**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Genetic and molecular characterization of type-IV glandular trichome
development in tomato (*Solanum lycopersicum* cv. Micro-Tom) and its
participation in arthropod resistance**

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Thesis presented to obtain the degree of Doctor in Science.
Area: Plant Physiology and Biochemistry

**Piracicaba
2020**

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Bachelor's in Biological Sciences

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Vendemiatti, Eloisa

Genetic and molecular characterization of type-IV glandular trichome development in tomato (*Solanum lycopersicum* cv. Micro-Tom) and its participation in arthropod resistance./ Eloisa Vendemiatti. - - Piracicaba, 2020.

110 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. Tricoma 2. Herbivoria 3. Tomateiro 4. *S. galapagense* 5. *S. pennellii* l.
Título

I dedicate this work to my grandfather Francisco Pissinato, for the pride he always felt for my achievements.

ACKNOWLEDGEMENTS

I want to express my gratitude to everyone who has contributed to this work. I am deeply grateful to all of you, especially:

My advisor, Prof. Lázaro E. Pereira Peres, for the guidance, motivation, patience, friendship, and trust placed in me in ten years of work together.

I am very grateful to “Luiz de Queiroz” College of Agriculture – University of São Paulo. It was twelve years of learning. In this place, I lived great opportunities, met people I will take for my lifetime, and grew up in a way I could have never imagined.

To the Plant Physiology and Biochemistry Program, especially Maria Solizete Granzio Silva, for their help, support, and availability whenever necessary.

I genuinely thank CAPES (Agency for the Support and Evaluation of Graduate Education – Finance Code 001) and FAPESP (São Paulo Research Foundation, process number 2016/22323-4) for the financial support throughout my doctorate studies.

To Prof. Vagner Benedito for his friendship, availability, patience, and shared knowledge.

To Prof. Severino Matias de Alencar, who gave us access to his laboratory, for his collaboration and help during the GC-MS experiments.

To Prof. Alain Tissier, Prof. Robert Last, Nick Bergau, and Paul Fiesel for their availability to help with LC-MS analyses and for the insights in our work.

To Lina Yang and Umesh Reddy for help with sequencing data analyses.

To dear Cassia Figueiredo for all the support and friendship offered to us. I also thank Eduardo Micotti da Gloria for his help in the last months of this work.

To Prof. Fábio Nogueira for all productive discussions.

To Prof. Agustin Zsögön for his availability, insights, and help on many occasions throughout my Ph.D.

To Dr. Walter Bernardi and Nunhems Vegetable Seeds for their help and availability with the herbivory experiments.

My friends Marcela Notini, Maisa Pinto, Mateus Vicente, Geraldo Silva, and Eder Silva for all their support and friendship that started in the lab but goes on for life.

I greatly thank my labmates, Gabriela Prants, Karla Gasparini, Lilian Pino, Diego Sevastian, Rodrigo Therezan, Mayara Carvalho, Jonata Freschi, Diego Britto, Carolina de Marchi Santiago, Letícia Frizzo, Flaviani Pierdoná and João Paulo Corrêa for the stimulating discussions, coffees and the friendship in the last years.

To my parents Leila and Vanderlei, my heartfelt gratitude for the love, prayers, patience, and sacrifice. I also express my thanks to my siblings Ligia and Gustavo, for their support and encouragement.

Finally, thanks to God for always being present in my life and giving me the strength to overcome all challenges.

"I was taught that the way of progress was neither swift nor easy."

Marie Curie

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RESUMO

Caracterização genética e molecular do desenvolvimento de tricomas glandulares tipo IV em tomateiro (*Solanum lycopersicum* cv. Micro-Tom) e sua participação na resistência a artrópodes

Os tricomas glandulares são apêndices epidérmicos capazes de produzir, estocar e liberar metabólitos de importância econômica e ecológica que, dentre outras funções, têm papel fundamental na defesa das plantas. A via de desenvolvimento desses tricomas ainda permanece obscura, uma vez que a maior parte dos estudos desenvolvidos envolve a planta modelo *Arabidopsis thaliana*, na qual os tricomas multicelulares são ausentes. O gênero *Solanum* possui uma grande diversidade de tipos de tricomas, em especial os glandulares (tipos I, IV, VI e VII). Espécies selvagens como *S. galapagense* e *S. pennellii* são fontes de recursos genéticos para o tomateiro (*S. lycopersicum*) por possuírem variações genéticas naturais que lhes conferem maior resistência ao ataque de herbívoros. Dentre essas variações, está a presença de tricomas glandulares do tipo IV, sendo eles fontes do aleloquímico acilaçúcar (AS), uma substância multifuncional no controle de herbívoros. No presente trabalho, avançou-se na elucidação das bases genéticas que controlam o desenvolvimento dos tricomas tipo IV, além de entender a relação dessa estrutura com a resistência a herbívoros. Para isso, criou-se duas linhas de introgressão nas quais a capacidade de desenvolver tricomas IV tanto de *S. galapagense* quanto de *S. pennellii* foi introgridida na cv. Micro-Tom. No primeiro capítulo, encontram-se os resultados da linhagem *Galapagos enhanced trichomes* (MT-Get), produzida a partir do cruzamento com *S. galapagense*. Embora, a linhagem tenha apresentado alta densidade de tricomas do tipo IV, as plantas continuaram suscetíveis à mosca branca (*Bemisia tabaci*). As análises de GC-MS, LC-MS e expressão gênica demonstraram que a presença de tricomas IV não é suficiente para alta produção de AS. Além disso, o mapeamento por sequenciamento de MT-Get revelou que cinco regiões cromossômicas contendo alelos de *S. galapagense* estão associadas ao fenótipo apresentado. Assim, esses resultados fornecem a base para a compreensão do desenvolvimento de tricomas glandulares, mostrando seu caráter poligênico em *S. galapagense*. Além disso, os resultados subsidiam a obtenção de tomateiros resistentes a insetos a partir de variedades com alta densidade de tricomas IV, a serem posteriormente complementada pela piramidação das vias metabólicas associadas à produção de AS. No segundo capítulo, a linhagem *Pennelli enhanced trichomes* (MT-Pet) foi obtida e analisada. Nela, uma única região cromossômica de *S. pennellii* está relacionada com a presença de tricomas IV. As plantas não apresentaram caracteres pleiotrópicos ligados à presença do tricoma IV e as análises de GC-MS e ensaio de Rodamina-B apontam uma ausência de AS. Embora a quantidade de tricomas IV seja bem menor que em Get, o caráter monogênico de Pet poderá facilitar o isolamento de um dos principais genes envolvidos no desenvolvimento dessa estrutura, assim contribuindo para desvendar a base genética da formação de tricomas glandulares, um via de desenvolvimento ainda praticamente desconhecida.

Palavras-chave: Herbivoria; Micro-Tom; *S. galapagense*, *S. pennellii*; Tricomas; Tomateiro;

ABSTRACT

Genetic and molecular characterization of type-IV glandular trichome development in tomato (*Solanum lycopersicum* cv. Micro-Tom) and its participation in arthropod resistance.

Glandular trichomes are epidermal appendages capable of producing, storing, and releasing metabolites of economic and ecological importance that, among other functions, have a fundamental role in plant defense. The developmental path of these trichomes remains unclear since most of the studies involve the model plant *Arabidopsis thaliana*, in which multicellular trichomes are absent. The genus *Solanum* has a great diversity of trichomes, especially glandular types (I, IV, VI, and VII). Wild species such as *S. galapagense* and *S. pennellii* are sources of genetic resources for tomato (*S. lycopersicum*) because they have natural genetic variations that provide them with greater resistance to herbivore attacks. Among these variations, there is the presence of type-IV glandular trichomes, a source of acylsugar (AS), a multifunctional substance in the herbivores control. In this work, we sought to elucidate the genetic bases that control the development of type-IV glandular trichomes, in addition to understand the relationship of this structure with herbivory resistance. For this, two introgression lines were created in which the ability of both *S. galapagense* and *S. pennellii* to develop type-IV trichomes was introgressed into the cv. Micro-Tom. In the first chapter, we explored the *Galapagos enhanced trichomes* (MT-Get) line, which was produced from a crossing with *S. galapagense*. Although the lineage had a high density of type-IV trichomes, the plants remained susceptible to whitefly (*Bemisia tabaci*). Analysis of GC-MS, LC-MS, and gene expression showed that the presence of type-IV trichomes was not enough for AS high production. Furthermore, MT-Get mapping-by-sequencing revealed that five chromosomal regions containing *S. galapagense* alleles are associated with this phenotype. Thus, these results provide the basis for understanding the development of glandular trichomes, showing their polygenic nature in *S. galapagense*. Besides, they also subsidize the creation of insect-resistant tomatoes from varieties with a high density of type-IV trichomes, to be later complemented by pyramidization with the metabolic pathways associated with AS production. In the second chapter, the line *Pennelli enhanced trichomes* (MT-Pet) was obtained. In it, a single chromosomal region of *S. pennellii* is related to the presence of type-IV trichomes. The plants did not show any pleiotropic traits linked to the presence of type-IV trichomes, but GC-MS analyses and Rhodamine-B assay revealed a lack of AS. Although the type-IV trichome density in MT-Pet is much lower than in MT-Get, the monogenic trait of MT-Pet may facilitate the isolation of a critical ontogenic gene, thus helping to unravel the genetic basis for the formation of glandular trichomes, a development path that is still virtually unknown.

Keywords: Herbivory; Micro-Tom; *S. galapagense*, *S. pennellii*; Trichomes; Tomato

1 INTRODUCTION

1.1 General aspects of trichomes

Trichomes are appendages that protrude from the epidermis of aboveground organs in most plant species. They may vary in density or shape from one species to another. Among the several classification systems, the most common one is according to the presence or absence of secretory glands into glandular and non-glandular or tector, respectively (Glas *et al.*, 2012; Schuurink & Tissier, 2019).

The function of these structures may be related to the organ bearing them (Werker, 2000). Protection against insect herbivory, providing pathogen resistance, reducing transpiration, and increasing tolerance to abiotic factors (ultraviolet light, freezing, and drought stress) are among the beneficial roles ascribed to trichomes (Hülkamp 2004; Wagner, 2004; Yang & Ye, 2013; Kim, 2018).

Considering plant herbivory resistance, which is the main focus of this work, trichomes can be divided into two functional categories: mechanic and chemical. Non-glandular trichomes play as a physical barrier that makes it difficult for small insects to land, move, and feed. In turn, the role of chemical defense provided by glandular trichomes (GTs) is to produce some substances that are repellent, toxic or label insects for recognition by their predators (Levin, 1973; Lara, 1991; Weinhold & Baldwin, 2011; Liu *et al.*, 2018; Kim, 2018).

In addition to their significance for insect resistance, GTs are also commercially important in a myriad of other ecological interactions and industrial uses. They are present in approximately 30% of all vascular plant species and can produce, store, and often secrete natural products, including chemodiverse types of volatile, semi-volatile, and non-volatile compounds (Liu *et al.*, 2018; Chalvin *et al.*, 2020). These products can be used as scents, pigments, medicines, and food additives. For example, GTs of *Artemisia annua* produce a sesquiterpene named artemisinin used for the treatment of malaria (Lommen *et al.*, 2006; Schuurink & Tissier, 2019); cannabinoids from *Cannabis sativa* are extensively studied for medicinal purposes, including anxiety, psychosis, nausea, inflammation, and immunomodulation (Happyana *et al.*, 2013; Andre *et al.*, 2016); ambrox is a perfume ingredient synthesized from sclareol, a diterpene extracted from *Salvia sclarea* (Chalvin *et al.*, 2020); plant products with organoleptic properties, such as lavender (*Lavandula pedunculata*), oregano (*Origanum vulgare*), hops (*Humulus lupulus*), and mints (*Mentha* spp.) are synthesized in the GTs on the leaves of plants from

diverse botanical families (McConkey *et al.*, 2000; Wang *et al.*, 2008; Zuzarte *et al.*, 2010; Shafiee-Hajiabad *et al.*, 2014).

Trichomes are an excellent model system to study cell fate and differentiation because they have an epidermal origin and, therefore, are easily accessible. They are extensively studied in *Arabidopsis thaliana*, although this species only develops one trichome type, which is described as unicellular and non-glandular (Hülkamp, 2004; Schuurink & Tissier, 2019). Given this scenario and the ecological, agronomical, and economic importance of multicellular trichomes, especially the glandular types, the study of the development of these structures in other models is essential to advance the current knowledge in this area.

1.2 Molecular basis of trichome development

As aforementioned, the plant model par excellence, *Arabidopsis thaliana*, only develops a single type of trichome. Most of the knowledge about molecular mechanisms of trichome development comes from this model (Balkunde *et al.*, 2010). Essentially, a protodermal cell is selected on the basis when the leaf primordium grows to 140 - 200 μm in length (Larkin *et al.*, 1996). A specific epidermal cell switches from mitosis to four endoreduplication cycles, thus allowing the progenitor trichome cell to enlarge rapidly, project perpendicularly to the leaf surface, and undergo two consecutive branching events (Hülkamp, 2004). This process can be divided into three phases: determination of the cell fate, cell replacement, and morphogenesis. Each one of these steps is coordinated by several genes, as summarized by Fambrini & Pugliesi (2019). In general, genes from three families activate these phases: WD40 (*TRANSPARENT TESTA GLABRA1*, *TTG1*), bHLH (*GLABRA3*, *ENHANCER OF GLABRA3*, *TRANSPARENT TESTA*, and *MYC-1*), and MYB (*GLABRA1*, *MYB23*, and *MYB5*). These proteins act in a partially redundant manner to form an activating complex known as MYB-bHLH-WD40 (MBW) that binds to the *GLABRA2* (*GL2*) promoter, which protein has a homeodomain required during morphogenesis phase (endoreduplication, branching, and cell maturation). The negative regulators already described in the literature are single-repeat MYB proteins (*CAPRICE*, *TRIPTYCHON*, *ENHANCED OF TRY*, *CPC 1, 2, 3*, and *TRICHOMELESS1* and *2*) (Yang & Ye, 2013; Hauser, 2014).

Despite detailed knowledge about the development of unicellular, non-glandular trichomes, some evidence indicates that the formation of multicellular, glandular

trichomes is probably controlled by a distinct gene network than that controlling trichome formation in *Arabidopsis*. Indeed, some *Arabidopsis* genes, such as *GL1*, were over-expressed in tobacco (*Nicotiana tabacum*), and did not affect trichome development (Yang & Ye, 2013).

So far, little information is known about the molecular aspects of the development of multicellular trichomes. The gene *MIXTA* codifies for an R2R3-MYB transcription factor involved in the development of multicellular trichomes in *Antirrhinum majus*, *Gossypium hirsutum*, *Mimulus guttatus*, *Populus trichopoda*, *Medicago truncatula*, and *Dendrobium crumenatum* (Brockington *et al.*, 2013). Other members of the R2R3-MYB subfamily have been characterized as positive regulators of multicellular trichome initiation: *CotMYBA*, and *GaMYB2* (cotton), *AaMYB1* and *AaMIXTA1* (*Artemisia annua*), and *SLMX1* (tomato) (Payne *et al.*, 1999; Serna & Martin, 2006; Yang & Ye, 2013; Ewas *et al.*, 2017; Matías-Hernández *et al.*, 2017; Shi *et al.*, 2018; Fambrini & Pugliesi, 2019).

In tomato, an HD-ZIP IV transcription factor, *WOOLLY* (*Wo*), appears to be a regulator of GT initiation when interacting with a B-type cyclin, *CycB2*. The same behavior is observed for *AaHD1* and *AaHD8* from *A. annua* (Yang *et al.*, 2011, 2015; Yan *et al.*, 2017). Another HD-ZIP IV involved in GT formation in *S. lycopersicum* is *CUTIN DEFICIENT 2* (*SICD2*) (Nadakuduti *et al.*, 2012; Chalvin *et al.*, 2020). The loss of function of *SICD2* leads to, among other phenotypes, a reduced GT density (Nadakuduti *et al.*, 2012). Moreover, the tomato genes *HAIR* (*SIH*, C2H2 zinc-finger family) and *MYELOCYTOMATOSIS-RELATED1* (*SIMYC1*, a bHLH protein) were recently described. *SIH* is a positive regulator of GT initiation, and *SIMYC1* regulates type-VI trichome development (Chang *et al.*, 2018; Xu *et al.*, 2018).

Therefore, despite the growing amount of information involving GT development gathered recently, the knowledge of the genetic pathways and molecular mechanisms remains incipient (Schuurink & Tissier, 2019).

1.3 Study of trichomes in the *Solanum* genus

In the *Solanum* genus, trichomes are classified into seven types: four glandular (I, IV, VI, VII) and three non-glandular (II, III, V) types (Luckwill, 1943; Channarayappa *et al.*, 1992; Simmons & Gurr, 2005) (**Fig. 1, Table 1**). The cultivated tomato presents several trichomes types covering almost the totality of its external surface. However, the

glandular trichomes of the cultivated tomato do not produce allelochemicals in sufficient quantities to render it resistant to the major pests of the crop, as seen in some of its wild relatives. Indeed, some wild tomato species produce high amounts of allelochemicals, such as sesquiterpenes and methyl ketones in *Solanum habrochaites* (Simmons & Gurr, 2005; Bleeker *et al.*, 2009), or acylsugars that cause the typical sticky leaves of *Solanum pennelli* and *Solanum galapagense* (Liedl *et al.*, 1995; Blauth *et al.*, 1998).

Fig. 1 Schematic representation of the trichome types on the epidermis of aboveground organs in the tomato clade (*Solanaceae*, section *Lycopersicon*). Glandular trichomes are the types I, IV, VI, and VII. Observe the structural similarity between the trichome types IV and V, as well as I and II.

Table 1. Distinctive trichome features, according to Luckwill (1943), and revised by Channarayappa *et al.* (1992) and Glas *et al.* (2012).

Type	Description			
	Base		Stalk	Gland
I	Globular multicellular	6–10 cells	2–3 mm	Single, small and round
II	Globular multicellular	6–10 cells	0.2–1.0 mm	Non-glandular
III	Flat unicellular	4–8 cells	0.4–1.0 mm	Non-glandular
IV	Flat unicellular	Up to 3 cells	0.2–0.4 mm	Single, small and round
V	Flat unicellular	Up to 3 cells	0.2–0.4 mm	Non-glandular
VI	Flat unicellular	2 cells	0.1–0.2 mm	4 gland cells
VII	Flat unicellular	Unicellular	0.05 mm	4–8 gland cells

Due to the structural variability of trichomes present in the genus *Solanum*, this work focused on exploring the development of type-IV trichomes. This trichome type holds a head that exudes a viscous material containing acylsugars, which have been consistently linked to broad-spectrum resistance to fungal pathogens (Luu *et al.*, 2017) as well as herbivorous arthropods (insects and mites) of tomato, including Lepidoptera (*Helicoverpa amigera*, *Spodoptera exigua*, *Phthorimeae operculella*), Acarina (*T. urticae*), and Hemiptera (*Myzus persicae*) (Goffreda *et al.*, 1990; Hawthorne *et al.*, 1992; Liedl *et al.*, 1995; Simmons & Gurr, 2005; Maluf *et al.*, 2010). Until recently, it was assumed that type-IV trichomes were absent on the leaves of the cultivated tomato. Vendemiatti *et al.* (2017), however, demonstrated that the cultivated tomato does develop these structures on cotyledonary and young leaves. This fact implies that the development of this structure is regulated ontogenically and that the tomato does have the genetic machinery to develop this type of glandular trichome. Type-IV trichomes have been identified in other *Solanaceae* crops and closely related species, such as *Capsicum* peppers (Kim *et al.*, 2011), *Petunia hybrida* (Sasse *et al.*, 2016), the medicinal *Datura wrightii* (Dam *et al.*, 1999), as well as the close relative to eggplant, *Solanum sisymbriifolium* (Hill *et al.*, 1997 – herein named trichome B). Therefore, the study of type-IV trichomes in tomato will potentially extend to other *Solanaceae* species of global economic importance as well.

The tomato (*Solanum lycopersicum*) has been proposed as an ideal model for the study of identity control of glandular trichomes (Goffreda *et al.*, 1988). Among the advantages of this model are the relatively small, diploid genome (950 Mb), which has already been sequenced and annotated (The Tomato Genome Consortium, 2012), self-compatibility and prolificity, the availability of genetic variation in many accessions covering the whole tomato clade (i.e., many closely related wild relatives), a saturated genetic map covering virtually all crop traits of interest, the existence of mutant and characterized introgression line collections (Eshed & Zamir, 1995; Pino-Nunes *et al.*, 2009), the availability of efficient transformation protocols (Sun *et al.*, 2006; Pino *et al.*, 2010; Zsögön *et al.*, 2018), and an extensive physiological and biochemical knowledge base amassed over many decades of breeding studies. Furthermore, the miniature cultivar Micro-Tom (MT) can be used in research for speed and efficient space use purposes. This cultivar developed by Scott (1989) was proposed as a model by Meissener *et al.* (1997) due to its small size (~15 cm) and short life cycle (90-120 days

from seed to seed). The use of this cultivar has allowed working in very limited spaces (up to 78 plants m⁻²) and to obtain relatively fast results (Campos *et al.*, 2010). Our research demonstrated that there are no differences in trichome development between the cultivar MT compared to other genotypes used in tomato research, such as Moneymaker and M82 (Vendemiatti *et al.*, 2017).

As a model, the tomato has advantages because it is one of the most important crops worldwide (Croizer *et al.*, 1997). The global production of tomato in 2018 was 182,22 million tons cultivated in 4,76 million ha (FAOSTAT, 2020). Nevertheless, this crop is highly susceptible to many pests and diseases, and its commercial production requires intense protection and high levels of input, including frequent applications of pesticides (Campos, 2009). Thus, in line with our goal to get insights into the development of glandular trichomes, we also study the genetic basis of insect resistance that is provided by glandular trichomes. This approach has the potential to reduce pesticide use and increase yields significantly, and therefore, increase crop sustainability as well as farmer profits, which are aspects negatively associated with agrochemical use (Simmons & Gurr, 2005).

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2 NATURAL GENETIC VARIATION FROM THE GALAPAGOS TOMATO (*Solanum galapagense*) REVEALS MULTIPLE STEPS NECESSARY FOR ACYLSUGAR-BASED INSECT RESISTANCE.

2.1 Abstract

Glandular trichomes are involved in the production of food- and medicine-relevant chemicals, besides being associated with pest resistance in plants. In some wild *Solanum* species closely related to the cultivated tomato (*S. lycopersicum*), the presence of type-IV glandular trichomes leads to the production of high levels of insecticide acylsugars (AS). Conversely, low AS production observed in the cultivated tomato is attributed to its incapacity to develop type-IV trichomes. Therefore, it is hypothesized that cultivated tomatoes engineered to harbor type-IV trichomes will be pest resistant. We introgressed the capability of *S. galapagense* to develop type-IV trichomes into the tomato cultivar Micro-Tom (MT), thus creating a line named "*Galapagos enhanced trichomes*" (*Get*). Although MT-*Get* presented a high density of type-IV trichomes on leaves, MT-*Get* did not differ from MT in its susceptibility to whitefly (*Bemisia tabaci*). GC-MS, LC-MS, and gene expression analyses demonstrated that the sole presence of type-IV trichomes is not sufficient for high AS production. Mapping-by-sequencing of MT-*Get* revealed that five chromosomal regions containing *S. galapagense* alleles are associated with the phenotype. Our findings provide the basis for the understanding of glandular trichome development and for molecular breeding of tomato varieties harboring a high density of type-IV trichomes, which needs to be further complemented through the stacking of the associated AS metabolic pathway to confer insect resistance.

Keywords: Acylsugars; Glandular trichome; Herbivory; *Solanum lycopersicum*.

2.2 Introduction

Glandular trichomes have called considerable attention due to their economic potential as sources of a vast array of secondary metabolites (Schimiller *et al.*, 2008; Tissier, 2012). Among such metabolites, many have industrial or medicinal value (Aharoni *et al.*, 2005; Maes *et al.*, 2011), whereas others are especially relevant in the protection against insect pests (Kang *et al.*, 2010; Glas *et al.*, 2012). The cultivated tomato and its wild relatives display considerable variation in trichome type, size and number. Eight morphologically distinct types were defined for the *Lycopersicon* clade (the cultivated tomato and its 16 closest wild relatives), out of which four are glandular: the types I, IV, VI, and VII (Luckwill, 1943; Glas *et al.*, 2012). Type-IV trichomes (and, to a lesser extent, type-I trichomes, which are rare on leaves) are sources of allelochemical

compounds called acylsugars (AS) (Kim *et al.*, 2012; Ghosh *et al.*, 2014; Ning *et al.*, 2015; Schillmiller *et al.*, 2015; Fan *et al.*, 2016).

In the genus *Solanum*, AS confer resistance to fungal pathogens (Luu *et al.*, 2017) and to multiple insect pests (Goffreda *et al.*, 1990; Hawthorne *et al.*, 1992; Liedl *et al.*, 1995), such as whiteflies (*Bemisia spp.*), which is a major tomato (*S. lycopersicum*) pest worldwide (Maluf *et al.*, 2010). AS deter insect and other arthropod attacks via distinct mechanisms, such as poisoning, sticking, and even “labeling” insects to increase predator recognition (Weinhold & Baldwin, 2011). AS consist of aliphatic acyl groups of variable chain lengths (C2 to C12) esterified to a glucose (G) or sucrose (S) moiety at four possible positions (Schillmiller *et al.*, 2012; Ning *et al.*, 2015; Fan *et al.*, 2019). For instance, the AS molecule called S4:23 (2,4,5,12) is a sucrose-based AS esterified with C2, C4, C5, and C12 acyl groups.

Type-IV trichomes have a single flat basal cell with a short two- or three-celled stalk (0.2-0.4 mm) and a round gland at the tip. They are particularly abundant in the wild species *S. galapagense*, *S. habrochaites*, and *S. pennellii* and are the site of biosynthesis and secretion of AS (Simmons & Gurr, 2005). These species produce much larger amounts of AS compared to the cultivated tomato (Fobes *et al.*, 1985; Schillmiller *et al.*, 2010). AS accumulation is considered to be the cause of the robust and multiple pest resistances of these wild tomato species (Mutschler *et al.*, 1996; Momotaz *et al.*, 2010; Rodriguez-Lopez *et al.*, 2011; Leckie *et al.*, 2012; Schillmiller *et al.*, 2012). It has been long assumed that the cultivated tomato did not develop type-IV trichomes (Luckwill, 1943; Simmons & Gurr, 2005; McDowell *et al.*, 2011; Glas *et al.*, 2012). However, we have recently demonstrated that they do appear in the early stages of plant development (from the cotyledons to the 3rd – 6th leaf, depending on the cultivar), being markers of juvenility in tomato (Vendemiatti *et al.*, 2017). Therefore, the lack of type-IV trichomes in the adult phase of the cultivated tomato explains the low presence of AS and susceptibility to herbivores.

Interestingly, the structural genes coding for the enzymes of the AS biosynthesis pathway are present in *S. lycopersicum* (Schillmiller *et al.*, 2012; Schillmiller *et al.*, 2015; Fan *et al.*, 2016; Smeda *et al.*, 2018). Indeed, four acylsugar acyltransferases (SLAT1–SLAT4) belonging to the BAHD acyltransferase superfamily were identified in the genomes of the cultivated tomato as well as its wild relatives (Schillmiller *et al.*, 2012; Schillmiller *et al.*, 2015; Fan *et al.*, 2016; Fan *et al.*, 2019). Biochemical characterization

studies revealed that these ASATs sequentially catalyze the esterification of acyl chains at different positions of a sucrose moiety to generate a tetra-acylsucrose. Therefore, the catalytic activities of ASATs with different substrates can explain the diverse structures of *Solanum* spp. acylsugars (Ghosh *et al.*, 2014; Fan *et al.*, 2016; Nadakuduti *et al.*, 2017). Altogether, these observations led to the hypothesis that a cultivated tomato line modified to harbor type-IV trichomes on its adult leaves would accumulate high AS levels and be naturally resistant to pests.

Here, we successfully introgressed the capability of developing type-IV trichomes on adult leaves from *S. galapagense* LA1401 into the tomato genetic model system, cv. Micro-Tom (MT) (Carvalho *et al.*, 2011). The introgressed MT line was named "*Galapagos enhanced trichomes*" (MT-*Get*) and showed a high density of type-IV trichomes on all leaves throughout plant development. However, the plants did not display increased resistance against whiteflies in a preliminary assay. Biochemical analyses of leaf samples with type-IV trichomes by LC-MS and GC-MS demonstrated that MT-*Get* did not accumulate high levels of AS, even though qRT-PCR analysis of *SlAT1-SlAT4* genes and a transgenic line with the cassette *pSlAT2::GFP* suggested that the type-IV trichomes were functional. This result led us to the conclusion that the developmental pathway for the presence of type-IV trichome on adult leaves and the high accumulation of AS are uncoupled, and that different and hitherto unknown components comprise the resistance trait. Mapping-by-sequencing of the MT-*Get* segregating population revealed the development of type-IV trichomes to be a genetically complex trait since at least five chromosomal regions containing *S. galapagense* alleles are likely necessary for the full phenotype. Our findings pave the way for molecular breeding of commercial varieties harboring a high density of type-IV trichomes on adult tomato leaves. However, further studies are needed to unveil additional genetic components involved in the developmental and biochemical pathways necessary to confer high AS production and insect resistance in the cultivated tomato.

2.3 Materials and Methods

2.3.1 Plant material, growth conditions and breeding scheme

Seeds of *Solanum galapagense* LA1401 were obtained by the Tomato Genetics Resource Center (TGRC - University of California). Micro-Tom (MT) seeds were donated by Dr. Avram Levy (Weizmann Institute of Science, Israel) and maintained through self-

pollination as a true-to-type cultivar since 1998. The “*Galapagos enhanced trichome*” (MT-*Get*) line was generated by the cross MT x *S. galapagense* LA1401 using MT as the female donor and as the recurrent parent in the further backcrosses necessary for introgression (**Fig. 1**). The process of introgression was based on visual screening on stereoscope for the presence of type-IV trichomes on adult leaves (5th leaf in the MT background; Vendemiatti *et al.*, 2017) and followed the procedure previously described by Pino *et al.* (2010). Plants were grown in a greenhouse under natural day-length conditions (Lombardi-Crestana *et al.*, 2012).

2.3.2 Trichome counting and phenotyping

Identification and counting of trichomes were followed as described by Vendemiatti *et al.* (2017). At least 8 individuals per genotype were sampled, and four different samples were analyzed per plant on each leaf surface. Photographs were taken using a Leica S8AP0 (Wetzlar, Germany) at 50x magnification, coupled to a Leica DFC295 camera (Wetzlar, Germany). Counting and length measurements of trichomes were performed on the images using the manufacturer’s analytical program (Leica Application Suite 4.0).

2.3.3 Scanning electron microscopy

Leaf samples were fixed in Karnovsky solution for 24 hours at 4°C. The material was then washed twice with 0.05 M cacodylate solution for 10 minutes and fixed again in osmium tetroxide for 1 hour. Subsequently, the samples were washed with distilled water and dehydrated in a series of acetone baths. The dehydrated samples were submitted to drying to the critical point, and subsequently gold plated. The observations were performed on a LEO 435 VP scanning electron microscope (SEMTech Solutions, Massachusetts).

2.3.4 Plant genetic transformation

Constructs containing the green fluorescent protein (GFP) reporter driven by *pSlAT2*, which directed the expression of GFP to the tip of trichomes types I and IV (Schillmiller *et al.*, 2012), were kindly provided by Dr. Robert Last (Michigan State University, USA). The constructs were introduced into *Agrobacterium tumefaciens* LBA4404 and used to transform MT and MT-*Get* as described by Pino *et al.* (2010).

Plants regenerated under kanamycin selection, were acclimated in a greenhouse, and cultivated as described above.

2.3.5 Fluorescence microscopy

For trichome-specific expression of GFP under the *SLAT2* promoter, analyses were carried out under a Nikon SMZ18 stereoscope attached to a Nikon DS-RI1 digital camera. Excitation at 480 nm and a 505 nm emission filter detected fluorescence specifically from GFP. For chloroplast fluorescence detection, trichomes were observed under a Carl Zeiss Axioskop 2 microscope coupled to Axiocam MRc Zeiss camera using 540/625 nm excitation/emission filter.

2.3.6 Mapping-by-Sequencing Analysis

A segregating BC₇F₂ population composed of 315 plants from the cross MT-*Get* (BC₆F_n) x MT was phenotyped for the presence of type-IV trichomes on adult (5th) leaves according to the methodology described above. Plants were classified into two populations: plants with leaves bearing type-IV trichomes (*Get*-like) and those with virtually no type-IV trichomes observed (MT-like). Five leaf discs (7-mm diameter) from each plant were collected and pooled to form each phenotypical population prior to the extraction of genomic DNA using the method described by Fulton *et al.* (1995). The genomic DNA was further purified with the MasterPure kit (Lucigen, MC85200) and submitted to sequencing on a HiSeq PE150bp (Illumina) at Novogene (<https://en.novogene.com>) with 30X depth. Fastq files for each population were concatenated and submitted to quality check by FastQC on the Galaxy platform (<https://usegalaxy.org>). Mapping of reads against the cv. Micro-Tom v.1 genome reference sequence (Sol_mic assembly: <http://gbf.toulouse.inra.fr/Genome>) was carried out with the “Map with BWA for Illumina” (v.1.2.3) software on Galaxy, using default parameters (Li & Durbin, 2009) to generate “.sam” files. The variant calling against the cv. Micro-Tom assembly was performed with Samtools Mpileup (v.1.8) (Li *et al.*, 2009) and BCFtools call (v.1.6) to generate VCF files. Further comparisons between *Get*-like variants (S1) against the MT-like population (S4) were performed with BCFtools isec (v.1.6) on the command line interface. The variant allelic frequencies were filtered per site relative to the MT genome sequence using the following parameters: i) total depth of reading (10<DP<100); ii) allele frequency (AF>=0.8), which is defined as the

alternative depth of reading (AD) divided by the total depth of reading (DP); and iii) number of variants per 1 Mbp window ≥ 30 (Mascher *et al.*, 2014; Garcia *et al.*, 2016). The analysis pipeline can be visualized in **Suppl. Figure S1**.

2.3.7 *Micro-Tom and S. galapagense allele genotyping*

Genomic DNA was extracted using the protocol described by Fulton *et al.* (1995). The DNA quantity and quality were determined using agarose gel electrophoresis and NanoDrop One spectrophotometer (Thermo Fisher Scientific). The genotyping was performed using CAPS markers that discriminate *Solanum lycopersicum* cv. MT and *Solanum galapagense* alleles (**Suppl. Table S1**). Each 12- μ L PCR reaction contained 1.0 μ L DNA, 1.2 μ L Taq buffer (10x), 1.5 μ L MgCl₂ (25 nM), 0.2 μ L dNTPs (10 mM), 0.4 μ L each primer (10 pM), 0.1 μ L Taq DNA polymerase (5U/ μ L - Thermo Fisher Scientific), and 7.2 μ L distilled water. The PCR programs were developed according to the optimum annealing temperatures and amplicon sizes of each primer set. The digestion reactions (10 μ L) contained 4.0 μ L PCR product, 1.0 μ L enzyme buffer (10x), 0.2 μ L restriction enzyme, and 4.8 μ L water. The products were analyzed on 1.5% (w/v) agarose gels.

2.3.8 *Plant phenotyping*

Forty-eight-day old plants were used for measuring the length of the main stem and the length of the secondary branches of the plant, and the branching index was calculated according to Morris *et al.* (2001).

MT and MT-*Get* plants were hand self-pollinated because the MT-*Get* genotype presented low fruit setting rates. Many ovaries were pollinated, but after fruit set confirmation (five days after pollination), we performed selective fruit thinning to allow only five fruits to develop on each plant. The agronomic performance of the plants was assessed 90 days after germination. The following fruit parameters were determined: total fruit weight per plant, average weight per fruit, and total soluble solids content in fruits (Brix). The total soluble solids (TSS) content was assessed in all fruits of the plants using a digital refractometer (PR-101, Atago, Tokyo, Japan).

2.3.9 *Herbivory test with Bemisia tabaci*

The experiment was conducted at the Nunhems Company's Research Station (Uberlândia, Brazil). Seeds were sown in plastic trays using coconut fiber substrate and

remained in greenhouse conditions until the transplant. The seedlings were then transplanted into 8-liter pots with substrate. Each pot received 5 plants of the same genotype. The plants were kept in a greenhouse until 23 days after transplanting. During the interval between transplanting and the beginning of inoculation, the plants received appropriate cultural treatments and fertilization. After 23 days, the pots were randomly placed in a greenhouse chamber (7m x 15m) highly infested with a whitefly population (*Bemisia tabaci*), where the insects are bred and kept exclusively for tomato resistance tests. The pots remained there for 7 days to allow egg laying on the leaves of the plants. After the inoculation period, a vase of each genotype, each containing 5 plants, was randomly collected and sent to the Hormonal Control of Plant Development Laboratory (ESALQ/USP). From these plants, 30 leaflets were collected to perform the counting of hatched nymphs.

2.3.10 Rhodamine-B assay

Leaflets of *S. galapagense* and MT-Get were submerged in a 0.1% aqueous solution of Rhodamine-B for one minute. Subsequently, four sequential baths were run in distilled water to remove the excess dye. The images of the stained trichomes were taken as previously described.

2.3.11 LC-MS/MS analysis of surface extracts

This experiment was carried out by the Glandular Trichomes and Isoprenoid Biosynthesis Research Group at the Leibniz Institute for Plant Biochemistry (Halle, Germany). The analysis of the semipolar metabolites extracted was performed on an LC-MS/MS system composed of an Acquity UPLC (Waters GmbH, Eschborn, Germany) and a TripleTOF 5600 mass spectrometer (SCIEX, Toronto, Canada). For the separation of the analytes, 5 µL of extracts were injected into a Nucleoshell RP 18 column (2.7 µm x 150 mm x 2 mm, Macherey-Nagel GmbH, Düren, Germany). A solvent system composed of A: 0.3 mM ammonium formate acidified with formic acid at pH 3, and B: acetonitrile, with the following gradient was used: 0-2 min: isocratic 95% A, 2-19 min: linear from 95% to 5% A, 19-22 min: isocratic 5% A, 22-22.01 min: linear from 5% A to 95% A, 22.01-24 min: isocratic 95% A. The flow rate was set to 400 µL/min throughout and the column temperature was 40°C. Analyte ionization was performed by electrospray ionization in negative mode with the following parameters: gas 1 = 60 psi, gas 2 = 70 psi, curtain gas =

35 psi, temperature = 600°C and ion spray voltage floating= -4500 V. CID fragment spectra were generated in SWATH mode (Hopfgartner *et al.*, 2012) with mass windows of 33 Da and rolling collision energies from -10 to -80 V with a collision energy spread of 15 V. The integration of the peak areas was performed by Multiquant (Version 2.0.2; SCIEX, Toronto, Canada).

2.3.12 GC-MS Acyl sugar quantification

This experiment was carried out at the Laboratory of Biochemistry and Instrumental Analysis of the Department of Agroindustry, Food, and Nutrition (ESALQ-USP). Leaves of the adult vegetative phase (5th leaf) were collected, and the extraction was conducted according to the methodology described by Leckie *et al.* (2013). The compounds were separated via GC-2010 gas chromatography (Shimadzu Corp., Kyoto, Japan) attached to a QP 2010 Plus mass spectrometer (Shimadzu Corp., Kyoto, Japan), using Helium as the charging gas. For the separation of acyl groups from the acyl-sugar molecules, hexane was injected into a DB-WAX apolar column (0.25 mm diameter, 30 m length, and 0.25 µm film thickness). The data obtained were analyzed using the software Lab Solutions-GCMS version 2.5 (Shimadzu Corp., Kyoto, Japan). The compound identification was based on the retention time of chromatographic peaks and fragments of the mass spectrometer, which were compared to the available standards and data libraries.

2.3.13 Gene expression analyses

The expression of key genes involved in the acylsugar biosynthesis pathway was performed by real-time PCR. Total RNA was isolated from leaf pools of the genotypes MT-*Get* and the wild species *S. galapagense* using the mirVana™ Isolation Kit (Ambion) according to the manufacturer's instructions. The RNA was quantified on a NanoDrop One UV-Vis Spectrophotometer (Thermo Scientific), and the RNA integrity was examined by gel electrophoresis. The total RNA was treated with TURBO DNA-free™ Kit (Invitrogen™) and subsequently used for cDNA synthesis using the SUPERScript™ IV 1st Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR (qPCR) reactions were conducted in a 10-µL total volume using a 2× GoTaq® qPCR Master Mix (Promega) and run on an ABI 7500 qPCR thermocycler (Applied Biosystems). The constitutive housekeeping genes actin (Solyc04g011500) and

elongation factor 1 alpha (EF-1 α , Solyc06g005060) were used as internal controls. We used three biological repetitions, each composed of 5 leaves, and three technical repetitions. The threshold cycle (C_T) was determined automatically by the instrument, and fold changes for each gene of interest were calculated using the equation $2^{-\Delta\Delta C_t}$ (Livak & Schmittgen, 2001). The qPCR primer sequences used are listed in **Supplemental Table S1**.

2.3.14 Statistical analyses

The LC-MS data were converted to the Log10 function before analysis. The statistical comparisons with ANOVA and the Student's *t*-test were performed using the SAS software. The remaining results were compared statistically by the Student's *t*-test using GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.4 Results

2.4.1 Introgression of the capacity to display type-IV trichomes on adult leaves from *Solanum galapagense* LA1401 into tomato (*S. lycopersicum* cv. Micro-Tom).

Solanum galapagense LA1401 was chosen as a source of type-IV trichomes for genetic introgression. This species is closely related to the cultivated tomato, and we noticed that, unlike tomato, it has a high density of type-IV trichomes on adult leaves, especially on the abaxial leaf surface (**Fig. 1a-c**). We have previously shown that the cultivated tomato plant produces type-IV trichomes only on cotyledons and juvenile leaves but not on adult leaves (Vendemiatti *et al.*, 2017). We, therefore, set out to introduce the genetic determinants controlling the capacity to bear type-IV trichomes on adult leaves from *S. galapagense* into the cultivated tomato.

Solanum lycopersicum cv. Micro-Tom was fertilized with pollen from *S. galapagense*. After self-fertilization of F₁ plants, we selected F₂ plants with type-IV trichomes on adult leaves. These plants were backcrossed (BC₁) using MT as the recurrent parent. The process was repeated five more times until a stable BC₆F_n line that no longer segregated for the trait was obtained. The introgression scheme is shown in **Fig. 1e**. This new line was called “Galapagos enhanced trichomes” (MT-Get).

During the trichome characterization of the F_1 plants, we observed a lower density of type-IV trichomes on both leaf surfaces (**Fig. 1d**), suggesting that the presence of this trichome type is a dominant or semi-dominant trait with quantitative components.

Fig. 1 *Solanum galapagense* (LA1401) trichome characterization. **(a, b)** Representative light microscopy showing type-IV glandular trichomes on the adaxial (a) and abaxial (b) sides of the leaf. Scale bar=250 μ m. **(c, d)** Density (trichomes per mm²) of type-IV trichomes on both leaf surfaces of the wild species ($n = 35$) (c) and F_1 plants (MT x *S. galapagense* LA1401) ($n=30$) (d). Data are means $\pm$ SEM. **(e)** Introgression scheme used to create the Micro-Tom (MT) line bearing type-IV trichomes on adult leaves. The line was designated “Galapagos enhanced trichomes” (*Get*). X= self-pollination, BC= backcross.

We confirmed the identity of type-IV trichomes on adult leaves of MT-*Get* using scanning electron microscopy (**Fig. 2**). The type-IV trichome is a structure up to 0.4 mm tall with a glandular cell at the tip, and a unicellular and flat base (Luckwill, 1943; Channarayappa *et al.*, 1992; Glas *et al.*, 2012). This description fits with the structures shown in **Fig. 2a-c**. Thus, these results confirmed the presence of type-IV trichomes on adult leaves of MT-*Get*, which are similar in morphology and size to those present in *S. galapagense* (**Fig. 2d**).

Fig. 2 (a-c) Scanning electron micrographs of abaxial surfaces of the 5th leaf of representative 25-day-old plants of the wild relative *Solanum galapagense* LA1401 (a), Micro-Tom (b), and the “Galapagos enhanced trichomes” line (MT-*Get*) (c). Scale bar=200 μ m. The arrows represent the different trichomes: pink for type IV, green for type V, and yellow for type VI. (d) Type-IV trichome stalk height and gland size comparisons between *S. galapagense* and MT-*Get*. Data are mean ($n=30$) $\pm$ SEM. The data are not statically different according to Student’s *t*-test ($P < 0.05$).

We next verified the functionality of the trichomes present in MT-*Get*. MT and MT-*Get* plants harboring the *GFP* gene under the control of type-IV/I-specific *SlAT2* promoter were generated. Both MT and MT-*Get* cotyledons, as well as MT-*Get* adult leaves, displayed type-IV trichomes expressing GFP (**Fig. 3**). Accordingly, the absence of type-IV trichomes on adult leaves of MT prevented GFP to be expressed (**Fig. 3f**). No GFP signal was detected in non-transgenic type-IV trichomes on the leaves of the MT-*Get* control (**Suppl. Fig. S2a, b**).

Fig. 3 p*SlAT2*::*GFP* expression (green fluorescence) at the tip cells of MT and MT-*Get* type-IV trichomes on: the cotyledons (a juvenile organ) from MT (**a, b**) and MT-*Get* (**c, d**); and the 5th leaf (an adult organ) from MT (**e, f**) and MT-*Get* (**g, h**). Note that MT leaves do not present fluorescence due to the absence of type-IV trichomes on adult organs. Scale bar= 100µm.

2.4.2 Phenotypic characterization and genetic mapping of the “Galapagos enhanced trichomes” (*Get*) introgression line

The leaf sequence of MT tomato from bottom to the top consists of a pair of embryonic leaves (cotyledons), a pair of juvenile leaves (1st and 2nd true leaves), and adult leaves (3rd to 6th upward) (Vendemiatti *et al.*, 2017). In order to further characterize MT-*Get* plants, we first determined trichome classes and their respective densities on adult leaves. When comparing MT-*Get* and the control MT, an inverse relationship between type-IV and -V trichomes on both leaf surfaces was observed (Fig.

4). This pattern of type-IV predominance in MT-*Get*, as opposed to type-V density in MT, was found in both juvenile as well as adult leaves (**Suppl. Fig. S3**). There is a lack of type-IV trichomes on MT adult leaves and, on the other hand, MT-*Get* tended to lack type-V trichomes on juvenile leaves (**Suppl. Fig. S3b**). Indeed, this inverse relationship between trichomes types IV and V had already been observed in different tomato cultivars in our previous work (Vendemiatti *et al.*, 2017).

Fig. 4 (a) Density (mm⁻²) of types-IV and -V trichomes on the adaxial surface of mature 5th leaves of 45-day-old plants of Micro-Tom (MT) and MT-*Get*. (b, c) Representative micrographs of the adaxial side of 5th leaves of 45-day-old MT (b) and MT-*Get* (c) plants. (d) Density (mm⁻²) of types-IV and -V trichomes on the abaxial surface of mature 5th leaves of 45-day-old plants of Micro-Tom (MT) and MT-*Get*. (e, f) Representative micrographs of the adaxial side of 5th leaves of 45-day-old MT (e) and MT-*Get* (f) plants. Data are means (n=40) $\pm$ SEM. Asterisks indicate a significant difference when compared with the reference sample according to the Student's *t*-test at $P < 0.001$ (***). Scale bars= 250 μ m.

When compared to *S. galapagense*, MT-*Get* displayed around 3.5-fold (abaxial) and 6.7-fold (adaxial) less type-IV trichomes (**Fig. 1b; Fig. 4**). *S. galapagense*, with only trichomes types I and VI, showed less trichome diversity than MT-*Get* (**Suppl. Fig. S4b**). Conversely, MT-*Get* bore trichomes types I, III, VI, and VII (**Suppl. Fig. S4b, c**) in addition to the types IV and V (**Fig. 4**).

In order to determine the genetic configuration of MT-*Get*, *i.e.*, the *S. galapagense* genome regions and alleles that were introgressed, we resorted to mapping-by-sequencing (Garcia *et al.*, 2016). This approach allows bulk genome sequencing of phenotypical groups from a segregating population in order to identify common loci responsible for a trait through the identification of distinct allelic frequencies between groups. Our results revealed that the MT-*Get* has a complex genetic composition: *S. galapagense* genome segments were found on the long arm of MT chromosomes 1, 2 and 3, the short arm of chromosome 5, and a large pericentromeric region of chromosome 6 (**Fig. 5**). The genomic coordinates of the genetic variation from *S. galapagense* present at high frequencies (≥ 0.8) in the *Get*-like phenotypical group of the MT-*Get* segregating population are provided in Supplemental Table S2. All of the regions present on chromosomes 1, 2, 3, 5, and 6, or smaller combinations thereof, may be involved in type-IV trichome formation. These results suggest that the *Get* trait has a polygenic basis, involving epistatic interactions among multiple genes that are located in all five segments derived from *S. galapagense*.

Fig. 5 (a) Allelic frequency indicating the chromosomal positions where MT-*Get* has introgressions from *S. galapagense* LA1401 compared to MT. (b) Representation of the corresponding positions of the chromosomal fragments from *S. galapagense* (pink bars) introgressed into MT-*Get*. The positions were based on the *Solanum lycopersicum* cv. Heinz reference genome sequence. Relevant genes (as discussed in the text) and their SGN (SolyC) ID numbers are represented. Note that MT-*Get* bears the MT-mutated alleles for *DWARF* (Bishop *et al.*, 1996) and *SP* (Pnueli *et al.*, 1998), which are determinant of the MT reduced plant size (Carvalho *et al.*, 2011). The presence of the MT allele at the *SP5G* locus also contributes to the reduced plant size of MT-*Get*, since the *S. galapagense*'s allele promotes additional vegetative growth due to a lack of flower induction under long days (Soyk *et al.*, 2017b). MT-*Get* also lacks *S. galapagense*'s alleles for genes conferring additional phenotypes distinct to this wild species, such as highly dissected leaves (*Pts*) (Kimura *et al.*, 2008) and β -carotene accumulating fruits (*B*) (Ronen *et al.*, 2000). The impact of the wild species alleles *EJ-2* and *FW3.2* on the MT-*Get* phenotype is discussed in the text.

We also verified whether the presence of known alleles from *S. galapagense* in the introgressed segments could contribute to additional, developmental differences independent of trichome traits by comparing the distinct genomic regions between MT-*Get* and MT. Notably, within the chromosome 3 segment, MT-*Get* harbors the *S. galapagense* alleles for the genes *EJ-2* (Soyk *et al.*, 2017a) and *FW3.2* (Chakrabarti *et al.*, 2013) (**Fig. 5**). The pleiotropic effects of non-domesticated alleles of the *FW3.2* gene, which codes for a *P450 monooxygenase*, are known to lead to a reduction in fruit weight and an increase in shoot branching (Chakrabarti *et al.*, 2013). Both phenotypes are present in MT-*Get* (**Fig. 6**), which holds the *S. galapagense*'s *fw3.2* allele. Another gene controlling fruit weight is *FW2.2* (Frary *et al.*, 2000), although it cannot be responsible for the smaller fruit of MT-*Get* compared to MT since both genotypes hold the same MT allele (**Fig. 5**). Lastly, the reduced size of MT-*Get* sepals is probably an effect of the *EJ-2* allele from *S. galapagense*, since this *MADS-box* gene controls the size of this floral whorl (Soyk *et al.*, 2017a).

Fig. 6 (a) Vegetative appearance of representative 35-day-old Micro-Tom (MT) and the MT-Get plants. (b) Branching Index values ($n = 15$). (c) Main stem height ($n = 15$). (d) Average fruit weight and sepal length of MT-Get ($n = 15$). (e) Fruits and sepals from MT, MT-Get, and *S. galapagense*. Note the small calyx and slightly smaller fruit of the MT-Get, which are the expected respective effects of *ej-2* and *fw3.2* alleles from *S. galapagense*. (f) PCR-based markers showing the presence of *S. galapagense* alleles at the *Ej-2* and *FW3.2* loci (see Fig. 5). Note that MT-Get has the same red fruit that is characteristic of MT due to an absence of the *S. galapagense*'s *B* allele (see Fig. 5), which is responsible for orange fruit color (Ronen *et al.*, 2000). The asterisks indicate significant statistical differences according to the Student's *t*-test at $P < 0.05$ (*) or $P < 0.001$ (***). Scale bar=5 cm.

2.4.3 Whitefly resistance test and trichome exudation in MT-Get plants

Based on the information that type-IV trichomes drive whitefly (*Bemisia tabaci*) resistance in both, *S. galapagense* and *S. pimpinellifolium* (Rodriguez-Lopez *et al.*, 2011; Firdaus *et al.*, 2012; Firdaus *et al.*, 2013; Vosman *et al.*, 2019), we verified whether MT-Get displayed an increased resistance to this insect. However, MT-Get did not differ from MT (Fig. 7a-c) in a preliminary assay based on the number of whitefly nymphs on leaves after exposure to a controlled greenhouse infested with whiteflies. We also observed that MT-Get did not display exudates at the tip of the type-IV trichome gland, which is a feature typical from *S. galapagense* that accounts for its sticky leaves and is regarded to

be the effect of acylsugar accumulation (Schilmiller *et al.*, 2008). The exudates from *S. galapagense* can be stained with Rhodamine B (Fig. 7d, e), which is a dye for acylsugars. In MT-*Get*, Rhodamine B staining was restricted to the area inside the gland. We also observed that, differently from *S. galapagense*, MT-*Get* leaves were not sticky. These results prompted us to profile the acylsugar accumulated and expression of the related type-IV trichome-specific biosynthesis genes in MT-*Get* leaves.

Fig. 7 Representative photographs of whitefly (*Bemisia tabaci*) nymphs infestation on Micro-Tom (a) and MT-*Get* (b) leaves. Scale bar=250 μ m. (c) Quantification of *Bemisia tabaci* nymphs in MT-*Get* compared to the control MT (n=30). The data are not statistically different according to the Student's *t*-test ($P < 0.05$). (d, e) Representative micrographs of *Solanum galapagense* droplets on type-IV trichomes (d) and their absence in MT-*Get* (e). In the inserts, trichomes dyed with Rhodamine-B reveal AS exudation.

2.4.4 Acylsugar accumulation and related gene expression in MT-Get

Since type-IV trichomes are sources of acylsugars (Goffreda *et al.*, 1989; Liedl *et al.*, 1995; Maluf *et al.*, 2010), it was assumed that this insecticide would accumulate on adult leaves of the cultivated tomato upon the introduction of the capacity to maintain type-IV trichomes. We, therefore, conducted a metabolic profile analysis using both liquid chromatography and mass spectrometry to assess the acylsugar accumulation on adult leaves of the MT-Get, compared to the parental *S. galapagense*.

S. galapagense showed peaks corresponding to a variety of sucrose (S)-based acylsugars with different acyl moieties, ranging from 2 to 12 carbons (C2 to C12) (**Fig. 8** and **Table 1**). Consistently, the acylsugar peaks were very attenuated or absent in the cultivated tomato (MT), which is already known to accumulate very low amounts of acylsugars (**Fig. 8**) (Blauth *et al.*, 1998). MT-Get, although accumulating more acylsugars than MT, showed dramatic quantitative and qualitative differences when compared to *S. galapagense*. Notably, MT-Get showed reduced levels of acylsugars harboring C10 and C12 moieties, such as S4:23 (2,4,5,12), S4:22 (2,5,5,10), and S4:24 (2,5,5,12) (**Fig. 8** and **Table 1**). The amounts of S4:23, S4:22, and S4:24 were 120, 42, and 18-fold lower in MT-Get than *S. galapagense*, respectively (**Table 1**). These differences are greater than those for type-IV trichome densities between *S. galapagense* and MT-Get, which were 3.5-fold (abaxial surface) and 6.7-fold (adaxial surface) (**Figs. 1, 4**).

Fig. 8 (a) Representative LC-MS chromatographic comparison between *Solanum galapagense* LA1401, Micro-Tom (MT), and MT-Get regarding acylsugar (AS) content. Peak area quantifications are shown in Table 1. (b) Signal intensity for each one of the AS analyzed in the three plant genotypes.

Table 1. Peak areas from ion chromatograms (LC-MS) of acylsugars from *S. galapagense*, MT-Get, and MT.

AS	<i>m/z</i>	Peak area			<i>S.galap./</i> MT-Get
		<i>S. galapagense</i>	MT-Get	MT	
S3:15 (5,5,10)	709.368	675.914 a	72.873 b	7.192 c	9.28
S3:22 (5,5,12)	737.403	9,877.349 a	1,767.007 b	145.07 c	5.59
S4:16 (2,4,5,5)	667.279	18,907.925 a	3,835.183 b	514.101 c	4.93
S4:17 (2,5,5,5)	681.297	61,140.597 a	22,592.369 b	2,509.92 c	2.71
S4:22 (2,5,5,10)	751.375	5,363.138 a	125.538 b	26.872 c	42.72
S4:23 (2,4,5,12)	765.396	5,864.023 a	48.632 b	26.809 c	120.58
S4:24 (2,5,5,12)	779.413	26,335.227 a	1,446.972 b	301.079 c	18.20

Acylsugars were identified according to their *m/z* ratios and retention time (n=4). For the sake of simplicity, values were divided by 1,000. Values followed by the same letter in each row do not differ statistically according to the Student's *t*-test ($P < 0.05$).

In the GC-MS analysis, detectable peaks of n-decanoate (C10) and n-dodecanoate (C12) were observed only for *S. galapagense* (**Suppl. Fig. S5, Suppl. Table S3**). These carboxylates are derived from C10 and C12 harboring acylsugars, which is in agreement with the higher amounts of the acylsugars S4:23 (2,4,5,12), S4:22 (2,5,5,10) and S4:24 (2,5,5,12) in *S. galapagense* found in the LS-MS analysis (**Fig. 8**). Only small quantities of methyl dodecanoate were detected in MT-Get (**Suppl. Table S3**), which indicates the presence of S3:22 (5,5,12) and S4:24 (2,5,5,12) in this genotype (**Fig. 8**).

We next evaluated the relative expression of the genes involved in acylsugar biosynthesis. There are four acyltransferases (ASAT) that act sequentially to esterify acyl chains in specific positions of the sugar moiety (Schillmiller *et al.*, 2012; Fan *et al.*, 2016). The expression levels of the four ASAT genes were higher in *S. galapagense* when compared to MT-Get (**Fig. 9**), which correlates with the differences in acylsugar content determined (**Fig. 8, Table 1**).

Fig. 9 Relative transcript accumulation of ASAT enzymes in leaf tissues of MT-Get, MT, and *S. galapagense*. qRT-PCR values are means $\pm$ SE of three biological samples. Each biological sample is an average of three technical replicates. Asterisks indicate a statistically significant difference when compared with the reference sample according to the Student's *t*-test at $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***).

The relative expression of the genes coding for acylhydrolase (ASH) enzymes was also quantified (**Suppl. Fig. S6a-d**). They are responsible for the removal of acyl chains from specific acylsugars positions, thus creating the substrate for the action of acyltransferases (Schilmiller *et al.*, 2016; Fan *et al.*, 2019). The relative expression of ASH1 was significantly higher when comparing MT-Get and *S. galapagense* (**Suppl. Fig. S6a**), which also reflects the differences in acylsugar content observed between these two genotypes.

Since MT-Get and *S. galapagense* seem to differ in the capacity to exudate acylsugars (**Fig. 7**), we assessed a putative efflux transporter, which may be responsible for acylsugar exudation in type-IV trichome tips. Notably, the ABC transporter previously associated with acylsugar exudation (Mandal *et al.*, 2019) presented a higher expression level in *S. galapagense* compared to MT-Get, which correlates with the differences in their exudation capacity (**Suppl. Fig. S6**).

2.5 Discussion

Type-IV trichomes represent an important mechanism for herbivore resistance in the *Solanum* genus and beyond. In the wild species *S. pennelli*, *S. galapagense*, and *S. habrochaites*, these structures are found at high densities, making them acylsugar accumulators (Simmons & Gurr, 2005; Mutschler *et al.*, 1996; Momotaz *et al.*, 2010; Leckie *et al.*, 2012; Schilmiller *et al.*, 2012). These specialized metabolites protect the plants via both, their toxicity as well as their stickiness, thereby trapping and immobilizing the insects or labeling them for recognition by their predators (Mirnezhad *et al.*, 2010; Weinhold & Baldwin, 2011; Vosman *et al.*, 2019; Schuurink & Tissier, 2019). Since type-IV trichomes are not found on the adult structures of the cultivated tomato (*S. lycopersicum*) (Vendemiatti *et al.*, 2017), obtaining and studying a line with this phenotype is a critical step towards a better understanding of insect resistance based on this type of trichome as well as elucidating the molecular mechanisms of glandular trichome development. We created this line by introgressing into *S. lycopersicum* cv. Micro-Tom the trait from *S. galapagense* LA1401, which is a wild species related to the cultivated tomato and highly resistant to whiteflies (Firdaus *et al.*, 2013). We named the introgression line “*Galapagos enhanced trichomes*” (MT-Get).

MT-*Get* displays several traits that overall look intermediate between both parents: it has less glandular trichomes than *S. galapagense*, but a higher diversity of trichome types, whereas the comparison with MT has the opposite trend: MT-*Get* has more glandular trichomes and less diversity (**Fig. 10a**). The reduction of the trichome diversity in *S. galapagense* and MT-*Get* is mainly represented by the reduced density (or absence) of trichomes types III, V, and VI. In the case of type-V trichomes, its inverse correlation with type-IV structures had already been pointed out in a previous study comparing juvenile and adult leaves of cultivated tomato cultivars (Vendemiatti *et al.*, 2017). This result suggests that both trichome types may have an overlapping ontogeny since they only differ anatomically by the presence/absence of a terminal gland (Luckwill, 1943; Glas *et al.*, 2012). The density reduction of trichomes types III and VI may also be a consequence of the increased number of type-IV trichomes via a general mechanism of trichome differentiation that maintains the identity of neighboring epidermal cells. The mechanism that prevents trichome formation in clusters is well known in *Arabidopsis* (Pesch & Hülkamp, 2011) and was recently also suggested for tomato (Schuurink & Tissier, 2019). Our findings reveal the complexity of studying trichome distribution as a trait since perturbations in one trichome type are likely to provoke a pleiotropic effect in the abundance of other types.

Fig. 10 (a) Schematic representation of trichome type distribution in each genotype used in this work. (b) Logarithmic scale representation of the results of total trichomes related to the total acylsugar content (types I and IV). (c) Sequential steps to obtain broad and durable insect-resistant tomatoes through the high production of acylsugars on tomato leaves.

Genetically, our analysis revealed that the development of type-IV trichomes is associated with multiple genes dispersed over several chromosomes. It is not clear yet whether all genomic fragments (loci) identified in this study are essential for type-IV trichome development. However, this result led us to hypothesize that the trait has a

polygenic inheritance and that some of the genes within these regions can explain the additional traits present in the introgressed lineage. One region found on chromosome 2 coincides with a previous QTL associating adult whitefly survival and the presence of type-IV trichomes in a segregating population (F₂ and F₃) from a crossing between the cultivated tomato and *S. galapagense* (Firdaus *et al.*, 2013). This is strong evidence that at least one gene that is necessary, but probably not sufficient for type-IV trichome formation, is located in this region of the genome. On chromosome 3, MT-*Get* harbors the *S. galapagense*'s wild alleles for the genes *EJ-2* and *FW3-2*, but they cannot be directly linked with type-IV trichome development. Beyond *S. galapagense*, other species in the tomato clade carry wild alleles of *EJ-2* and *FW3-2*, but not all of them show type-IV trichomes on mature leaves, such as the *S. cheesmaniae* accessions LA0521 and LA1139, and *S. pimpinellifolium* LA4645 (Simmons & Gurr, 2005; Bitew, 2018). Nevertheless, this fact does not rule out the hypothesis that these genes may be epistatic enhancers of other genes, which together may result in the MT-*Get* phenotype.

The initial hypothesis that herbivore resistance was coupled with type-IV trichome presence was not borne out by our data. The MT-*Get* line did not show improved resistance to *Bemisia tabaci*, the main insect controlled by the presence of type-IV trichomes in the wild species (Maluf *et al.*, 2010). Our results show that MT-*Get* plants, although having a reasonable density of type-IV trichomes, do not produce acylsugars on the same scale as *S. galapagense* (**Fig. 10b**). We propose that the main difference is the accumulation of acylsugars with long-chain carbon groups (10 and 12 carbons). These results suggest that among the genes controlling the AS metabolic pathway in *S. galapagense*, some could be related to the esterification of C10 and C12 acyl groups, which may have repercussions for insect resistance.

In our evaluation of the known structural genes of the acylsugar pathway, we noticed that *ASAT1* (Solyc12g006330), *ASAT2* (Solyc04g012020), *ASAT3* (Solyc11g067270), and *ASAT4* (Solyc01g105580) are not contained within any of the introgressed regions (See Fig. 5). This means that *S. galapagense* and MT-*Get* have distinct alleles for these genes, including probably their *cis*-regulatory elements. Therefore, the effect of both *cis* and *trans* elements regulating the expression of *ASAT* genes may explain the higher expression of *S. galapagense* compared to MT-*Get*. However, we cannot exclude that the differences in transcript accumulation also reflect the enriched content of type-IV trichome-derived RNAs in *S. galapagense* due to their

higher density compared to that of MT-*Get* (**Fig. 1b, 4**). On the other hand, the difference in *ASAT4* expression between MT-*Get* and *S. galapagense* is far beyond the magnitude of the trichome density difference between these genotypes. It is interesting to note that the higher expression of *ASAT2* in *S. galapagense* is consistent with the observation of the increased levels of C10-12 acyl groups in the wild parental genotype (**Fig. 8**). We propose that the enzyme encoded by *ASAT2* from *S. galapagense* may be able to esterify more efficiently long acyl chains (up to 12 carbons) in the R3 position of the sucrose backbone (Fan *et al.*, 2016; cf. **Fig. 9**).

Since acylsugars are non-volatile compounds, they are stored in the glands, and by a mechanism that is not yet clear, they drip out of the glandular tip (Schuurink & Tissier, 2019). This phenomenon was observed for *S. galapagense* (**Fig. 7d-e**) and may sustain the positive feedback responsible for AS production. Recently, a comparative transcriptomic analysis of *Solanum pennellii* accessions with distinct AS contents found that the expression levels of most AS metabolic genes were positively correlated with AS accumulation (Mandal *et al.*, 2019). Among the differentially expressed genes (DEGs), three genes putatively encoding ATP-binding cassette (ABC) transporters were upregulated in the accessions with high AS content. Furthermore, Demissie *et al.* (2019) described an ABC transporter strongly expressed in the glandular trichomes of *Lavandula angustifolia* (Lamiaceae). Based on this information, we verified the relative expression of an ABC transporter (Solyc03g005860) in our material. The same pattern of *ASAT* expression was observed: its gene expression was closely related to the type-IV trichome density on the leaves (**Suppl. Fig. S6**). Whereas this result suggests that this ABC transporter may be involved in AS exudation, the definitive explanation of the differences in AS exudation capacity between *S. galapagense* and MT-*Get* remains to be further investigated.

Regarding the quantitative differences in AS content, we initially expected that type-IV trichomes of *S. galapagense* would have the capacity to accumulate the amounts of sugar moieties necessary to be acylated in the gland tip. Studies using radiolabeled carbon in tobacco showed that isolated trichome glands might be metabolically independent to produce the main exudates, but only when adequately supplied with carbon sources (Kandra & Wagner, 1988). Earlier transcription analyses performed with expressed sequence tags (EST) indicated that trichomes could work with simple biochemical input while having few biochemical pathways of the primary and

specialized metabolisms locally and highly activated (Schilmiller *et al.*, 2008). Although type-IV trichomes contain chloroplasts (**Suppl. Fig. S2c**), these probably are not in sufficient number to sustain the primary as well as the specialized metabolisms occurring in the cells of this structure (Schilmiller *et al.*, 2008; Balcke *et al.*, 2017). Therefore, the differences in AS accumulation are unlikely to be fully explained by genes related to modifications of the acyl moiety, such as *ASATs* and *ASHs*. Still, instead, there could be additional unknown genes involved in sugar metabolism or transport that can transform the trichome gland into a stronger sink.

2.6 Concluding remarks

The results obtained here and their implications can be summarized in a model in which the transference of insect resistance from a wild species into the cultivated tomato requires at least three steps: i) Favorable alleles necessary to build the specific glandular trichomes, such as in *MT-Get*; ii) Favorable alleles necessary for specific metabolic pathways (*e.g.*, different compositions of acyl groups and capacity to accumulate the sugar moiety), and iii) Favorable alleles necessary to transform glandular trichomes into exudation structures, such as transmembrane transporters (**Fig. 10c**). The *MT-Get* introgression line presented here is the starting point of a challenging, long-sought breeding goal – the introduction of a trait in tomato for effective, broad, and long-lasting insect resistance. Altogether, this study demonstrates that glandular trichome development along with the metabolite production pathway and exudation are partially uncoupled at the genetic level. The *MT-Get* genotype represents a valuable resource for further studies involving the biochemical manipulation of type-IV trichome content through either genetic introgression or transgenic approaches. *In toto*, *MT-Get* is the first step to creating a tomato plant that naturally produces a substance that actively kills pests. In other words, we have created plants that carry the weapons, which can now be used to learn how to load them with the appropriate metabolic ammunition.

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2.7 Supporting information

Suppl. Fig. S1 Mapping-by-sequencing bioinformatic analysis pipeline.

Suppl. Fig. S2 (a-b) Absence of green fluorescence in non-transgenic MT-Get type-IV trichomes. (c) Fluorescence of type-IV trichomes in *S. galapagense*.

Suppl. Fig. S3 Ontogenetic sequence of type-IV and -V trichomes on both leaf surfaces of Micro-Tom (MT) and MT-Get.

Suppl. Fig. S4 (a) Counting of others trichomes types in *Solanum galapagense*. (b, c) Density (mm^{-2}) of additional trichome types on both leaf surfaces of Micro-Tom (MT) and MT-Get.

Suppl. Fig. S5 (a) GC-MS profile comparison between *Solanum galapagense* LA1401, Micro-Tom (MT) and MT-Get regarding acyl groups content. **(b)** Full mass spectrum scan showing the relative abundance of ions for acyl group peaks found on extracts analyzed by GC-MS.

Suppl. Fig. S6 Relative transcript accumulation of genes coding for ASAT enzymes (a-f) and ABC transporter (g-h) in leaf tissues of MT-Get and *S. galapagense*.

Suppl. Table S1. Oligonucleotide sequences used in this work.

Primer	Sequence	Access	Utilization	Reference
<i>FW2.2</i>	GGTGGTGTGATGTGGAGTGAGTG	Solyc02g090730	CAPS marker	This work
	GGCAGATACATAGTGAGGAGGAAC			
<i>DWARF</i>	TGATCCATATTCGTTCAATCCA	Solyc02g089730	CAPS marker	This work
	CGTGATTATGTTAGCGGGAAT			
<i>FW3.2</i>	GAGATAACGGGTAAATAGAGT	Solyc03g114940	CAPS marker	This work
	TAGTTAGGATAGTTATAGTTTGC			
<i>EJ-2</i>	CACAATTCATGCTGGATCAGC	Solyc03g114840	CAPS marker	This work
	CGGAGTAATCTATTAGATTCTGC			
<i>SP5G</i>	TAATTACGCAGTGACGAAGCA	Solyc05g053850	CAPS marker	This work
	TTGACACAGAGTTTCGAGAACG			
<i>SP</i>	GGGTTGAAGTTCATGGTGGT	Solyc06g074350	CAPS marker	This work
	AGTGCCTGGAATGTCTGTGA			
<i>PTS</i>	AGGAAGTGATTGACCCATGC	Solyc06g072480	CAPS marker	This work
	CCCCAAACACCACTATCTAAGC			
<i>ASAT1</i>	GGGAGGCCAAGACAAGTTGATA	Solyc12g006330	qPCR	Fan <i>et al.</i> , 2016
	TGGAGAAGCAAACCTGAAGAAAATC			
<i>ASAT2</i>	GACTCCATTTCGTCATCTTTACTTC	Solyc04g012020	qPCR	
	TTTGACTTCTTCCTTCTCCTTTCTTA			

<i>ASAT3</i>	TTTCTTCCCTTTACCGTCTGAA TGAACAAGTGCTGAGGCAAC	Solyc11g067270	qPCR	This work
<i>ASAT4</i>	GGTGGTCGTGATGTCCCTAA GCCCTCCTTGTTAGCAGTTG	Solyc01g105580	qPCR	This work
<i>ASH1</i>	TCTTTCATCCAACGTGATTAACATTT ATCTACACCACAGAACAACACTACCAATA	Solyc05g051660	qPCR	This work
<i>ASH2</i>	GCGACCCACTGAATAGCATC GGCGGAGGATTTGTATTAGGA	Solyc05g051670	qPCR	This work
<i>ASH3</i>	CTACACTCAAATCAACTTCCATACCATACA ATAGAACCGTTCTGACTCGACCATTT	Solyc09g075710	qPCR	Schillmiller <i>et al.</i> , 2016
<i>ABC transporter</i>	TCCGAAGGGATGATGGAG GCAGAAGACCAAATACAGGGTAA	Solyc03g005860	qPCR	This work
<i>ACTIN</i>	GGTCCCTCTATTGTCCACAG TGCATCTCTGGTCCAGTAGGA	Solyc04g011500	qPCR	Silva <i>et al.</i> , 2018
<i>EF1α</i>	AAGCCCATGGTTGTTGAGAC TTCTTGACAACACCCACAGC	Solyc06g005060	qPCR	Pinto <i>et al.</i> , 2017

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Suppl. Table S2. Genomic coordinates of the genetic variation from *S. galapagense* present at high frequencies (≥ 0.8) in the *Get*-like phenotypical group of the MT-*Get* segregating population.

Chromosome	Corresponding Region (bp)
1	82,558,349 – 83,597,817
2	53,604,856 - 55,297,272
3	61,495,951 - 65,007,167
5	19,540,000 - 19,637,400
6	3,688 - 38,647,717

Suppl. Table S3. GC-MS content of acyl groups in *S. galapagense*, MT-*Get* and Micro-Tom (MT).

RT	Acyl Groups Content	Genotypes		
		<i>S. galapagense</i>	MT- <i>Get</i>	MT
1	2-Methyl Butanoate	0,77	0,00	0,00
2	3-Methyl Butanoate	2,86	0,00	0,00
3	Methyl Decanoate	0,18	0,00	0,00
4	Methyl Dodecanoate	2,92	0,06	0,00

Results are presented as mg/g dry weight; n = 8

3 A SINGLE *Solanum pennellii*'s CHROMOSOMAL REGION CONTROLS TYPE-IV GLANDULAR TRICHOME DEVELOPMENT AND HAS NO PLEIOTROPIC EFFECT ON GROWTH AND YIELD OF THE CULTIVATED TOMATO.

3.1 Abstract

Glandular trichomes are epidermal structures which study is gaining momentum since they are sources of several secondary metabolites of economic and ecological importance. Related wild *Solanum* species are considered genetic resources for tomato (*S. lycopersicum*), which have natural genetic variations that provide resistance to herbivores attack. Among them, the presence of type-IV glandular trichomes confers insect resistance, since they are sources of the natural insecticide acyl sugars (AS). The absence of type-IV glandular trichomes in the cultivated tomato is regarded as one of the causes of its susceptibility to insects. In this work, we created a near-isogenic line (NIL) in the Micro-Tom (MT) cultivar that harbors type-IV trichomes on adult leaves. The NIL was named *Pennellii enhanced trichomes* (MT-Pet) because it was derived from crosses with *S. pennellii*. Despite the presence of type-IV trichomes, which is considered to be a juvenile character, MT-Pet did not present pleiotropic phenotypes and showed the same fruit yield of MT. GC-MS and the Rhodamine-B assay demonstrated that the presence of type-IV trichomes was not sufficient to increase AS accumulation in MT-Pet. The only locus introgressed was mapped in the pericentromeric region of chromosome 3, and its eventual cloning will contribute to gain insights into trichome development and breeding for insect resistance.

Keywords: Glandular trichome; *Solanum pennellii*; *Solanum lycopersicum*

3.2 Introduction

Trichomes are epidermal structures involved in the plant's defense against abiotic and biotic stresses (Mauricio & Rausher, 1997; Peiffer *et al.*, 2009; Yang & Ye, 2013). Among the different types of trichomes, the glandular ones are the most important ecologically and economically. Their gland cells are comparable to biochemical factories capable of producing, storing and secreting secondary metabolites (*e.g.*, terpenoids, methyl ketones, acylsugars, flavonoids) (Fridman *et al.*, 2005; Besser *et al.*, 2009; Sallaud *et al.*, 2009; Kang *et al.*, 2014). Some of these metabolites have industrial and medicinal applications (Schilmiller *et al.*, 2008; Huchelmann *et al.*, 2017; Chalvin *et al.*, 2020), and most of them act as pathogen deterrents or natural insecticides (Tissier, 2012; Huchelmann *et al.*, 2017). Therefore, the elucidation of the

developmental pathways of glandular trichome (GT) formation and their metabolic specificities is of increasing interest and scientific relevance (Schuurink & Tissier, 2019).

The study of GT development requires genetic model systems because *Arabidopsis thaliana* is devoid of such structures (Hauser *et al.*, 2001). Based on the diversity of GTs found in the genus *Solanum*, tomato (*S. lycopersicum*) has been proposed as one of the best models to study the development of GTs (Goffreda *et al.*, 1988). Among the GTs types present in the *Solanum* genus, the type-IV is one of the most important for herbivory resistance. Type-IV trichomes are capable of producing and storing non-volatile secondary metabolites called acylsugars (AS). These products are doubly effective against herbivores: besides being toxic, their sticky feature is capable of imprisoning and suffocating small arthropods (Puterka *et al.*, 2003; Wagner *et al.*, 2004). Initially, type-IV trichomes were only described in wild *Solanum* species related to the cultivated tomato, such as *S. galapagense*, *S. habrochaites*, *S. pennellii* and some accessions of *S. pimpinellifolium* (Luckwill, 1943; Simmons & Gurr, 2005; McDowell *et al.*, 2010; Glas *et al.*, 2012). It was further shown that type-IV trichomes could be found in cotyledons and the first pairs of leaves of the cultivated tomato. Hence, type-IV trichomes are now proposed as markers of the juvenile phase of vegetative development in tomato (Vendemiatti *et al.*, 2017). Therefore, unraveling the genetic and molecular determinants of the evolutionary divergences that allow the development of type-IV trichomes on the adult leaves of wild tomato relatives can advance our knowledge about plant defense, development, and adaptation. However, no monogenic natural variation controlling type-IV trichomes formation was discovered so far.

Here, a near-isogenic line (NIL) of tomato harboring type-IV trichomes on adult leaves were obtained by the introgression of a single chromosomal region from *S. pennellii* LA716 into the tomato model system, cultivar Micro-Tom (MT). The NIL was named *Pennellii enhanced trichomes* (MT-*Pet*) and was subjected to an extensive phenotypic characterization. Although MT-*Pet* presented an increased density of type-IV trichomes, GC-MS measurements, and the Rhodamine B assay showed that the presence of type-IV trichomes is not sufficient for accumulation of AS. Interestingly, besides the differences in trichome densities and the notion that the presence of type-IV trichomes is linked to juvenility, MT-*Pet* did not present any other pleiotropic phenotypes or yield penalties. The discovery of the *PET* molecular identity will provide for a better

understanding of glandular trichome development and will help in the development of tomato varieties with natural insecticide production.

3.3 Material and Methods

3.3.1 Plant Material

All plant material used in the experiments was grown in the greenhouse of the Laboratory of Hormonal Control of Plant Development, belonging to the Department of Biological Sciences, ESALQ-USP. The plants were grown in a greenhouse under natural day-length conditions and automated irrigation (Lombardi-Crestana *et al.*, 2012). The genotypes used to perform this work are described in **Table 1**. The introgression that resulted in the NIL MT-*Pet* was performed according to the procedure described by Pino *et al.* (2010) and is schematically depicted in **Fig. 3**.

Table 1- Description of the genotypes used in this work.

<i>Genotype</i>	<i>Effect/ Genic Function</i>	<i>Source</i>	<i>Reference</i>
<i>S. pennellii</i> LA716	Wild species of tomato from Peru	TGRC*	Correll, 1962
ILs	Introgressions lines of <i>S. pennellii</i> chromosome fragments in cv. M82	TGRC*	Eshed & Zamir, 1995
M82	<i>S. lycopersicum</i> cultivar	LA3475	-
Micro-Tom (MT)	<i>S. lycopersicum</i> cultivar	LA3911	Scott, 1989
<i>Pennellii</i> <i>enhanced</i> <i>trichomes</i> (MT- <i>Pet</i>)	MT plants with a significant amount of type-IV trichomes from <i>S. pennellii</i>	-	-
<i>Galapagos</i> <i>enhanced</i> <i>trichomes</i> (MT- <i>Get</i>)	MT plants with a high amount of type-IV trichomes from <i>S. galapagense</i>	-	Vendemiatti <i>et al.</i> , in prep

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3.3.2 Trichome counting and phenotyping

The protocol used in this work was described by Vendemiatti *et al.* (2017). At least 8 individuals per genotype were sampled, and four different samples were

analyzed per plant on each leaf surface. Photographs were taken using a Leica S8AP0 (Wetzlar, Germany) at 50x magnification, coupled to a Leica DFC295 camera (Wetzlar, Germany). Counting and length measurements of the trichomes were performed on the images using the manufacturer's analytical program (Leica Application Suite 4.0).

3.3.3 *Scanning electron microscopy*

Leaf samples were fixed in Karnovsky solution for 24 hours at 4°C. After that, the material was washed twice in 0.05M cacodylate solution for 10 minutes and again fixed in osmium tetroxide for 1 hour. Subsequently, the samples were washed with distilled water and dehydrated in a series of acetone. The dehydrated samples were submitted to drying to the critical point and were gold plated. The observations were performed on a LEO 435 VP scanning electron microscope (SEM Tech Solutions, Massachusetts).

3.3.4 *Plant phenotyping*

Plants at 55 days after germination were split into aerial parts (leaves, stem, and fruits) and roots to determine the dry mass (after drying in an oven at $\pm 60^{\circ}\text{C}$ for 70 hours). In addition, measurements were taken of the main and secondary branches, number of inflorescences, and number of leaves in order to characterize the plant size, ramification index (Morris *et al.*, 2001), and determination time of the lineages in comparison with the MT control. The yield of the genotypes was evaluated based on the total weight of fruits per plant as well as the average fruit weight at the end of the growing cycle. The content of soluble solids of fruits ($^{\circ}\text{Brix}$) was determined using a digital refractometer (Atago PR-101 α , Bellevue, WA).

3.3.5 *GC-MS acylsugar quantification*

The experiments of chemical compound quantification were carried out at the Laboratory of Biochemistry and Instrumental Analysis of the Department of Agroindustry, Food, and Nutrition (ESALQ-USP).

Leaves of the adult vegetative phase (fifth leaf from the cotyledons; Vendemiatti *et al.*, 2017) were collected, and the extraction was carried out according to the methodology described by Leckie *et al.* (2013). The compounds were separated via GC-2010 gas chromatography (Shimadzu Corp., Kyoto, Japan) attached to a QP 2010 Plus mass spectrometer (Shimadzu Corp., Kyoto, Japan), using Helium as the charging gas.

For the separation of acyl groups from acylsugars, the hexane portion was injected into a DB-WAX apolar column (0.25 mm in diameter, 30 m in length, with 0.25 μm film thickness). The data obtained were analyzed using software version 2.5 Lab Solutions-GCMS (Shimadzu Corp., Kyoto, Japan). The identification of the compounds was based on the retention time of the chromatographic peaks and fragments of the mass spectrometer, which were compared to the available standards and data libraries.

3.3.6 Rhodamine B assay

Leaflets of *S. pennellii* LA716 and MT-Pet were submerged in a 0.5% aqueous solution of Rhodamine B for one minute. Subsequently, four sequential baths were run in distilled water to remove the excess dye. The images of the stained trichomes were collected as previously described.

3.3.7 Statistics analysis

The data were compared statistically by the *t*-test using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com.

3.4 Results and Discussion

3.4.1 Introgression of a chromosome 3 fragment from *S. pennellii* LA716 into *S. lycopersicum* cv. Micro-Tom (MT) controlling type-IV trichome formation.

The large amount of type-IV trichomes on adult leaves of *S. pennellii* and the possible association of this trichome type with the high levels of the natural insecticide acylsugars prompted us to investigate the genetic bases of this phenotype, which diverges from the cultivated tomato. We determined the density of type-IV trichomes on the *S. pennellii* LA716 leaves. Type-IV trichomes are found mainly on the leaf abaxial side in *S. pennellii* at a density of around 150 mm^{-2} (**Fig. 1c**). Although these trichomes are present at a high density on the abaxial surface, the difference of the density between the two surfaces of the leaf is smaller in *S. pennellii* than that found in other species, such as *S. galapagense* (Vendemiatti *et al.*, *in prep*). While the abaxial surface of *S. galapagense* leaves has around 6.5-fold more type-IV trichome than its adaxial surface, the difference in *S. pennellii* is only 1.14-fold (Vendemiatti *et al.*, *in prep*). It is known that the stomata

distribution between the two surfaces in *S. pennellii* is more equivalent (amphistomatic) when compared to tomato (Chitwood *et al.*, 2013). Therefore, it might be that *S. pennellii* has genetic determinants that lead to a smaller difference between abaxial and adaxial surfaces regarding different epidermal structures.

A small frequency of trichomes types V and VI was also observed (**Suppl. Fig. S1**). The higher proportion of type-IV trichomes in relation to the other types corroborates with data from the literature. For example, Slocombe *et al.* (2008) showed that mature leaves of *S. pennellii*, although presenting less type-IV trichomes than our measurements in the same species, have just a small number of type-VI trichomes (2% - 3% total).

When compared to other works, our trichome counts for *S. pennellii* can vary from 3-10-fold more type-IV trichomes, as can be seen in **Suppl. Table S1**. The large variation in the density of trichomes already described may be related to different quantification methodologies, plant growth conditions, and age of the samples analyzed.

Fig. 1 *Solanum pennellii* LA716 trichome characterization. **(a-b)** Representative image showing type-IV glandular trichomes on the adaxial (a) and abaxial (b) leaf surface. Scale bar= 250 μ m. **(c)** Type-IV trichomes density (mm⁻²) in both leaf surfaces. Data are means (n = 25) $\pm$ SEM.

In order to identify the chromosomal fragment(s) involved in the formation of type-IV trichomes, we performed a preliminary screening using the *S. pennellii* LA716 introgression lines (ILs) (Eshed; Zamir, 1995). The ILs are a set of genotypes, each containing a small chromosomal segment of *S. pennellii* LA716 introgressed and mapped into the tomato cultivar M82. As observed in **Fig. 2**, 47 ILs and the control cultivar M82 were analyzed. In this first analysis, several ILs presented the type-IV trichome, including lines covering the whole chromosome 3. Taking into account that type-IV trichome presence is associated with tomato juvenility (Vendemiatti *et al.*, 2017), we performed a second characterization of the ILs for the chromosome 3 using only leaves of the adult phase of vegetative development (from the tenth leaf thereafter). In this second screening, only the IL3-2 consistently presented type-IV trichomes (**Fig. 2**). It is interesting to note that Muir *et al.* (2014) also found a trichome density QTL (*td3.1*) in IL3-2, although the specific type of trichome was not reported.

Fig. 2 Screening for type-IV trichomes on the 5th leaf of *Solanum pennellii* LA716 x *S. lycopersicum* M82 introgression lines (ILs) (Eshed and Zamir, 1995). Error bars represent means $\pm$ SEM ($n = 10$) for each surface. In the insert, the type-IV trichome density on the 10th leaf of the ILs of chromosome 3 and the control M82 is shown. Error bars represent means $\pm$ SEM ($n = 30$) for each side of the leaf.

Based on these findings, we started the introgression of *S. pennellii* 's chromosomal segment present in the IL3-2 into the cultivar Micro-Tom (MT). After self-fertilization of F_1 plants, we searched for F_2 plants with the characteristic dwarfism of MT (Campos *et al.*, 2010) along with type-IV trichome on leaves from the adult phase of vegetative development (the 5th leaf, Vendemiatti *et al.*, 2017). These plants were backcrossed (BC) with the recurrent parental MT. The process was repeated five times, until obtaining a BC_6F_n stable and not segregating line for the trait selected. The introgression scheme is shown in **Fig. 3**. The resulted near-isogenic line (NIL) was named “*Pennellii enhanced trichomes*” (MT-Pet).

Fig. 3 Crossing and backcrossing (BC) sequence to create a near-isogenic line (NIL) with type-IV trichomes in the Micro-Tom (MT) background. The resulted NIL was denominated “*Pennellii enhanced trichomes*” (Pet). The IL-3-2, which is derived from *Solanum pennellii* accession LA716, was used as the source of alleles for type-IV trichomes. The “X” symbol means self-pollination.

3.4.2 Phenotypic characterization of “Pennelli enhanced trichomes” (MT-Pet)

We next performed the characterization of the MT-Pet. To confirm the identity of type-IV trichomes present in the NIL, we carried out scanning electron microscopy analyses of *S. pennellii* LA716 (**Fig. 4a**), IL3-2 (**Fig. 4b**), Micro-Tom (**Fig. 4c**) and MT-Pet (**Fig. 4d**). Type-IV trichomes are described as having a single flattened basal cell with a short two- or three-celled stalk (0.2 - 0.4 mm) and a gland on the tip. (**Fig. 1a**; Luckwill, 1943; Channarayappa *et al.*, 1992; Glas *et al.*, 2012). The results confirmed the identity of type-IV in MT-Pet, which have basically the same phenotype presented in *S. pennellii* and IL3-2, and the absence of this trichome type on mature leaves of MT. However, although the trichome stalk is essentially of the same size among the genotypes analyzed (**Fig. 4e**), the gland size of type-IV trichomes present in IL3-2 and MT-Pet is significantly smaller than that of the parental *S. pennellii* (**Fig. 4f**). This result may be an indication that other factors controlling the size of the gland have not been introgressed, including genes specifically controlling gland development or metabolic pathways necessary to fill the gland with metabolites (*e.g.*, AS).

Fig. 4 Scanning electron micrographs of abaxial surfaces of representative leaves from *S. pennellii* LA716 (a), IL3-2 (b), Micro-Tom (c), and “*Pennellii enhanced trichomes*” (MT-Pet) (d). Scale bar=100 μm. (e–f) Type-IV trichome characterization. Stalk height (e) and gland size (f) comparisons between *S. pennellii*, IL3-2, and MT-Pet. Data are means ($n=30$) $\pm$ SEM. Asterisks indicate the means significantly differed from the control *S. pennellii*, according to the Student’s *t*-test ($P<0.001$). Black dots represent outliers.

We further determined the trichome density of the new line obtained. The MT-*Pet* plants had fewer type-IV trichomes (**Fig. 5**) than the wild parental on both leaves side (**Fig. 1b**). Considering the average of type-IV trichomes present on both leaf surfaces of the genotypes, MT-*Pet* displayed around 30-fold fewer trichomes than *S. pennellii* (**Fig. 1b**; **Fig. 5**). On the other hand, MT-*Pet* has a greater diversity of trichome types than *S. pennellii* (**Suppl. Fig. S1**).

Fig. 5 Density of trichome types IV and V on the adaxial (**a**) and abaxial (**d**) surfaces of mature 5th leaves from 30-day-old plants. Data are means ($n=30$) $\pm$ SEM. (**b-c**) Representative micrographs of the adaxial surface of MT (**b**) and MT-*Pet* (**c**) leaves. (**e-f**) Representative micrographs of the abaxial surface of MT (**e**) and MT-*Pet* (**f**) leaves. Scale bars= 250 μ m. Asterisks indicate the means are significantly different from the MT control, according to Student's *t*-test ($P<0.001$).

When compared to the MT control, an inverse relationship is observed between the densities of trichomes type IV and V on both surfaces (**Fig. 5**). This negative relation was also noticed by Vendemiatti *et al.* (2017) when characterizing the trichome densities of juvenile leaves of the cultivated tomato. The fact that type-IV and -V trichomes differ from each other only by the presence of a gland on the tip of type-IV

trichomes (Luckwill, 1943; Glas *et al.*, 2012) and their opposite pattern of distribution suggests an ontogenic relationship between them.

The number of type-IV trichomes on MT-*Pet* leaves is significantly lower when compared with MT-*Get*, a line harboring type-IV trichomes introgressed into MT from *S. galapagense* LA1401 (**Suppl. Fig. S2a**). In this comparison, we also observed that the gland of type-IV trichomes from MT-*Pet* is smaller than that of type-IV trichomes from MT-*Get* (**Suppl. Fig. S2b**).

The observation of the plant phenotype showed that MT-*Pet* did not present any obvious pleiotropic effects when compared to the MT control (**Fig. 6a**). Hence, MT-*Pet* and MT plants have the same height (**Fig. 6a-b**; **Suppl. Fig. S3a**) and branching pattern (**Fig. 6c**) and get determined at the same time (**Fig. 6d**). There were also no significant differences in the dry weights of shoots and roots (**Suppl. Fig. S3c**). The MT-*Pet* presents fewer inflorescences (**Suppl. Fig. S3b**), and fruits than MT (**Suppl. Fig. S4a**), but the MT-*Pet* fruits are bigger and have more soluble solids content (%Brix) (**Suppl. Fig. S4b-c**). Thereby, the genotypes are not statically different in yield parameters (**Suppl. Fig. S4d**).

Fig. 6 (a) Phenotype of 40-day-old Micro-Tom (MT) and MT-*Pet* plants. (b) Plant height (n=12). (c) Branching Index (n=24). (d) Number of leaves until the first inflorescence (n=24). The phenotypic characterization of MT-*Pet* plants and the MT control was performed on 50-day old plants. The results are not significantly different according to the Student's *t*-test ($P < 0.05$). Black dots represent outliers. Scale bar = 2.5 cm.

Previously, in the MT-*Get* lineage harboring the type-IV trichome of *S. galapagense*, we saw that at least 5 chromosomal fragments might be involved in the type-IV development, which resulted in many pleiotropic effects. The MT-*Get* also has less type-IV trichomes than the wild parent, but the difference is smaller than when comparing MT-*Pet* and its wild parent. This result indicates that the fragment introgressed from IL3-2 may be just one, although a major factor (See **Fig. 2**), of the genetic components from *S. pennellii* involved in the development of the type-IV

trichomes. From our previous results, we know that one of the genetic determinants of the presence of type-IV trichome in *S. galapagense* is also found on chromosome 3. However, apparently, that locus is not correspondent to the one found here in *S. pennellii*. In *S. galapagense*, the region is close to the *FW3.2* (Chakrabarti *et al.*, 2013) and *EJ-2* (Soyk *et al.*, 2017) genes, which bring very distinct characteristics to the plants that carry their “non-domesticated” alleles, such as increasing branching (and a slight reduction in the fruit weight) and small sepal size, respectively. MT-*Pet* is not more branched than MT (**Fig. 6d**) and does not display a small sepal size phenotype, as MT-*Get* does (**Suppl. Fig. S5**).

Altogether, these results suggest that, unlike the MT-*Get* (Vendemiatti *et al.*, *in prep*), MT-*Pet* presents a cleaner introgression, with fewer additional chromosomal portions other than that harboring the presumably *Pet* allele. This finding prompted us to carry out a fine mapping of *Pet*.

3.4.3 Mapping of the “*Pennellii* enhanced trichomes” trait

The chromosomal segment harboring the *Pet* locus is present in IL3-2 but absent in IL3-3 (see Fig. 2, inset). This region corresponds to BIND-3C, which is divided into two segments: BIND-3C.1, comprising of 110 genes, and BIND-3C.2, with 493 genes (**Fig. 7**; Chitwood *et al.*, 2013). Therefore, at least 603 genes (**Suppl. Table S2**) are candidates to be the causative factor of the capacity to bear type-IV trichomes on adult leaves.

Two well-known natural allelic variations from *S. pennellii* are located on the chromosomal region that defines IL3-2, the parental of MT-*Pet*. The first one is *SP3D* (Solyc03g063100) (Carmel-Goren *et al.*, 2003), which allele from *S. pennellii* confers early flowering and thus reduces the number of leaves for the first inflorescence to appear (termination). MT-*Pet* did not differ from MT in its termination (**Fig. 6b**), which indicates that it does not have the *S. pennellii*’s *SP3D* allele. This result is consistent with the fact that *Pet* is located in BIND-3C and not BIND-3D.2 that harbors *SP3D*. The second allelic variation is *yellow flesh* (*r*), which corresponds to the *PHYTOENE SYNTHASE1* (*PSY1*) gene (Solyc03g031860). The dominant *R* allele from *S. pennellii* confers a yellow color to the fruit when introgressed into the cultivated tomato (Pinto *et al.*, 2017). Also, the *R* allele co-segregates with the *Rg3C* allele from *S. pennellii*, which is a natural genetic variation that enhances *in vitro* organogenesis (Pinto *et al.*, 2017). Since the *PSY1* and

Rg3C loci belong to BINd3C.2, one would expect MT-*Pet* to bear yellow fruit and present a high *in vitro* organogenic capacity. However, MT-*Pet* has nor enhanced organogenic capacity (data not shown) neither yellow fruits (**Suppl. Fig. S6**). This finding is evidence that the introgressed segment from *S. pennellii* recombined in MT-*Pet*, and suggests that it has a narrower region introgressed into MT that can be further delimited in order to pinpoint the causative gene of *Pet*.

Fig. 7 Genetic mapping of the *Pet* natural allelic variation. Chromosomal fragments related to the parent IL3-2 are found in BINd-3C, according to Chitwood *et al.* (2013) (BIN refers to the chromosomal sub-regions of the ILs). Therefore, BINd-3C is divided into two segments: the first is called BINd-3C.1 and comprises of 110 genes, whereas the second is BINd-3C.2, which is composed of 493 genes. The annotations of the 603 candidate genes are presented in **Suppl. Table S2**.

3.4.4 The presence of type-IV trichomes in MT-*Pet* is not sufficient for high acylsugar accumulation

S. pennellii is one of the highest producers of acylsugar in the *Solanum* family, with up to 25% of the leaf dry weight being comprised of this natural insecticide (Fobes *et al.*, 1985). Since type-IV trichomes are the primary site of AS production (Slocombe *et al.*, 2008), one would expect that the introgression of the capacity to harbor type-IV trichomes could bring together an increased AS production. However, the GC-MS analysis showed that MT-*Pet* did not accumulate the acyl groups characteristic of the *S. pennellii* parental (**Fig. 8a**). An indirect test to predict the acylsugar accumulation and exudation is Rhodamine B staining (**Fig. 8b and c**), which is a dye for acylsugars (Weinhold & Baldwin, 2011). In MT-*Pet*, Rhodamine B did not stain inside or outside the trichome. However, the trichomes from *S. pennellii* accumulated pigments inside the

gland, on the external surface of the stem, and leaf epidermis, indicating a high production of AS and its exudation. This result shows that there are additional genetic components from *S. pennellii* necessary for AS accumulation but were not introgressed in MT-*Pet*. Additionally, the lack of AS in the MT-*Pet* trichome gland might also explain, at least in part, its smaller size (**Fig. 4f**).

Blauth *et al.* (1999) did not identify any QTL for acylsugar fatty acid composition in an intraspecific population of *S. pennellii*. Indeed, the dependence of this trait on multiple chromosomal regions, including those necessary for type-IV trichome development, could limit the detection of the QTL analysis. On the other hand, once identified, the introgression of all discrete components necessary for AS accumulation, including that found in MT-*Pet*, will likely provide for further staking to incorporate the desired trait. Such further genetic determinants are likely to be the *S. pennellii* alleles of genes involved in the metabolic pathway that produces AS (Fan *et al.*, 2019).

Fig. 8 (a) GC-MS profile comparison between *Solanum pennellii* LA716, Micro-Tom (MT), and MT-Pet regarding acyl group content. The y-axis represents arbitrary units. The identity of each peak is shown in Table 2. **(b-c)** Rhodamine-B assay for acylsugars dyed in *S. pennellii* (b) and MT-Pet (c).

Table 2. Description of acyl groups founded in *S. pennellii* LA716, MT-Pet, and Micro-Tom (MT) (See **Fig. 8a**).

Peek	Acyl Groups Content
1	2-Methyl Butanoic Acid
2	Methyl Pentadecanoic Acid
3	Methyl Decanoate
4	Methyl Hexadecanoic Acid
5	Methyl Dodecanoate
6	3-Methyl Hexadecanoic Acid
7	Methyl 9,12,15-Octadecatrienoic Acid
8	Methyl Octadecanoic Acid
9	Methyl 9,12-Octadecadienoic Acid
10	Methyl 9,12,15-Octadecatrienoic Acid

3.5 Conclusion

Our results show that alleles at the *Pet* locus are necessary and sufficient to confer type-IV trichome development on adult leaves of the cultivated tomato. On the other hand, *Pet* is not sufficient to reproduce the high AS content characteristic of the insect-resistant wild tomato relatives. The future cloning of the culprit gene(s) in MT-*Pet* will improve our understanding of glandular trichome development. Regardless of the genetic identity of *Pet*, the finding that it has no yield penalty opens the possibility to use it, in combination with genes controlling AS biosynthesis, for the development of insect-resistant crops.

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3.6 Supplementary Material

Suppl. Fig. S1 (a) Density (mm⁻²) of other trichome types on both leaf surfaces of *S. pennellii*. Data are means ($n = 25$) $\pm$ SEM. (b) Density (mm⁻²) of other trichomes types on the adaxial and abaxial sides of the mature 5th leaf in 30-day-old plants from the genotypes MT-*Pet* and the control Micro-Tom (MT). Data are means ($n=30$) $\pm$ SEM. Asterisks indicate mean significantly different from the MT control, according to the Student's *t*-test [$P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)].

Suppl. Fig. S2 (a) Type-IV and V trichome density in MT-*Pet* and MT-*Get*. (b) Type-IV trichome height and gland size comparison between MT-*Pet* and MT-*Get*. Data are means ($n=30$) $\pm$ SEM. Asterisks indicate statistical difference according to the Student's *t*-test [$P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***)].

Suppl. Fig. S3 Additional phenotypic characterization of MT-Pet plants. **(a)** Internode length (mm). **(b)** Number of inflorescences. **(c)** Root and shoot dry matter (g). Measurements were performed in plants (50 days after sowing). Data are means $\pm$ SEM ($n = 24$). Asterisks indicate statistical difference according to the Student's t -test ($P < 0.001$).

Suppl. Fig. S4 Productivity parameters in Micro-Tom (MT) and “*Pennellii enhanced trichomes*” (MT-Pet). **(a)** Average of the total number of fruits in each genotype. **(b)** Average fruit weight of fruits. **(c)** Quantification of total soluble solids (°Brix). **(d)** Productivity of fruits per plant. Data are means $\pm$ SEM (n=12). Asterisks indicate the statistical differences according to the Student's *t*-test [$P < 0.05$ (*), $P < 0.01$ (**)].

Suppl. Fig. S5 Sepal comparison between the introgression lines MT-*Pet* (which does not harbor the wild *EJ-2* allele from *S. pennellii*) and MT-*Get* (that harbors the wild allele from *S. galapagense* for *EJ-2*).

Suppl. Fig. S6 Representative ripe fruits of MT-*Pet*. Note that MT-*Pet* does not harbor the dominant wild allele *R* (yellow flesh) from *S. pennellii*, which confers yellow fruits in the IL3-2.

Suppl. Table S1. Density variation of type-IV trichomes in *Solanum pennellii* LA716 cited in the scientific literature.

Reference	Value of type-IV trichome density
Simmons; Gurr, 2004	53.11 mm ²
Slocombe <i>et al.</i> , 2008	10 mm ⁻²
Lucini <i>et al.</i> , 2015	17.4 ± 0.41 mm ⁻² (adaxial surface);
	16.5 ± 0.64 mm ⁻² (abaxial surface);
Bitew, 2018	23,69 mm ⁻²
This work	131,06 ± 11,72 mm ⁻² (adaxial surface);
	150,14 ± 8,84 mm ⁻² (adaxial surface);

Suppl. Table S2. Gene list of BINd-3C corresponding to “*Pennellii* enhanced trichomes” (MT-*Pet*) introgression.

Bin	ID	Description
3C.1	Solyc03g006910	BHLH transcription factor-like
3C.1	Solyc03g006920	Receptor kinase-like protein
3C.1	Solyc03g006930	Catalytic/ protein phosphatase type 2C
3C.1	Solyc03g006940	Catalytic/ protein phosphatase type 2C
3C.1	Solyc03g006950	Protein phosphatase 2C
3C.1	Solyc03g006960	Protein phosphatase 2C
3C.1	Solyc03g006970	Subtilisin-like protease
3C.1	Solyc03g006980	Alpha-L-fucosidase 1
3C.1	Solyc03g006990	Pentatricopeptide repeat-containing protein
3C.1	Solyc03g007000	Peptidase M48 Ste24p
3C.1	Solyc03g007010	ATP-dependent DEAD-box RNA helicase DeaD
3C.1	Solyc03g007020	MADS-box transcription factor-like protein
3C.1	Solyc03g007030	CDGSH iron sulfur domain-containing protein 1
3C.1	Solyc03g007040	Glutathione-regulated potassium-efflux system protein
3C.1	Solyc03g007050	Receptor like kinase, RLK
3C.1	Solyc03g007060	Unknown Protein
3C.1	Solyc03g007070	1-aminocyclopropane-1-carboxylate synthase
3C.1	Solyc03g007080	Genomic DNA chromosome 5 P1 clone MIO24
3C.1	Solyc03g007090	At5g51670-like protein (Fragment)
3C.1	Solyc03g007100	Cleavage and polyadenylation specificity factor
3C.1	Solyc03g007110	ATP-dependent Clp protease ATP-binding subunit ClpC
3C.1	Solyc03g007120	Leaf senescence protein-like
3C.1	Solyc03g007130	Microtubule-associated protein MAP65-1a
3C.1	Solyc03g007140	B3 domain-containing protein Os01g0905400
3C.1	Solyc03g007150	AT-hook motif nuclear localized protein 1
3C.1	Solyc03g007160	Spermine oxidase
3C.1	Solyc03g007170	FK506-binding protein 4
3C.1	Solyc03g007180	Unknown Protein
3C.1	Solyc03g007190	SPFH domain / Band 7 family protein
3C.1	Solyc03g007200	Oxidoreductase zinc-containing alcohol dehydrogenase family
3C.1	Solyc03g007210	Receptor serine/threonine kinase
3C.1	Solyc03g007220	Membrane receptor-like protein 1
3C.1	Solyc03g007230	Protein phosphatase 2C
3C.1	Solyc03g007240	Spermidine synthase 1
3C.1	Solyc03g007250	CNGC5-like protein (Fragment)
3C.1	Solyc03g007260	Cyclic nucleotide-gated ion channel 1
3C.1	Solyc03g007270	Protein phosphatase 2C
3C.1	Solyc03g007280	Chaperone protein DnaJ
3C.1	Solyc03g007290	Trehalose 6-phosphate phosphatase
3C.1	Solyc03g007300	50S ribosomal protein L16
3C.1	Solyc03g007310	Abscisic acid receptor PYL8
3C.1	Solyc03g007320	Polypyrimidine tract-binding protein 1-like
3C.1	Solyc03g007330	Cell division protease ftsH homolog
3C.1	Solyc03g007340	Unknown Protein
3C.1	Solyc03g007350	Pentatricopeptide repeat-containing protein
3C.1	Solyc03g007360	Nodulin MtN3 family protein

3C.1	Solyc03g007370	RNA polymerase sigma factor
3C.1	Solyc03g007380	WRKY transcription factor 27
3C.1	Solyc03g007390	Pentatricopeptide repeat-containing protein
3C.1	Solyc03g007400	Unknown Protein
3C.1	Solyc03g007410	BHLH transcription factor
3C.1	Solyc03g007420	Genomic DNA chromosome 5 TAC clone K19E1
3C.1	Solyc03g007430	Mitochondrial carrier family
3C.1	Solyc03g007440	Unknown Protein
3C.1	Solyc03g007450	Unknown Protein
3C.1	Solyc03g007460	Ethylene-responsive transcription factor 4
3C.1	Solyc03g007470	Ubiquitin-conjugating enzyme E2 10
3C.1	Solyc03g007480	Myosin
3C.1	Solyc03g007490	Kinesin like protein
3C.1	Solyc03g007500	Unknown Protein
3C.1	Solyc03g007510	HAT dimerization domain-containing protein-like
3C.1	Solyc03g007520	Proline-rich cell wall protein-like
3C.1	Solyc03g007530	Multidrug resistance protein ABC transporter family
3C.1	Solyc03g007540	Unknown Protein
3C.1	Solyc03g007550	Unknown Protein
3C.1	Solyc03g007560	Pre-mRNA-splicing factor CLF1
3C.1	Solyc03g007570	Magnesium and cobalt efflux protein corC
3C.1	Solyc03g007580	Genomic DNA chromosome 5 BAC clone F6N7
3C.1	Solyc03g007590	Unknown Protein
3C.1	Solyc03g007600	Pentatricopeptide repeat-containing protein
3C.1	Solyc03g007610	Chaperone protein dnaJ
3C.1	Solyc03g007620	Os01g0225300 protein (Fragment)
3C.1	Solyc03g007640	WRKY transcription factor 22
3C.1	Solyc03g007650	Chaperone protein dnaJ 3
3C.1	Solyc03g007660	NADH dehydrogenase
3C.1	Solyc03g007670	SGT1
3C.1	Solyc03g007680	Trafficking protein particle complex subunit 4
3C.1	Solyc03g007690	ABC transporter G family member 8
3C.1	Solyc03g007700	Unknown Protein
3C.1	Solyc03g007710	Unknown Protein
3C.1	Solyc03g007720	Unknown Protein
3C.1	Solyc03g007730	Beta-ocimene synthase
3C.1	Solyc03g007740	Reticulon family protein
3C.1	Solyc03g007750	F-box family protein
3C.1	Solyc03g007760	Cell division protease ftsH
3C.1	Solyc03g007770	C4-dicarboxylate transporter/
3C.1	Solyc03g007780	Unknown Protein
3C.1	Solyc03g007790	Receptor-like protein kinase
3C.1	Solyc03g007800	Topoisomerase 1-associated factor 1
3C.1	Solyc03g007810	Pyruvate kinase
3C.1	Solyc03g007820	Unknown Protein
3C.1	Solyc03g007830	Threonine endopeptidase
3C.1	Solyc03g007840	Pentatricopeptide repeat-containing protein
3C.1	Solyc03g007850	Pre-mRNA-splicing factor CLF1
3C.1	Solyc03g007860	ABC-type Co2+ transport system permease component

3C.1	Solyc03g007870	Heavy metal-associated domain
3C.1	Solyc03g007880	Unknown Protein
3C.1	Solyc03g007890	Heat shock protein 90 (Fragment)
3C.1	Solyc03g007900	Glutamine cyclotransferase-like
3C.1	Solyc03g007910	Receptor-like serine/threonine kinase
3C.1	Solyc03g007920	Glutamine cyclotransferase-like
3C.1	Solyc03g007930	Unknown Protein
3C.1	Solyc03g007940	Glycoside hydrolase family 28 protein
3C.1	Solyc03g007950	Glycoside hydrolase family 28 protein
3C.1	Solyc03g007960	Beta-carotene hydroxylase 2
3C.1	Solyc03g007970	UDP-N-acetylmuramate-alanine ligase
3C.1	Solyc03g007980	Septum site-determining protein MinD
3C.1	Solyc03g007990	Unknown Protein
3C.1	Solyc03g008000	Genomic DNA chromosome 5 BAC clone F6N7
3C.1	Solyc03g008010	PPPDE peptidase domain-containing protein 1
3C.2	Solyc03g025100	Unknown Protein
3C.2	Solyc03g025110	Unknown Protein
3C.2	Solyc03g025120	Unknown Protein
3C.2	Solyc03g025130	Unknown Protein
3C.2	Solyc03g025140	Unknown Protein
3C.2	Solyc03g025150	Unknown Protein
3C.2	Solyc03g025160	Unknown Protein
3C.2	Solyc03g025170	GRAS family transcription factor
3C.2	Solyc03g025180	Unknown Protein
3C.2	Solyc03g025190	Multidrug resistance protein mdtK
3C.2	Solyc03g025200	Multidrug resistance protein mdtK
3C.2	Solyc03g025210	Mate efflux family protein
3C.2	Solyc03g025220	Multidrug resistance protein mdtK
3C.2	Solyc03g025230	Multidrug resistance protein mdtK
3C.2	Solyc03g025240	Multidrug resistance protein mdtK
3C.2	Solyc03g025250	Multidrug resistance protein mdtK
3C.2	Solyc03g025260	Retinoblastoma-binding protein
3C.2	Solyc03g025270	rRNA 2'-O-methyltransferase fibrillarin
3C.2	Solyc03g025280	RNA-binding protein
3C.2	Solyc03g025290	Hydroxyproline-rich glycoprotein family protein
3C.2	Solyc03g025300	Unknown Protein
3C.2	Solyc03g025310	Sec-independent protein translocase protein tatA/E
3C.2	Solyc03g025320	Hydroxycinnamoyl transferase
3C.2	Solyc03g025330	Unknown Protein
3C.2	Solyc03g025340	C2 domain-containing protein
3C.2	Solyc03g025350	Citrate transporter family protein
3C.2	Solyc03g025360	Protein serine/threonine kinase
3C.2	Solyc03g025370	Short-chain dehydrogenase/reductase family protein
3C.2	Solyc03g025380	Peroxidase
3C.2	Solyc03g025390	Retinol dehydrogenase 12
3C.2	Solyc03g025400	Retinol dehydrogenase 12
3C.2	Solyc03g025410	Dehydrogenase/reductase SDR family member 13
3C.2	Solyc03g025420	SCF ubiquitin ligase skp1 component

3C.2	Solyc03g025430	Genomic DNA chromosome 5 TAC clone K24M7
3C.2	Solyc03g025440	Zinc finger family protein
3C.2	Solyc03g025450	Receptor-like protein kinase
3C.2	Solyc03g025460	Myosin heavy chain-related-like
3C.2	Solyc03g025470	Kinesin-like protein
3C.2	Solyc03g025480	Unknown Protein
3C.2	Solyc03g025490	Gibberellin 20-oxidase-like protein
3C.2	Solyc03g025500	Prolyl 4-hydroxylase alpha subunit-like protein
3C.2	Solyc03g025510	Calmodulin-binding heat shock protein
3C.2	Solyc03g025520	60S ribosomal protein L36
3C.2	Solyc03g025530	S-layer domain protein
3C.2	Solyc03g025540	Cleavage and polyadenylation specificity factor
3C.2	Solyc03g025550	Signal peptide peptidase family protein
3C.2	Solyc03g025560	Undecaprenyl pyrophosphate synthase
3C.2	Solyc03g025570	DNA-directed RNA polymerase subunit H
3C.2	Solyc03g025580	Pectinacetylesterase (Fragment)
3C.2	Solyc03g025590	Tripeptidyl peptidase II
3C.2	Solyc03g025600	Pectinacetylesterase like protein (Fragment)
3C.2	Solyc03g025610	Subtilisin-like serine protease
3C.2	Solyc03g025620	Tripeptidyl peptidase II
3C.2	Solyc03g025630	Tripeptidyl-peptidase 2
3C.2	Solyc03g025640	Cytochrome P450
3C.2	Solyc03g025650	Glucan-endo-1 3-beta-glucosidase
3C.2	Solyc03g025660	Self-incompatibility protein (Fragment)
3C.2	Solyc03g025670	PAR-1c protein
3C.2	Solyc03g025680	PAR-1c protein
3C.2	Solyc03g025690	Unknown Protein
3C.2	Solyc03g025700	Pentatricopeptide repeat-containing protein
3C.2	Solyc03g025710	GCN5-related N-acetyltransferase
3C.2	Solyc03g025720	Long-chain-fatty-acid--CoA ligase
3C.2	Solyc03g025730	Tubulin beta chain
3C.2	Solyc03g025740	CCHC zinc finger protein
3C.2	Solyc03g025750	Actin depolymerizing factor 10
3C.2	Solyc03g025760	Glycosyltransferase CAZy family GT90 (Fragment)
3C.2	Solyc03g025770	Glycosyltransferase CAZy family GT90 (Fragment)
3C.2	Solyc03g025780	Glycosyltransferase CAZy family GT90 (Fragment)
3C.2	Solyc03g025790	Metal ion binding protein
3C.2	Solyc03g025800	Os03g0111400 protein (Fragment)
3C.2	Solyc03g025810	Low-temperature-induced 65 kDa protein
3C.2	Solyc03g025820	Genomic DNA chromosome 5 TAC clone K24M7
3C.2	Solyc03g025830	Os03g0107400 protein (Fragment)
3C.2	Solyc03g025840	Cytochrome b561
3C.2	Solyc03g025850	Remorin 1
3C.2	Solyc03g025860	Vesicle-trafficking protein SEC22b
3C.2	Solyc03g025870	MYB transcription factor
3C.2	Solyc03g025880	Cleavage and polyadenylation specificity factor
3C.2	Solyc03g025890	DNA mismatch repair protein mutS
3C.2	Solyc03g025900	Flap structure-specific endonuclease
3C.2	Solyc03g025910	Unknown Protein

3C.2	Solyc03g025920	F-box family protein
3C.2	Solyc03g025930	F-box family protein
3C.2	Solyc03g025940	F-box family protein
3C.2	Solyc03g025950	Membrane-associated progesterone receptor
3C.2	Solyc03g025960	Unknown Protein
3C.2	Solyc03g025970	Methyl binding domain protein
3C.2	Solyc03g025980	Peptidyl-prolyl cis-trans isomerase (Fragment)
3C.2	Solyc03g025990	Hexulose-6-phosphate isomerase
3C.2	Solyc03g026000	Unknown Protein
3C.2	Solyc03g026010	Transmembrane protein 161B
3C.2	Solyc03g026020	Heat stress transcription factor
3C.2	Solyc03g026030	Os10g0578600 protein (Fragment)
3C.2	Solyc03g026040	Receptor like kinase, RLK
3C.2	Solyc03g026050	Terminal flower 1 (Fragment)
3C.2	Solyc03g026060	Ankyrin repeat-rich protein
3C.2	Solyc03g026070	Homeobox-leucine zipper protein ATHB-8
3C.2	Solyc03g026080	Rhomboid family protein
3C.2	Solyc03g026090	Non-specific lipid-transfer protein
3C.2	Solyc03g026100	tRNA-specific adenosine deaminase-like protein 3
3C.2	Solyc03g026110	Calmodulin binding protein
3C.2	Solyc03g026120	Dehydration-responsive family protein
3C.2	Solyc03g026130	Cytochrome P450
3C.2	Solyc03g026140	Cytochrome P450
3C.2	Solyc03g026150	Ring-h2 finger protein
3C.2	Solyc03g026160	F-box family protein
3C.2	Solyc03g026170	F-box family protein
3C.2	Solyc03g026180	Unknown Protein
3C.2	Solyc03g026190	Transcriptional corepressor SEUSS
3C.2	Solyc03g026200	Os10g0522500 protein (Fragment)
3C.2	Solyc03g026210	Dihydrodipicolinate reductase family protein
3C.2	Solyc03g026220	BCL-2 binding anthanogene-1
3C.2	Solyc03g026230	Multidrug resistance protein mdtK
3C.2	Solyc03g026240	Arginine/serine-rich splicing factor 31
3C.2	Solyc03g026250	Response regulator 11
3C.2	Solyc03g026260	TraB family protein
3C.2	Solyc03g026270	Ethylene-responsive transcription factor 4
3C.2	Solyc03g026280	CRT binding factor 2
3C.2	Solyc03g124110	CRT binding factor 2
3C.2	Solyc03g026290	Post-GPI attachment to proteins factor 3
3C.2	Solyc03g026300	Post-GPI attachment to proteins factor 3
3C.2	Solyc03g026310	ABC transporter permease/ATP-binding protein
3C.2	Solyc03g026320	Lipid A export ATP-binding/permease protein MsbA
3C.2	Solyc03g026330	Uncharacterized conserved protein
3C.2	Solyc03g026340	Calcium dependent protein kinase 17
3C.2	Solyc03g026350	WD-40 repeat family protein
3C.2	Solyc03g026360	Peptidoglycan-binding LysM domain
3C.2	Solyc03g026370	Peptidoglycan-binding LysM domain
3C.2	Solyc03g026400	F-box family protein
3C.2	Solyc03g026410	F-box family protein

3C.2	Solyc03g031410	Unknown Protein
3C.2	Solyc03g031420	Molybdenum cofactor sulfurase
3C.2	Solyc03g031430	Urease accessory protein UreF
3C.2	Solyc03g031440	Flavoprotein wrbA
3C.2	Solyc03g031450	BHLH transcription factor-like protein
3C.2	Solyc03g031460	Pentatricopeptide repeat-containing protein
3C.2	Solyc03g031470	Dihydroflavonol 4-reductase
3C.2	Solyc03g031480	HR7 protein
3C.2	Solyc03g031490	HR7 protein
3C.2	Solyc03g031500	HR7 protein
3C.2	Solyc03g031510	HR7 protein
3C.2	Solyc03g031520	Fruit-specific protein
3C.2	Solyc03g031530	Os01g0786800 protein (Fragment)
3C.2	Solyc03g031540	Receptor-like protein kinase
3C.2	Solyc03g031550	Cysteine-rich receptor-like protein kinase
3C.2	Solyc03g031560	UDP-3-O-
3C.2	Solyc03g031570	Dopamine beta-monooxygenase
3C.2	Solyc03g031580	Stig1 (Fragment)
3C.2	Solyc03g031590	C4-dicarboxylate transporter
3C.2	Solyc03g031600	Peroxisomal membrane protein PMP22
3C.2	Solyc03g031610	Unknown Protein
3C.2	Solyc03g031620	Phosphoadenosine phosphosulfate reductase
3C.2	Solyc03g031630	Calcium-binding EF-hand family protein-like
3C.2	Solyc03g031640	Unknown Protein
3C.2	Solyc03g031650	Autophagy-related protein 8
3C.2	Solyc03g031660	Unknown Protein
3C.2	Solyc03g031670	Calcium-dependent protein kinase
3C.2	Solyc03g031680	Uncharacterized GPI-anchored protein At1g61900
3C.2	Solyc03g031690	DNA-directed RNA polymerases I, II, and III subunit
3C.2	Solyc03g031700	ABC transporter FeS assembly protein SufB
3C.2	Solyc03g031710	Genomic DNA chromosome 5 P1 clone MBG8
3C.2	Solyc03g031720	RNA Binding Protein 45
3C.2	Solyc03g031730	Beta-glucosidase 47
3C.2	Solyc03g031740	Glucose-6-phosphate/phosphate translocator 2
3C.2	Solyc03g031750	Vacuolar protein sorting-associated protein 28
3C.2	Solyc03g031760	Homeobox-leucine zipper protein ATHB-14
3C.2	Solyc03g031770	Rop-interactive crib motif-containing protein 1
3C.2	Solyc03g031780	Unknown Protein
3C.2	Solyc03g031790	Magnesium transporter protein 1
3C.2	Solyc03g031800	Xyloglucan endotransglucosylase/hydrolase 1
3C.2	Solyc03g031810	Translation initiation factor SUI1
3C.2	Solyc03g031820	Tyrosyl-DNA phosphodiesterase 1
3C.2	Solyc03g031830	Zinc finger family protein
3C.2	Solyc03g031840	Expansin
3C.2	Solyc03g031850	Unknown Protein
3C.2	Solyc03g031860	Phytoene synthase 1
3C.2	Solyc03g031870	Acyl-CoA synthetase/AMP-acid ligase
3C.2	Solyc03g031880	Amine oxidase family protein
3C.2	Solyc03g031890	Cold induced protein-like

3C.2	Solyc03g031900	Helicase sen1
3C.2	Solyc03g031910	Helicase sen1
3C.2	Solyc03g031920	Yellow stripe-like protein 2.1 (Fragment)
3C.2	Solyc03g031930	Unknown Protein
3C.2	Solyc03g031940	Acyl-CoA synthetase/AMP-acid ligase II
3C.2	Solyc03g031950	Unknown Protein
3C.2	Solyc03g031960	Unknown Protein
3C.2	Solyc03g031970	Auxin response factor 8-1
3C.2	Solyc03g031980	Receptor-like kinase
3C.2	Solyc03g031990	Auxin efflux carrier family protein
3C.2	Solyc03g032000	Thioredoxin reductase
3C.2	Solyc03g032010	Hydroxy-methylglutaryl-coenzyme A reductase
3C.2	Solyc03g032020	Hydroxy-methylglutaryl-coenzyme A reductase
3C.2	Solyc03g032030	Mitochondrial phosphate carrier protein
3C.2	Solyc03g032040	MFS family major facilitator transporter
3C.2	Solyc03g032050	Genomic DNA chromosome 5 TAC clone K2A18
3C.2	Solyc03g032060	Ring finger protein
3C.2	Solyc03g032070	Holliday junction ATP-dependent DNA helicase ruvB
3C.2	Solyc03g032080	Auxin efflux carrier family protein
3C.2	Solyc03g032090	Amino acid transporter family protein
3C.2	Solyc03g032100	Unknown Protein
3C.2	Solyc03g032110	Unknown Protein
3C.2	Solyc03g032120	Nucleoside-triphosphatase THEP1
3C.2	Solyc03g032130	High mobility group protein
3C.2	Solyc03g032140	Heterogeneous nuclear ribonucleoprotein H1
3C.2	Solyc03g032150	Serine/threonine kinase-like protein ABC1063
3C.2	Solyc03g032160	Ubiquilin-1
3C.2	Solyc03g032170	Unknown Protein
3C.2	Solyc03g032180	CCA-adding enzyme
3C.2	Solyc03g032190	Cyclin B2
3C.2	Solyc03g032200	Unknown Protein
3C.2	Solyc03g032210	Acyl-CoA synthetase/AMP-acid ligase II
3C.2	Solyc03g032220	Caffeoyl-CoA O-methyltransferase
3C.2	Solyc03g032230	CM0216.330.nc protein
3C.2	Solyc03g032240	Cation/H(+) antiporter 2
3C.2	Solyc03g033240	Os11g0148200 protein (Fragment)
3C.2	Solyc03g033250	Tracheary element differentiation-related 6
3C.2	Solyc03g033260	Maltose excess protein 1, chloroplastic
3C.2	Solyc03g033270	Maltose excess protein 1-like, chloroplastic
3C.2	Solyc03g033280	Tracheary element differentiation-related 6
3C.2	Solyc03g033290	Tracheary element differentiation-related 6
3C.2	Solyc03g033300	Ammonium transporter
3C.2	Solyc03g033310	RNA-binding protein
3C.2	Solyc03g033320	Prolyl 4-hydroxylase alpha subunit-like protein
3C.2	Solyc03g033330	RING finger protein 44
3C.2	Solyc03g033340	Protein phosphatase 2C
3C.2	Solyc03g033350	Aspartyl protease family protein
3C.2	Solyc03g033360	Aspartic proteinase nepenthesin II-like
3C.2	Solyc03g033370	PreY mitochondrial

3C.2	Solyc03g033380	Ran-binding protein 9
3C.2	Solyc03g033390	F-box family protein
3C.2	Solyc03g033400	Vacuolar protein sorting 35
3C.2	Solyc03g033410	Ubiquitin-conjugating enzyme E2 10
3C.2	Solyc03g033420	Rho-related GTP-binding protein RhoC
3C.2	Solyc03g033430	26S proteasome non-ATPase regulatory subunit 4
3C.2	Solyc03g033440	ATP synthase subunit alpha
3C.2	Solyc03g033450	Ribosomal RNA small subunit methyltransferase B
3C.2	Solyc03g033460	Oligopeptide transporter 9
3C.2	Solyc03g033470	AGAP009131-PA (Fragment)
3C.2	Solyc03g033480	Zinc finger HIT domain-containing protein 1
3C.2	Solyc03g033490	Thaumatococcus-like protein
3C.2	Solyc03g033500	Cold shock protein-1
3C.2	Solyc03g033510	Armadillo/beta-catenin repeat family protein
3C.2	Solyc03g033520	Maturase (Fragment)
3C.2	Solyc03g033530	DNA lyase
3C.2	Solyc03g033540	Calcium-dependent protein kinase
3C.2	Solyc03g033550	Unknown Protein
3C.2	Solyc03g033560	Zinc finger protein
3C.2	Solyc03g033570	Agamous-like MADS-box protein AGL62
3C.2	Solyc03g033580	Calcium-dependent protein kinase CPK1
3C.2	Solyc03g033590	Auxin-induced SAUR-like protein
3C.2	Solyc03g033600	Small nuclear ribonucleoprotein Sm D3
3C.2	Solyc03g033610	Receptor like kinase, RLK
3C.2	Solyc03g033620	Serine carboxypeptidase S28 family protein
3C.2	Solyc03g033630	Zinc finger CCH domain-containing protein 44
3C.2	Solyc03g033640	DUF726 domain-containing protein
3C.2	Solyc03g033650	Unknown Protein
3C.2	Solyc03g033660	GATA transcription factor 9
3C.2	Solyc03g033670	Unknown Protein
3C.2	Solyc03g033680	Short internode related sequence 5
3C.2	Solyc03g033690	Peroxidase
3C.2	Solyc03g033700	Peroxidase
3C.2	Solyc03g033710	Peroxidase
3C.2	Solyc03g033720	Genomic DNA chromosome 3 P1 clone MDJ14
3C.2	Solyc03g033730	BZIP transcription factor
3C.2	Solyc03g033740	Unknown Protein
3C.2	Solyc03g033750	BCS1 protein-like protein
3C.2	Solyc03g033760	Unknown Protein
3C.2	Solyc03g033770	BCS1 protein-like protein
3C.2	Solyc03g033780	Unknown Protein
3C.2	Solyc03g033790	Cell division protease ftsH homolog 3
3C.2	Solyc03g033800	Unknown Protein
3C.2	Solyc03g033810	Unknown Protein
3C.2	Solyc03g033820	26S protease regulatory subunit 4 homolog
3C.2	Solyc03g033830	Unknown Protein
3C.2	Solyc03g033840	26S protease regulatory subunit 6B homolog
3C.2	Solyc03g033850	Syntaxin 81
3C.2	Solyc03g033860	Genomic DNA chromosome 5 TAC clone K1F13

3C.2	Solyc03g033870	Unknown Protein
3C.2	Solyc03g033880	MADS-box transcription factor 31
3C.2	Solyc03g033890	MADS-box protein-like
3C.2	Solyc03g033900	MADS-box protein
3C.2	Solyc03g033910	Unknown Protein
3C.2	Solyc03g033920	Unknown Protein
3C.2	Solyc03g033930	Unknown Protein
3C.2	Solyc03g033940	MADS-box protein
3C.2	Solyc03g033950	Pre-mRNA-splicing factor CWC26
3C.2	Solyc03g033960	Unknown Protein
3C.2	Solyc03g033970	Phenylcoumaran benzylic ether reductase 3
3C.2	Solyc03g033980	Tubby-like F-box protein 5
3C.2	Solyc03g033990	Cytochrome c-type biogenesis protein CcmE
3C.2	Solyc03g034000	Transcription factor
3C.2	Solyc03g034010	U-box domain-containing protein 5
3C.2	Solyc03g034020	U-box domain-containing protein 5
3C.2	Solyc03g034030	Ce-LEA
3C.2	Solyc03g034040	Unknown Protein
3C.2	Solyc03g034050	FAM96B protein
3C.2	Solyc03g034060	Receptor-like protein kinase At5g59670
3C.2	Solyc03g034070	P-166-4_1 (Fragment)
3C.2	Solyc03g034080	Acidic leucine-rich nuclear phosphoprotein 32 family
3C.2	Solyc03g034090	BAC19.4
3C.2	Solyc03g034100	Plant-specific domain TIGR01568 family protein
3C.2	Solyc03g034110	Homeobox-leucine zipper protein ATHB-40
3C.2	Solyc03g034120	Homeobox-leucine zipper protein ATHB-40
3C.2	Solyc03g034130	Homeobox-leucine zipper protein ATHB-40
3C.2	Solyc03g034140	Flavoprotein wrbA
3C.2	Solyc03g034150	Homeobox leucine zipper protein
3C.2	Solyc03g034160	Unknown Protein
3C.2	Solyc03g034170	Uncharacterized conserved membrane protein
3C.2	Solyc03g034180	14-3-3 protein beta/alpha-1
3C.2	Solyc03g034190	Ribosomal protein PSRP-3/Ycf65
3C.2	Solyc03g034200	KH domain containing protein expressed
3C.2	Solyc03g034210	Sentrin-specific protease 2
3C.2	Solyc03g034220	Ribulose biphosphate carboxylase small chain
3C.2	Solyc03g034230	Unknown Protein
3C.2	Solyc03g034240	Chromosome 18 contig 1 DNA sequence
3C.2	Solyc03g034250	Similarity the predicted <i>A. niger</i> protein contains a neutral zinc
3C.2	Solyc03g034260	Agamous-like MADS-box protein AGL62
3C.2	Solyc03g034270	Abhydrolase domain-containing protein FAM108B1
3C.2	Solyc03g034280	GPI-anchored protein
3C.2	Solyc03g034290	Pentatricopeptide repeat-containing protein
3C.2	Solyc03g034300	Unknown Protein
3C.2	Solyc03g034310	Unknown Protein
3C.2	Solyc03g034320	Class I glutamine amidotransferase
3C.2	Solyc03g034330	Lipid transfer protein
3C.2	Solyc03g034340	Ulp1 protease family
3C.2	Solyc03g034360	Unknown Protein

3C.2	Solyc03g034370	Unknown Protein
3C.2	Solyc03g034380	Lipid transfer protein
3C.2	Solyc03g034390	Lipid transfer protein
3C.2	Solyc03g034400	Multidrug resistance protein mdtK
3C.2	Solyc03g034410	cDNA clone J100026I16 full insert sequence
3C.2	Solyc03g034420	SLL2-S9-protein-like
3C.2	Solyc03g034430	Eukaryotic translation initiation factor 5
3C.2	Solyc03g034440	Eukaryotic translation initiation factor 5
3C.2	Solyc03g034450	E3 ubiquitin-protein ligase sina
3C.2	Solyc03g034460	E3 ubiquitin-protein ligase sina
3C.2	Solyc03g036460	E3 ubiquitin-protein ligase SINA-like 10
3C.2	Solyc03g036470	Phenylalanine ammonia-lyase
3C.2	Solyc03g036480	Phenylalanine ammonia-lyase
3C.2	Solyc03g037490	E3 ubiquitin-protein ligase SINA-like 10
3C.2	Solyc03g042500	B3 domain-containing protein At1g49475
3C.2	Solyc03g042510	B3 domain-containing protein At1g49475
3C.2	Solyc03g042520	E3 ubiquitin-protein ligase SINA-like 10
3C.2	Solyc03g042530	Unknown Protein
3C.2	Solyc03g042540	HAT family dimerization domain containing protein
3C.2	Solyc03g042550	E3 ubiquitin-protein ligase sina
3C.2	Solyc03g042560	Phenylalanine ammonia-lyase
3C.2	Solyc03g043570	Unknown Protein
3C.2	Solyc03g043580	B3 domain-containing protein At1g49475
3C.2	Solyc03g043590	Seven in absentia family protein
3C.2	Solyc03g043600	E3 ubiquitin-protein ligase SINA-like 10
3C.2	Solyc03g043610	ATP synthase subunit a
3C.2	Solyc03g043620	Unknown Protein
3C.2	Solyc03g043630	Unknown Protein
3C.2	Solyc03g043640	Metal ion binding protein
3C.2	Solyc03g043650	Ulp1 protease family
3C.2	Solyc03g043660	Pro-apoptotic serine protease nma111-like protein
3C.2	Solyc03g043670	Transposon Ty1-BL Gag-Pol polyprotein
3C.2	Solyc03g043680	Gag-Pol polyprotein
3C.2	Solyc03g043690	Retrovirus-related Pol polyprotein from transposon
3C.2	Solyc03g043700	Armadillo/beta-catenin repeat family protein
3C.2	Solyc03g043710	Receptor protein kinase-like protein
3C.2	Solyc03g043720	BRICK1
3C.2	Solyc03g043730	Unknown Protein
3C.2	Solyc03g043740	Hydroxyproline-rich glycoprotein
3C.2	Solyc03g043750	FAD-dependent pyridine nucleotide-disulphide oxidoreductase
3C.2	Solyc03g043760	Expressed protein (Fragment)
3C.2	Solyc03g043770	Receptor like kinase, RLK
3C.2	Solyc03g043780	Unknown Protein
3C.2	Solyc03g043790	Unknown Protein
3C.2	Solyc03g043810	Unknown Protein
3C.2	Solyc03g043820	DNA binding protein
3C.2	Solyc03g043830	DNA binding protein
3C.2	Solyc03g043840	DNA binding protein
3C.2	Solyc03g043850	Cytochrome c oxidase subunit VIa family protein

3C.2	Solyc03g043860	Nudix hydrolase 1
3C.2	Solyc03g043870	Ubiquitin ligase protein FANCL
3C.2	Solyc03g043880	Branched-chain amino acid aminotransferase
3C.2	Solyc03g043890	1-aminocyclopropane-1-carboxylate synthase
3C.2	Solyc03g043900	BZIP transcription factor family protein
3C.2	Solyc03g043910	BSD domain containing protein
3C.2	Solyc03g043920	Alpha-6-galactosyltransferase
3C.2	Solyc03g043930	Adiponectin receptor protein 2
3C.2	Solyc03g043940	Unknown Protein
3C.2	Solyc03g043950	Amino-acid N-acetyltransferase
3C.2	Solyc03g043960	ADP-ribosylation factor-like protein
3C.2	Solyc03g043970	Glycosyl hydrolase family protein 43
3C.2	Solyc03g043980	Unknown Protein
3C.2	Solyc03g043990	Beta-glucanase-like protein
3C.2	Solyc03g044000	Ribosomal protein L26
3C.2	Solyc03g044010	Mitochondrial porin (Voltage-dependent anion channel)
3C.2	Solyc03g044020	Condensin-2 complex subunit G2
3C.2	Solyc03g044030	Pre-mRNA-splicing factor SYF1
3C.2	Solyc03g044040	Pre-mRNA-splicing factor SYF1
3C.2	Solyc03g044050	Unknown Protein
3C.2	Solyc03g044060	Formin 3
3C.2	Solyc03g044070	Gag-Pol polyprotein
3C.2	Solyc03g044080	Unknown Protein
3C.2	Solyc03g044090	Phototropic-responsive NPH3 family protein
3C.2	Solyc03g044100	Peroxidase 5
3C.2	Solyc03g044110	B3 domain-containing protein LOC_Os12g40080
3C.2	Solyc03g044120	Aromatic L-amino acid decarboxylase
3C.2	Solyc03g044140	Protein kinase 2
3C.2	Solyc03g044150	Subtilisin-like protease
3C.2	Solyc03g044160	Pto-like, Serine/threonine kinase protein
3C.2	Solyc03g044170	Gag-pol polyprotein (Fragment)
3C.2	Solyc03g044180	ABC transporter family protein
3C.2	Solyc03g044190	FAD-dependent oxidoreductase
3C.2	Solyc03g044200	Alcohol dehydrogenase
3C.2	Solyc03g044210	Vicilin (Fragment)
3C.2	Solyc03g044220	SCF E3 ubiquitin ligase complex F-box protein grrA
3C.2	Solyc03g044230	Cathepsin B-like cysteine proteinase
3C.2	Solyc03g044240	Cysteine proteinase
3C.2	Solyc03g044250	Unknown Protein
3C.2	Solyc03g044260	Ubiquitin-conjugating enzyme E2 I
3C.2	Solyc03g044270	tRNA-dihydrouridine synthase 1-like protein
3C.2	Solyc03g044280	Cysteine proteinase
3C.2	Solyc03g044290	Unknown Protein
3C.2	Solyc03g044300	AP2-like ethylene-responsive transcription factor
3C.2	Solyc03g044310	Transposase family protein
3C.2	Solyc03g044320	Unknown Protein
3C.2	Solyc03g044330	Acetolactate synthase
3C.2	Solyc03g044340	Unknown Protein
3C.2	Solyc03g044350	Chaperone dnaJ 3

3C.2	Solyc03g044360	Mutator-like transposase 53847-56139
3C.2	Solyc03g044370	OTU domain containing protein
3C.2	Solyc03g044380	Histone-lysine N-methyltransferase MEDEA
3C.2	Solyc03g044390	Mutator-like transposase
3C.2	Solyc03g044400	Ulp1 protease family
3C.2	Solyc03g044410	Unknown Protein
3C.2	Solyc03g044420	Unknown Protein
3C.2	Solyc03g044430	Nicotinate phosphoribosyltransferase-like protein
3C.2	Solyc03g044440	Ribosomal protein L22 family protein
3C.2	Solyc03g044450	Unknown Protein
3C.2	Solyc03g044460	BHLH transcription factor
3C.2	Solyc03g044470	Red chlorophyll catabolite reductase (Fragment)
3C.2	Solyc03g044480	Unknown Protein
3C.2	Solyc03g044490	HAT family dimerization domain containing protein
3C.2	Solyc03g044500	Mutator-like transposase
3C.2	Solyc03g044510	Unknown Protein
3C.2	Solyc03g044520	Unknown Protein
3C.2	Solyc03g044530	Unknown Protein
3C.2	Solyc03g044540	Unknown Protein
3C.2	Solyc03g044550	Unknown Protein
3C.2	Solyc03g044560	Unknown Protein
3C.2	Solyc03g044570	Transposon Ty1-A Gag-Pol polyprotein
3C.2	Solyc03g044580	Unknown Protein
3C.2	Solyc03g044590	Unknown Protein
3C.2	Solyc03g044600	Unknown Protein
3C.2	Solyc03g044610	Mutator-like transposase
3C.2	Solyc03g044620	Transmembrane protein 131
3C.2	Solyc03g044630	Conserved domain protein
3C.2	Solyc03g044640	Unknown Protein
3C.2	Solyc03g044660	Dihydrodipicolinate synthase 2
3C.2	Solyc03g044670	Phosphoglycerate dehydrogenase-like protein
3C.2	Solyc03g044690	Protein FAR1-RELATED SEQUENCE 5
3C.2	Solyc03g044700	Unknown Protein
3C.2	Solyc03g044710	Patatin-like protein 3
3C.2	Solyc03g044720	Pinoresinol-lariciresinol reductase
3C.2	Solyc03g044730	Ulp1 protease family
3C.2	Solyc03g044740	Methyl jasmonate esterase
3C.2	Solyc03g044790	Alpha-hydroxynitrile lyase
3C.2	Solyc03g044800	Methyl jasmonate esterase
3C.2	Solyc03g044810	Alpha-hydroxynitrile lyase
3C.2	Solyc03g044820	Methyl jasmonate esterase
3C.2	Solyc03g044830	Guanine nucleotide-binding protein
3C.2	Solyc03g044840	Helicase-like protein
3C.2	Solyc03g044850	Unknown Protein
3C.2	Solyc03g044860	Os01g0686000 protein (Fragment)
3C.2	Solyc03g044870	Unknown Protein
3C.2	Solyc03g044880	Exostosin family protein
3C.2	Solyc03g044890	L-galactose-1-phosphate phosphatase 3
3C.2	Solyc03g044900	Calmodulin-like protein 41

3C.2	Solyc03g044910	GNS1/SUR4 membrane family protein
3C.2	Solyc03g044920	Unknown Protein
3C.2	Solyc03g044930	Unknown Protein
3C.2	Solyc03g044940	Transposase (Fragment)
3C.2	Solyc03g044950	Coiled-coil domain-containing protein 109A
3C.2	Solyc03g044960	Coiled-coil domain-containing protein 109A
3C.2	Solyc03g044970	Unknown Protein
3C.2	Solyc03g044980	Unknown Protein
3C.2	Solyc03g044990	Unknown Protein
3C.2	Solyc03g045000	Expressed protein having alternate splicing products
3C.2	Solyc03g045010	Aromatic L-amino acid decarboxylase
3C.2	Solyc03g045020	Aromatic L-amino acid decarboxylase
3C.2	Solyc03g045030	CYCLOIDEA-like protein (Fragment)
3C.2	Solyc03g045040	Hydroxycinnamoyl-CoA
3C.2	Solyc03g045050	Nuclear matrix constituent protein 1
3C.2	Solyc03g045060	Zinc finger CCHC domain-containing protein 8
3C.2	Solyc03g045070	Ammonium transporter
3C.2	Solyc03g045080	Receptor-like protein kinase 2
3C.2	Solyc03g045090	cDNA clone 001-043-A08 full insert sequence

4 General Conclusions

The results of this work indicate that different genes in the Solanaceae clade control the development of type-IV trichomes. We do not know the identity of the genes yet, but our data points out that at least six loci (five from *S. galapagense* and one from *S. pennellii*) are involved in this glandular trichome type development. The near-isogenic lines produced during this work will facilitate the mapping and subsequent cloning of the genes involved in this pathway, a fact that will shed light on the still-incipient knowledge we have about the development of multicellular trichomes in plants.

Regarding the association of type-IV trichomes with herbivory, we discovered a partial decoupling between the presence of these trichomes and the production and exudation of acylsugars. We hypothesized that in order to obtain a lineage resistant to herbivorous insects based on the presence of this trichome type, it is necessary to pyramid specific loci related to the acylsugar biosynthesis pathway, as well as potential transporters involved with the exudation of the compound to the leaf surface.

The prospection of genes related to trichome development, and the biosynthesis and the dripping of acylsugars, as well as obtaining a resistant lineage using lines containing type-IV trichomes throughout the plant's development, will make it possible to get a breeding toolkit for creating an effective insect-resistant tomato and, through their highly conserved synteny, also other Solanaceae crops (eggplant, peppers, tobacco, petunia).