) were transformed into *Agrobacterium* and agroinfiltrated into the *odorless-2* trichome mutant by leaf infiltration. The transient expression of the genes within the expression cassettes wase verified by RT-PCR, while their epidermis-specific expression was visualized by fluorescence microscopy. The infiltrated leaves of *odorless-2* mutant had the two sesquiterpene mixtures produced predominately in the epidermis tissue.

2) The wingless aphid on the infiltrated leaves had longevity and fecundity reduced, confirming that the engineering of the defensive trait in the epidermis is a feasible strategy for improving the resistance of cultivated tomato to the potato aphid.

3) The binary vectors with the *AtSUC2* promoter and the *SlTPS9* promoter were cocultured into explants of a c.v. MP1 tomato line by *Agrobacterium*-mediated plant transformation. However, the explants co-incubated with the *Agrobacterium* suspension failed to develop, possibly due to the selection gene in the expression cassettes not being expressed.

6.2 Discussion and further perspective

Plant defense mechanisms against insect herbivores are generally divided into three categories, *i.e.*, antixenosis, antibiosis, and tolerance (Bitenc and Milevoj 2002), with the former two frequently discussed in plant-aphid interaction (Smith and Chuang 2014; Aznar-Fernández and Rubiales 2018). Antixenotic resistance relates to the non-preference properties of plants in the form of morphology, phenology, and chemistry, in order to reject the acceptance and colonization of pests. Antibiotic resistance describes the ability of plants to inhibit or disturb the biological and physiological processes in herbivores after colonization, and it could be represented by the low growth rate, reproduction, and high mortality of the herbivores.

In this project, the two sesquiterpene mixtures not only reduced the attraction of winged aphids to plant odors in olfactometric choice assays, but also restrained the feeding and salivation activities of the wingless aphid in feeding experiments. These at least demonstrated an antixenosis action conferred by the two sesquiterpene mixtures. On the other hand, the reduction of longevity and fecundity of wingless aphids were usually interpreted as results of antibiosis action in previous studies (Daryanto et al. 2017; Aznar-Fernández and Rubiales 2018). Nevertheless, our study lacks confirmative evidence supporting physiological disturbance and subsequent impacts on behavioral performance, after the aphid contacts or uptakes the sesquiterpene mixtures. Further directions following the project might explore the potential effects of the two sesquiterpene mixtures on the aphid nervous system and aphid-bacterium endosymbiosis. Terpenes as lipophilic secondary metabolites are well-known insect neurotoxins that cause paralysis and death. Being an acetylcholinesterase (AChE) inhibitor that disturbs the neuro-neuronal and neuro-muscular junctions is a common neurotoxic action by terpenes and related compounds (Regnault-Roger et al. 2011; López and Pascual-Villalobos 2015). Besides, terpenes are also ligands of octopamine receptors, competing with octopamine, a neurotransmitter, in binding to its receptor (Jankowska et al. 2018). The binding activity of terpenes modifies cAMP and calcium in nervous cells, such that the final neuron activities are modified (Enan 2001; Kostyukovsky et al. 2002). On the other hand, studies reporting the effects of natural terpenoids on aphid endosymbiosis are limited. It has been noticed that azadirachtin, an oxidized tetranortriterpenoid naturally occurring in neem oil, declined the symbiont population in brown planthopper (*Nilaparvata lugens* Stål) (Raguraman and Saxena 1994). The

triterpenoid azadirachtin also strongly degenerates bacterial endosymbionts in the green peach aphid (*Myzus persicae* Sulzer), decreasing the production of endosymbiotically synthesized protein symbionin, while acting as a feeding deterrent to eventually inhibit aphid development (van den Heuvel et al. 1994).

In this project, the production of defensive traits in the epidermis was found to be a relevant strategy to improve the resistance of cultivated tomato by altering their interaction with the potato aphid. However, it is still unclear whether such tissue-specific metabolic engineering strategy would apply to glandular trichomes and companion cells of the cultivated tomato, which is supposed to be devoid of effective defense mechanisms. Toward an efficient approach to control the pest population in the greenhouse, it is necessary to create more transgenic lines with stably expressed genes in each of the three tissues, to compare efficiencies of defense for the different transgenic lines, and event to optimize the defense by additional crossing. Nevertheless, it is also worth noticing that the transgenic plants with defensive sesquiterpenes produced in epidermis and companion cells but having the attractive TPS-20 derived monoterpenes emitted from glandular trichomes, might exhibit 'attract and deter' phenotypes to the aphid, making plants attractive to the aphid while inhibiting its settling. Aphids mostly spread plant viruses through nonpersistent transmission, whereby viruses attach transiently to the probing mouthpart (Pirone and Harris 1977). Thus, the "attract and deter" phenotypes would likely encourage the spread of viral inoculum by aphids from infected plants to neighboring uninfected tomato plants, accelerating the virus transmission. Thus, the plant-aphid-virus interaction could be evaluated by more experiments by tracking the spread of the aphid-transmitted virus before a sustainable and effective aphid control strategy is defined.

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