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Research Article

Woolly mutation with the *Get02* locus overcomes the polygenic nature of trichome-based pest resistance in tomato

Eloisa Vendemiatti,[1] Inty Omar Hernández-De Lira,[1] Roxane Snijders,[2] Tanmayee Torne-Srivastava,[3] Rodrigo Therezan,[2] Gabriela Simioni Prants,[4] Carlos Lopez-Ortiz,[5] Umesh K. Reddy,[5] Petra Bleeker,[2] Craig A. Schenck,[3] Lázaro Eustáquio Pereira Peres,[4,*] Vagner Augusto Benedito[1,*]

1 Division of Plant and Soil Sciences, Davis College of Agriculture, Natural Resources, and Design, West Virginia University, Morgantown, WV 26506-6108, USA
2 Swammerdam Institute for Life Sciences, Green Life Sciences Research Cluster, The University of Amsterdam, Amsterdam 1090 GE, The Netherlands
3 Department of Biochemistry, Interdisciplinary Plant Group, University of Missouri, Columbia, MO 65211, USA
4 Department of Biological Sciences, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Piracicaba, SP 13418-900, Brazil
5 Department of Biology, West Virginia State University, Institute, WV 25112-1000, USA

*Authors for correspondence: lazaro.peres@usp.br (L.E.P.P.), vagner.benedito@mail.wvu.edu (V.A.B.)
The author responsible for the distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is: Vagner Augusto Benedito (vagner.benedito@mail.wvu.edu).

Abstract

Type-IV glandular trichomes, which only occur in the juvenile developmental phase of the cultivated tomato (*Solanum lycopersicum*), produce acylsugars that broadly protect against arthropod herbivory. Previously, we introgressed the capacity to retain type-IV trichomes in the adult phase from the wild tomato, *Solanum galapagense*, into the cultivated species cv. Micro-Tom (MT). The resulting MT-*Galapagos enhanced trichome* (MT-*Get*) introgression line contained 5 loci associated with enhancing the density of type-IV trichomes in adult plants. We genetically dissected MT-*Get* and obtained a subline containing only the locus on Chromosome 2 (MT-*Get02*). This genotype displayed about half the density of type-IV trichomes compared to the wild progenitor. However, when we stacked the gain-of-function allele of *WOOLLY*, which encodes a homeodomain leucine zipper IV transcription factor, *Get02*/*Wo* exhibited double the number of type-IV trichomes compared to *S. galapagense*. This discovery corroborates previous reports positioning *WOOLLY* as a master regulator of trichome development. Acylsugar levels in *Get02*/*Wo* were comparable to the wild progenitor, although the composition of acylsugar types differed, especially regarding fewer types with medium-length acyl chains. Agronomical parameters of *Get02*/*Wo*, including yield, were comparable to MT. Pest resistance assays showed enhanced protection against silverleaf whitefly (*Bemisia tabaci*), tobacco hornworm (*Manduca sexta*), and the fungus *Septoria lycopersici*. However, resistance levels did not reach those of the wild progenitor, suggesting the specificity of acylsugar types in the pest resistance mechanism. Our findings in trichome-mediated resistance advance the development of robust, naturally resistant tomato varieties, harnessing the potential of natural genetic variation. Moreover, by manipulating only 2 loci, we achieved exceptional results for a highly complex, polygenic trait, such as herbivory resistance in tomato.

Introduction

Despite their minute size, plant trichomes are the first site of contact with many environmental factors and provide substantial ecological advantages. These hair-like structures help plants tolerate biotic and abiotic stresses by protecting against herbivorous insects and microorganisms, excessive ultraviolet light, heat, drought, and even heavy metals

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(Hülskamp 2004; Wagner 2004; Yang and Ye 2013; Koul et al. 2021; Zhang et al. 2021). In particular, glandular trichomes are a critical defense mechanism against arthropod herbivores, which warrants the study of their development and biochemistry (Bar and Shtein 2019).

Insect–plant interactions have been extensively studied, and the tomato (*Solanum lycopersicum*) is among the best-documented model systems (Price et al. 1980; Kennedy 2003; Wagner 2004; Prasifka 2015; Kaur et al. 2023). Trichome-based defenses are critical in several wild tomato species (Kennedy 2003; Simmons and Gurr 2005; Maluf et al. 2010; Firdaus et al. 2012; Salinas et al. 2013; Rakha et al. 2017; Vosman et al. 2019; Kortbeek et al. 2021).

Luckwill (1943) classified 7 different types of trichomes (I to VII) in the *Solanum* genus, comprising 4 glandular (I, IV, VI, and VII) and 3 non-glandular types (II, III, and V) (Fig. 1). Our work focuses on type-IV glandular trichomes, which are elongated multicellular structures consisting of a single flat basal cell, a short uniseriate 2- or 3-celled stalk (0.2 to 0.4 mm), and a spherical gland at the tip (Glas et al. 2012). They differ from the type-V non-glandular trichome only by the presence of the apical gland, and many studies reported a negative association between the density of these 2 trichome types (Fernández-Muñoz et al. 2003; Simmons and Gurr 2005; Vendemiatti et al. 2017; Vendemiatti et al. 2022).

Type-IV trichomes produce acylsugars, a structurally diverse class of compounds resulting from the acylation of a sugar core (Mutschler et al. 1996; Schilmiller et al. 2008; Leckie et al. 2012; Vosman et al. 2019; Feng et al. 2021; Fiesel et al. 2022). Acylsugars are especially effective against arthropod herbivory, including Lepidoptera (*Helicoverpa armigera*, *Spodoptera exigua*, and *Phthorimaea operculella*), Acarina (*Tetranychus urticae*), and Hemiptera (*Myzus persicae*) (Goffreda et al. 1990; Hawthorne et al. 1992; Liedl et al. 1995; Simmons and Gurr 2005; Maluf et al. 2010). Acylsugars are also consistently associated with broad-spectrum resistance against fungal pathogens (Luu et al. 2017). Hence, a trichome-focused approach to breeding for pest-resistant tomato varieties has been pursued for several years, but the optimal genetic combination has yet to be achieved.

Type-I trichomes are another source of acylsugars. They differ from type-IV structures by their longer stalk length (2 to 3 mm) and globular multicellular base (Luckwill 1943; Glas et al. 2012; Schilmiller et al. 2012). Type-I trichomes are sparsely distributed across cultivated and wild tomato species belonging to the *Lycopersicon* and *Lycopersicoides* sections of the *Solanum* genus (Glas et al. 2012; Knapp and Peralta 2016), rendering them less effective sources of this specialized metabolite. In contrast, type-IV trichomes are persistently abundant during the entire lifecycle of some wild tomato species, including *Solanum habrochaites*, *Solanum galapagense*, and *Solanum pennellii*. While type-IV trichomes were previously believed to be absent in cultivated tomatoes (Luckwill 1943; Simmons and Gurr 2005), we demonstrated their presence on cotyledons and young leaves, linking this trait to juvenility (Vendemiatti et al. 2017). In that same study, we also noted that the mutant *Woolly* exhibits a high density of type-IV trichomes on its juvenile leaves. *WOOLLY*, identified as a homeodomain leucine zipper IV transcription factor, was initially characterized as a regulator of type-I trichome formation (Yang, Li, Zhang, Wang, and Ye 2011). However, recent findings about the function of this gene recognized its role in controlling the development of various trichome types in a dosage-dependent manner (Wu et al. 2023). This insight into *WOOLLY*'s function enhances our comprehension of trichome development and opens up promising avenues for its application in molecular breeding.

Aiming to introduce the ability of *S. galapagense* to retain type-IV trichomes in the adult life stage into a cultivated tomato, the tomato introgression line Micro-Tom (MT)-*Galapagos enhanced trichomes* (MT-*Get*) was developed in the genetic background *S. lycopersicum* cv. MT (Vendemiatti et al. 2022; Carvalho et al. 2011). This MT-*Get* genotype exhibits type-IV trichomes throughout all developmental stages. A mapping-by-sequencing approach revealed that MT-*Get* contains 5 introgressed loci. Although some individual loci were sufficient for the appearance of type-IV trichomes, 5 loci from *S. galapagense* were associated with increased type-IV trichome density. However, MT-*Get* plants did not exhibit the same level of resistance to whiteflies (*Bemisia tabaci*) as observed in *S. galapagense* (Firdaus et al. 2012, 2013; Vosman et al. 2019; Vendemiatti et al. 2022). Since both the density of type-IV trichomes and the levels of acylsugars in MT-*Get* were inferior to those observed in *S. galapagense*, it was concluded that there are additional genetic factors linked to insect resistance in *S. galapagense*, which is likely to be a complex, polygenic trait.

Considering the quantitative importance of acylsugars and type-IV trichomes in pest resistance, we explored gene interactions to enhance the density of type-IV trichomes in *S. lycopersicum*. To this end, we dissected the MT-*Get* line into sublines, each carrying a unique wild species fragment. Among these sublines, the genotype harboring Chromosome 2 (MT-*Get02*) was shown to be the major one controlling type-IV trichome development (Vendemiatti et al. 2022). Subsequently, we stacked a mild, nonembryo-lethal *Woolly* allele in homozygosity (Wo^m) (Yang, Li, Zhang, Luo, et al. 2011) using the introgression line MT-Wo^m in the same genetic background. Surprisingly, this double variant *Get02*/*Wo* displayed nearly twice the density of type-IV trichomes on the leaves of adult plants compared to *S. galapagense* (the description of these materials is summarized in Supplementary Table S1). We further explored acylsugar content in these genotypes and assessed resistance against relevant pests and pathogens for tomato cultivation: *B. tabaci* (whiteflies), *Manduca sexta* (caterpillars), and the fungus *Septoria lycopersici*—the etiological agent of *Septoria* leaf spot (SLS) disease. Our observations showed a compelling synergistic interplay between *Woolly* and the *Get02* locus, critically influencing type-IV trichome development and modulating acylsugar biosynthesis in tomato.

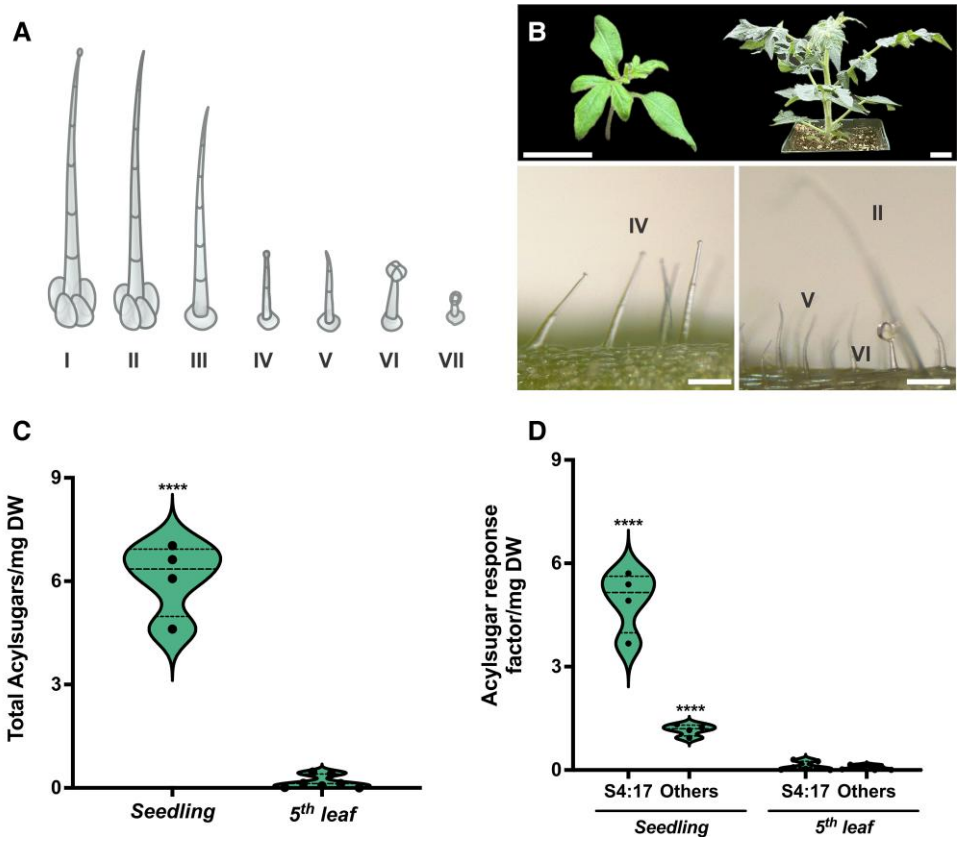

Figure 1. Trichome types and acylsugar profile in the juvenile stage of tomato. **A)** Trichome classification in the *Solanum* genus, according to Luckwill (1943). **B)** Trichome diversity on seedlings and the fifth leaf (adult stage) of *S. lycopersicum* cv. MT. In this upper panel, images were digitally extracted for comparison (scale bars = 2 cm). **C)** Comparison of total acylsugar concentrations between seedling and adult leaf stages and **D)** contribution of the most prominent acylsugar (S4:17) profile comparison in MT stages. Data are means ± SEM ($n \geq 4$ biological replicates). Acylsugar nomenclature (S4:17) indicates sucrose core, 4 total acylations totaling 17 carbons in those acyl chains of lengths 2, 5, 5, and 5. For **C)** and **D)**, asterisks indicate a significant difference between the groups according to the Student's *t*-test at $P < 0.0001$ (****). Scale bar = 100 μm. For the violin plots, the horizontal lines represent the median and quartiles.

Furthermore, our results indicate that a combination of mutations and natural genetic variation may offer a viable solution for overcoming the polygenic nature of pest resistance.

Results

Acylsugar biosynthesis in type-IV trichomes present on juvenile leaves of the cultivated tomato

Since type-IV trichomes are present on cotyledons of the cultivated tomato, but not on adult leaves (Vendemiatti et al. 2017) (Fig. 1), we first assessed whether these glands actively synthesize acylsugars by profiling these compounds in *S. lycopersicum* cv. MT seedlings. Our data show that MT seedlings produce these metabolites but that the adult leaves contain only negligible amounts (Fig. 1). The latter is likely synthesized in type-I trichomes, which are sparsely present on the adult leaves. The most abundant acylsugars to accumulate on MT cotyledons was a tetra-acylated sucrose (S4:17) containing 3 short chains and an acetyl group (2, 5, 5, and 5; see Fig. 1 legend) (Fig. 1). Interestingly, juvenile leaves exhibit a different acylsugar profile compared to leaves developed at the adult plant stage. While acylsugars with a 12-carbon-long acyl chain were present in cotyledons, this type was absent in later leaves (Supplementary Fig. S1).

Type-IV trichome density in the *Get02/Wo* double variant tomato line

Previously, we identified 5 chromosomal segments in the introgression line MT-*Get* inherited from the wild progenitor *S. galapagense* (LA1401), associated with the persistent development of type-IV trichomes throughout plant life. One of the sublines resulting from this genetic dissection of MT-*Get* contained only one of these segments and was named MT-*Get02* (Fig. 2), as this is the locus on the long arm of Chromosome 2 of *S. galapagense*. This segment spans the coordinates 51,924,000 to 52,413,200, corresponding to the *S. lycopersicum* genome assembly (version SL4.0). This locus contains 203 annotated genes and was previously associated with a quantitative trait locus (QTL) for whitefly resistance (Firdaus et al. 2012; Firdaus et al. 2013).

Wo^m is a mild gain-of-function mutation in the gene *WOOLLY* (*Solyc02g080260*) that increases the density of

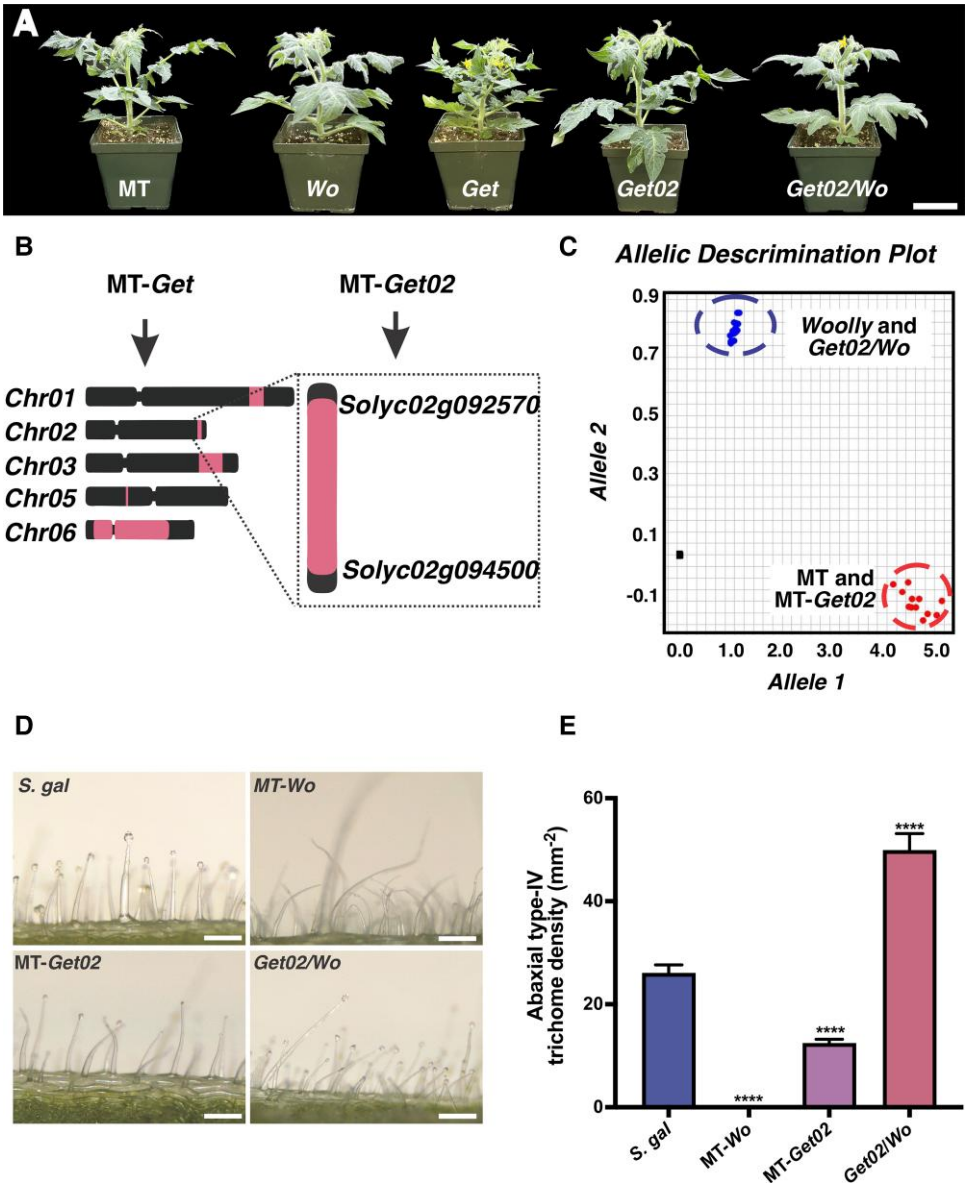

Figure 2. Phenotypic characterization of tomato introgression lines in the cv. MT background. **A)** Comparison of *S. lycopersicum* cv. MT, MT-*Wo*, MT-*Get*, MT-*Get02*, and the double variant *Get02/Wo*. In this panel, images were digitally extracted for comparison. Scale bar = 5 cm. **B)** Approximate locations of the introgressions in MT-*Get* and MT-*Get02* chromosomes. The segments highlighted in pink are related to the *S. galapagense* fragments in the MT genome. **C)** PACE analysis shows that MT-*Get02* Plants #4 and #35 have the Wo^m gain-of-function mutation in homozygosis (blue dots), whereas MT and the other MT-*Get02* plants tested have the wild-type *Wo* allele in homozygosis (red dots). The black dot represents the negative control. **D)** Representative leaf abaxial surface images show the presence/absence of type-IV trichomes. Scale bar = 100 μm. **E)** Quantification of type-IV trichomes in the tomato lines used in this work. Data are the means of 50 biological replicates ± SEM. Asterisks indicate significant differences compared to *S. galapagense* (*S. gal*), according to Dunnett's test at $P < 0.0001$ (****).

digitate trichomes (mainly types III and IV; Vendemiatti et al. 2017) and results in a hairy phenotype (Fig. 2; Yang, Li, Zhang, Luo, et al. 2011; Wu et al. 2023). This gene is located 9.16-Mb upstream of the MT-*Get02* locus. To investigate the combined effect of the *Get02* locus and *Wo* mutation on the type-IV trichome pathway, we sought to produce a double variant line, combining MT-*Get02* with a near-isogenic line in the cv. MT background that contains the mild Wo^m mutation originating from the accession LA0715 (*S. lycopersicum*). While strong *Wo* alleles are lethal to embryos in a homozygous state (Yang, Li, Zhang, Luo, et al. 2011), the Wo^m allele is also a spontaneous, viable mutation in the cv. Rutgers (LA0258).

To obtain the double Get02/Wo line, an F_2 segregating population resulting from the crossing between MT-*Get02* and MT-Wo^m was screened for type-IV trichome density (Supplementary Fig. S2). Out of 98 plants screened, 18.37% exhibited a significant increase in type-IV trichomes

compared to the parental MT-*Get02* (Supplementary Fig. S2). This observation fits with the expected Mendelian proportion of 3/16 (18.75%) for independent segregation of 2 genes, where one contains 1 allele in homozygosity (*Get02*) and the other in heterozygosity (Wo^m), even though both loci are located on the long arm of Chromosome 2.

Among the F_2 plants identified to exhibit high densities of type-IV trichomes, 1 plant (Line #4 in Supplementary Fig. S2 developed nearly twice the number of trichomes compared to *S. galapagense*; Fig. 2). Interestingly, this plant was sticky to the touch, indicative of the presence of acylsugars. Besides Plant #4, we also selected Plant #35 and confirmed both contained the 2 loci, *Get02* and Wo^m. Self-fertilization of Plants #4 and #35 produced F_3 seeds, whose seedlings were subsequently screened for both loci in homozygosity. The presence of the homozygous *Get02* alleles was confirmed using cleaved amplified polymorphic sequence (CAPS) markers and the Wo^m homozygous alleles by PCR allele competitive extension (PACE) genotyping (Fig. 2), thus establishing the *Get02/Wo* double variant lines. Next, we analyzed the acylsugar profile in homozygous plants derived from the double *Get02/Wo* lines. We tested the performance of this material in herbivory assays with whiteflies and caterpillars and fungal disease exposure.

Get02/Wo acylsugar profiling

Due to the stickiness of *Get02/Wo* leaves, suggestive of high acylsugar production, we profiled this lineage by both liquid and gas chromatography coupled with mass spectrometry (LC-MS and GC-MS) (Fig. 3; Supplementary Figs. S1 and S3). *Get02/Wo* produced similar amounts of total acylsugars as *m/z* peaks in *S. galapagense* (Fig. 3). However, the acylsugar (AS) composition of *Get02/Wo* contains mainly tetra-acylated sucrose (S4:17, *m/z* 681.3; Fig. 3). Compared to *S. galapagense*, *Get02/Wo* produces significantly lower concentrations of S3:22(5,5,12) and S4:24(2,5,5,12) structures.

Furthermore, GC-MS analysis was further performed to evaluate the composition of acyl chains in each genotype. Our results revealed that the main difference in mature leaves of the cultivated tomato (MT) compared to *S. galapagense* is the lack of acylsugars with medium acyl chains (10 to 12 carbons in length). However, acylsugars containing 12-carbon-long acyl chains are produced in all introgression lines with type-IV trichomes and, to a lesser extent, in MT seedlings (Supplementary Fig. S3).

Pest resistance assessment in *Get02/Wo*

To investigate the biological effect of the altered acylsugar profile in our material, we performed bioassays with relevant biotic interactors of tomato. We conducted 2 no-choice assays to evaluate the survival and oviposition rates of the pest insect *B. tabaci* among the genotypes (Fig. 4; Supplementary Fig. S4). As expected, significantly fewer eggs were deposited on *S. galapagense* compared to MT. On the other hand, MT-*Get*, the single-locus introgressions of MT-*Wo*, and MT-*Get02* did not result in altered oviposition rates compared to *S. galapagense*. In contrast, only a slight reduction in oviposition was observed in the double variant *Get02/Wo*, but this difference was not statistically significant (Fig. 4). A second experiment confirmed significantly fewer eggs were laid on *Get02/Wo* when compared to MT in Petri dish assays (Supplementary Fig. S4). The experiment also showed no significant difference in the adult whitefly survival rate among the genotypes in this setting. These results suggest that *Get02/Wo* can resist whitefly infestation by hampering oviposition.

We also evaluated the effect of increasing plant acylsugars on the development rate of *M. sexta* caterpillars when fed MT-*Wo* compared to *Get02/Wo* leaves. Due to experimental limitations, only 2 genotypes could be analyzed. Thus, we decide to compare the MT-*Wo*, which has a high density of non-glandular trichomes and a negligible amount of acylsugars (Fig. 3), to the *Get02/Wo*, the genotype with a high density of type-IV trichomes accumulating high amounts of acylsugars. After 7 d, the *Get02/Wo*-fed caterpillars were significantly smaller than those provided with MT-*Wo* leaves (Fig. 4). These results suggest that the amount and the acylsugars composition in *Get02/Wo* can delay the growth of caterpillars.

Acylsugars have also contributed to pathogen resistance based on lower spore germination and smaller necrotic lesions (Luu et al. 2017). Thus, we tested the resistance of the *Get02/Wo* to SLS infection, a well-established study system in our laboratory. Our evaluation showed that the wild species *S. galapagense* has a medium-high tolerance (3.0/5 score) to this fungus. The incubation period of the fungus was delayed by 1 d compared to the MT control (Fig. 4). On the *Get02/Wo* plants, the incubation time was delayed by 2 d. Plants displayed prolonged tolerance throughout the pathogen exponential growth phase compared to the wild species. However, *Get02/Wo* plants could not maintain this resistance throughout the infection. With these results, we conclude that the *Get02/Wo* lineage has a medium level of resistance (2.5/5) for SLS compared to the cv. MT (1.6/5 score).

Agronomical parameters in *Get02/Wo*

Get02/Wo was derived from MT-*Get*, and it exhibits several traits that could affect agronomical performance, such as reduced fruit weight (Vendemiatti et al. 2022). Therefore, we evaluated *Get02/Wo* performance also because there is a concern that breeding based on high trichome densities and enhanced specialized metabolism may negatively impact yield due to the deviation of energy from primary metabolism that contributes to fruit size and, partially, to taste (Tieman et al. 2017). *Get02/Wo* plants have the same height and flowering time as MT (Supplementary Fig. S5). In contrast, MT-*Get* plants are smaller and take longer to flower than MT and *Get02/Wo* (Supplementary Fig. S5; see also Fig. 2). There was no statistical difference in stem diameter among the lines (Supplementary Fig. S5).

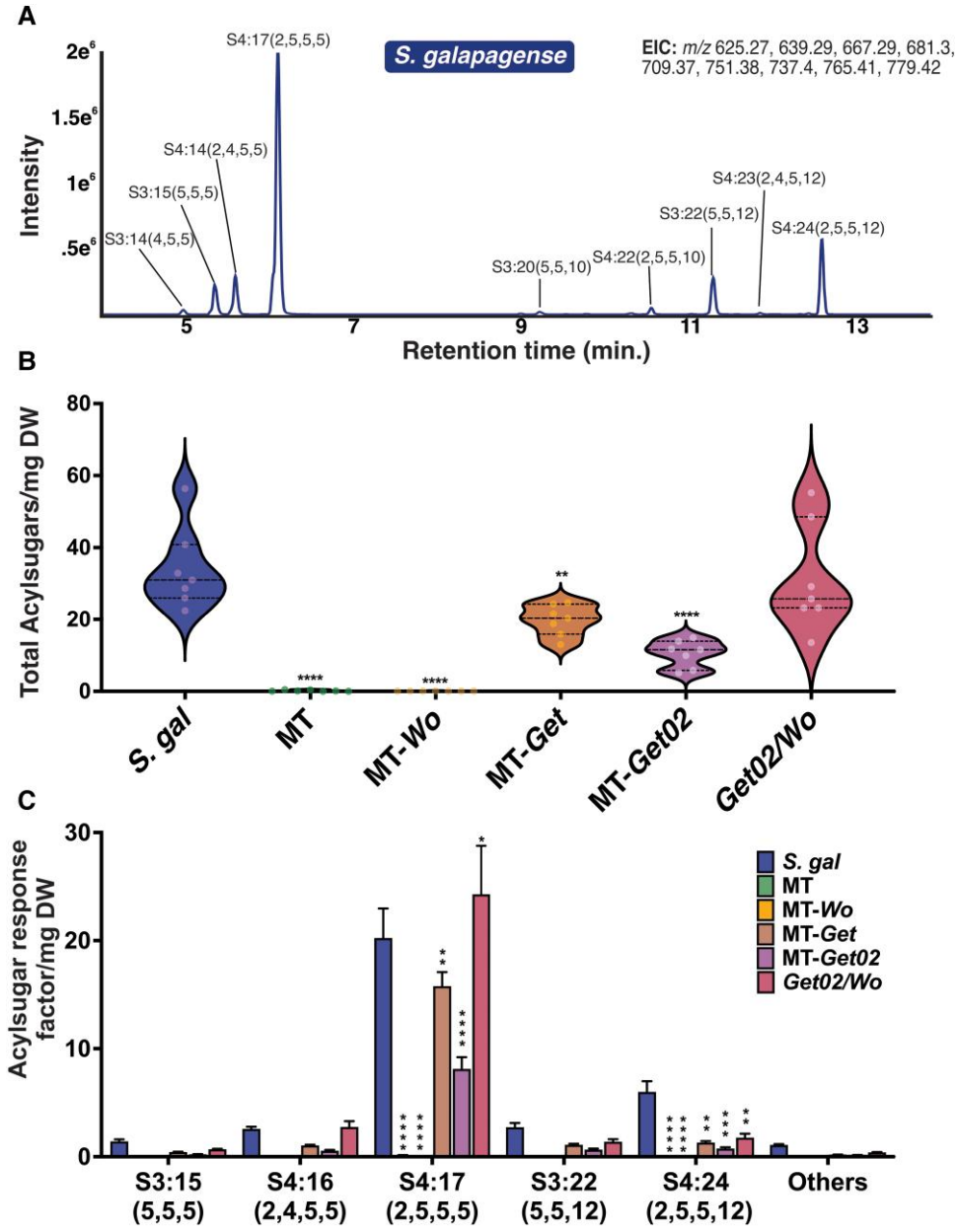

Figure 3. Acylsugar profiles in the study material. **A)** Acylsugar profiles of leaf extracts of *S. galapagense* and its derivative introgression lines in cv. MT. **B)** Total acylsugar quantification across the introgression lines. Individual acylsugars were quantified based on the peak area of the formate adduct and corrected for the internal standard (telmisartan) and the dry leaf weight. **C)** *Sum* of the main individual acylsugar types quantified **A)**, resulting in the total acylsugars for each line. Data are the means of ≥5 biological replicates ± SEM. Asterisks indicate significant differences compared with *S. galapagense*, according to Dunnett's test at $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) for the statistical analysis of **B)** and **C)**. For the violin plots, the horizontal lines represent the median and quartiles.

MT-*Get* and MT-*Get02* produced the lowest total fruit weight and also the lowest fruit soluble solids content (Brix), respectively, compared to all other genotypes analyzed (Fig. 5). Interestingly, MT-*Wo* showed the highest Brix among the genotypes evaluated. Our double variant line *Get02/Wo* did not show significant differences in total fruit weight and Brix, and this way, it is very similar to the control MT (Fig. 5).

Discussion

The *Get02* natural genetic variation enables the occurrence of type-IV trichomes in the adult phase of tomato development

In 2005, Simmons and Gurr proposed a trichome-based resistance to minimize pesticide use by introducing trichome traits from wild accessions into cultivated tomatoes. Such

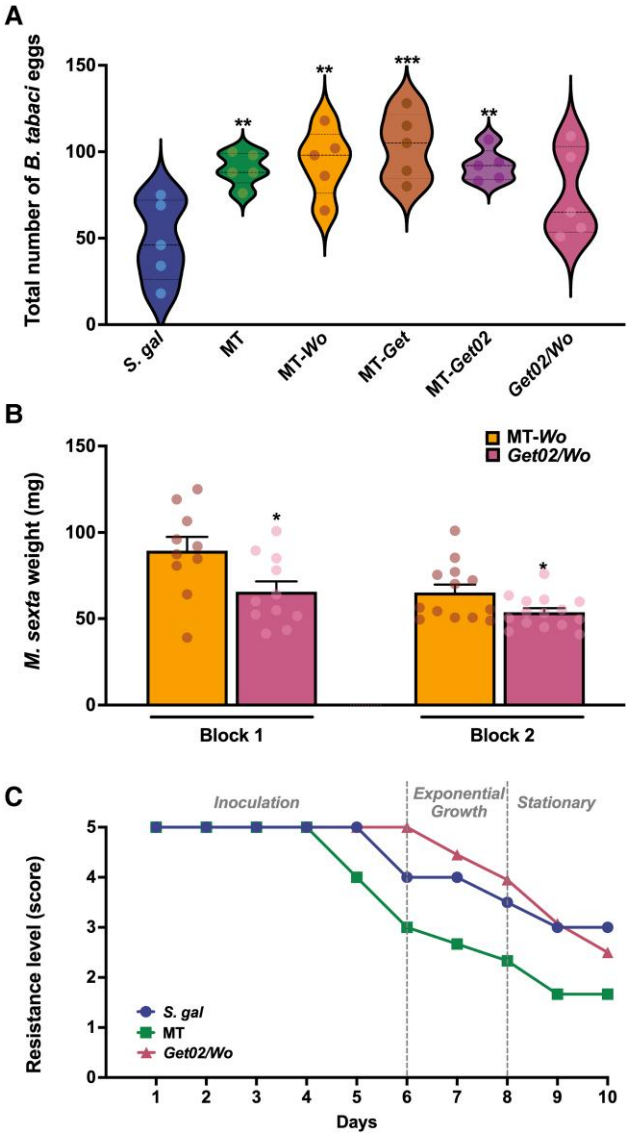

Figure 4. Insect resistance assessment. **A)** Total number of *B. tabaci* eggs, 2 d after infestation on leaves of different genotypes using clip cages. Data are means of 5 biological replicates ± SEM. $P < 0.01$ (**) and $P < 0.001$ (***) according to Dunnett's *t*-test compared to *S. galapagense* (*S. gal*). In the violin plots, the horizontal lines represent the medians and quartiles. **B)** Weight of *M. sexta* larvae feeding on MT-*Wo* and *Get02*/*Wo* for 7 d. Each block represents an experimental repetition. Data are means of at least 10 replicates ± SEM in each block. Asterisks indicate a significant difference between the groups according to the Student's *t*-test at $P < 0.05$ (*). C) Infestation score of SLS in MT, *S. galapagense*, and *Get02*/*Wo* plants during 10 d of infestation.

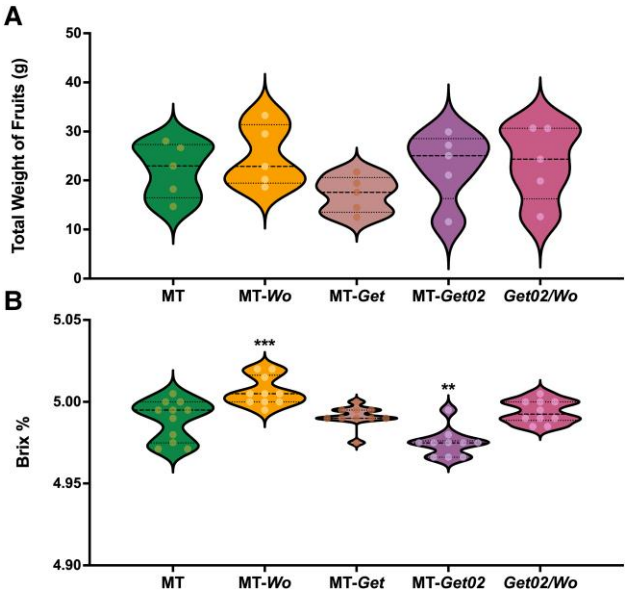

Figure 5. Impact of genetic introgressions on yield parameters. **A)** Total weight of fruits (g) produced by each genotype. Data are means of 5 biological replicates ± SEM. According to Dunnett's *t*-test and using MT as a control, the data are not significantly different at $P < 0.05$. **B)** Soluble solids content (% Brix) for each genotype. Data are the mean of 10 fruits per genotype ± SEM. $P < 0.01$ (**) and $P < 0.001$ (***) according to Dunnett's *t*-test using MT as a control. In the violin plots, the horizontal lines represent the medians and quartiles.

an approach is grounded in the understanding that glandular trichomes and their specialized metabolites contribute to pest and pathogen resistance (Weinhold and Baldwin 2011; Firdaus et al. 2013; Luu et al. 2017; Rakha et al. 2017; Feng et al. 2021). While numerous studies have delved into this field, a commercial tomato line with trichome-based resistance has yet to be achieved. The primary forms of resistance are based on metabolites produced in type-IV and type-VI trichomes. In terms of pest resistance deriving from type-IV trichomes—the most predominant type of acylsugar-producing trichomes in some wild tomato species—the lack of these structures in adult plants of cultivated tomato accessions seems to be the main obstacle in developing plants with broad, enduring resistance against arthropod herbivores.

In a previous study, we showed that cultivated tomatoes display type-IV trichomes in the juvenile phase of plant development (Vendemiatti et al. 2017). Herein, we confirm they are metabolically active and accumulate acylsugars (Fig. 1; Supplementary Fig. S3). However, the intricate genetics dictating their persistence in the adult phase of the plant could be the obstacle preventing the success in obtaining a commercial tomato line with trichome-based pest resistance (Vendemiatti et al. 2022; Mutschler et al. 2023). Understanding the key genetic factors that could stabilize type-IV trichome development in the cultivated tomato prompted us to transfer this trait from *S. galapagense* to the tomato model system MT, creating the multilocus MT-*Get* lineage (Vendemiatti et al. 2022). In the present work, we advanced the dissection of the MT-*Get* into derivative sublines, each with a single locus from the wild progenitor. We initiated this exploration with MT-*Get02*, the subline that carries the fragment from Chromosome 2 from *S. galapagense*. This decision was based on the preliminary data from Vendemiatti et al. (2022) that attributed this locus as critical for the persistent development of type-IV trichomes in MT-*Get*.

Further reinforcing our choice, insect resistance in previous studies has been correlated with a locus in the same genomic region. Therefore, we updated the coordinates on Chromosome 2 from the QTLs outlined by Salinas et al. (2013) and Smeda et al. (2016) using the latest version of the *S. lycopersicum* cv. Heinz genome assembly (SL4.0; Supplementary Table S2). We also compared these ranges with the introgression obtained by Vendemiatti et al. (2022). We are currently using this approach to identify the culprit gene on Chromosome 2 involved with trichome development and pest resistance.

Since type-IV trichomes are present in the mature phase of different tomato wild species, it is plausible that such species employ a unique set of genes, potentially a genetic network, influencing the presence of type-IV trichomes at a specific stage of the plant lifecycle (Maliepaard et al. 1995; Momotaz et al. 2010; Leckie et al. 2012; Firdaus et al. 2013; Smeda et al. 2016; Mata-Nicolás et al. 2021; Vendemiatti et al. 2022). Interestingly, the genomic region encompassed by *Get02* has been associated with type-IV trichome development in 3 wild species of tomato: *S. habrochaites*, *S. pennellii*, and some accessions of *Solanum pimpinellifolium* (Maliepaard et al. 1995; Momotaz et al. 2010; Salinas et al. 2013; Smeda et al. 2016). Altogether, these pieces of information suggest the existence of a locus on Chromosome 2 driving the type-IV trichome developmental pathway in the *Solanum* genus.

The *Woolly* mutation plays a pivotal role in increasing the density of type-IV trichomes in the adult phase when combined with the Get02 locus

The MT-*Get02* subline mirrored the MT overall appearance (Fig. 2), except for the persistence of type-IV trichomes in the adult phase. Notably, unlike MT-*Get*, which carried 5 introgression sections, MT-*Get02* has no yield penalties (Fig. 5). To enhance the density of type-IV trichomes in MT-*Get02*, we examined its interaction with *Woolly* (*Wo*), a spontaneous tomato mutation initially linked with type-I trichome formation (Yang, Li, Zhang, Luo, et al. 2011) and further characterized as having a high density of type-IV trichomes in the juvenile phase (Vendemiatti et al. 2017). The increased density of type-IV trichome in the adult phase of *Get02/Wo* supports the notion that *Wo* can enhance type-IV trichome formation (Vendemiatti et al. 2017), and it suggests that *Get* loci play a role in controlling juvenility (Vendemiatti et al. 2022). Interestingly, recent research has shown that the *Woolly* mutant can increase the density of all digitate trichome types (Wu et al. 2023), including types I, II, III, IV, and V. While types I, II, and III are sparsely present in all phases of tomato development, types IV and V predominate in the juvenile and adult phases, respectively, with their densities showing a negative correlation throughout tomato development (Vendemiatti et al. 2017). Since type-IV and type-V trichomes differ morphologically solely by the presence of a single-cell gland at the tip of the former, they likely share the same initial developmental pathway. Therefore, determining the genetic identity of *Get02* may contribute to our understanding of the genetic network downstream of *WOOLLY* in specifying different digitate trichome types (Wu et al. 2023). According to the model proposed by Wu et al. (2023), elevated *WOOLLY* levels result in digitate trichome differentiation. It is possible to observe a trend toward an increase in type-V trichomes in *Get02/Wo* compared to MT-*Get02* (Supplementary Fig. S6). In contrast, when *WOOLLY* is expressed at low levels, the formation of peltate trichomes (types VI and VII) is increased. This phenomenon is also evident in *Get02/Wo*, which exhibits a reduced density of peltate (mainly type-VI) trichomes (Supplementary Fig. S6). Type-VI trichomes also produce natural insecticides, such as plastid-derived sesquiterpenes and methyl ketones, which are absent in cultivated tomatoes but present in the wild species *S. habrochaites* and *S. habrochaites* f. *glabratum* (Sallaud et al. 2009; Therezan et al. 2021). These findings suggest that it may be challenging to engineer type-IV and type-VI trichome-based pest resistance in the same tomato genotype. We also observed the presence of branched trichomes in MT-*Wo*^{m} and Get02/Wo (Supplementary Fig. S6), which is consistent with our previous findings (Vendemiatti et al. 2017). This resembles the trichomes present in *Arabidopsis thaliana*, where branching is associated with the extent of DNA endoreduplication (Schwab et al. 2000).

The *Get02/Wo* genotype produces elevated levels of acylsugars, with a molecular profile distinct from that of the wild parental *S. galapagense* but capable of imparting pest resistance

Get02/Wo plants are sticky to the touch, indicating high levels of acylsugars, which are probably able to exudate from type-IV trichomes (Supplementary Fig. S7). The higher density of type-IV trichomes in *Get02/Wo*, which surpasses the trichome density of the wild progenitor, is likely the reason behind its increased production of acylsugars (Fig. 3). However, the composition of individual acylsugar structural variants differed considerably. Notably, *Get02/Wo* synthesized significantly more S4:17 than *S. galapagense* (Fig. 3). This acylsugar type is predominant in *S. lycopersicum* cv. M82 (Kim et al. 2012; Schilmiller et al. 2015; Fan et al. 2016), and, interestingly, it is also the most abundant type in *Get02/Wo*. An important question that arises from this is the effectiveness of these acylsugar types for pest resistance. Testing this hypothesis has proven challenging because cultivated tomatoes, such as M82, only produce a minimal amount of acylsugars, likely attributable to the absence of type-IV trichomes and the sparse density of type-I trichomes.

We conducted pest evaluation performance to assess the impact of the *Get02/Wo* trichome and acylsugar profile on 2 insect orders with distinct feeding mechanisms: whiteflies (*B. tabaci*) and caterpillars (*M. sexta*). This choice was based on the literature that shows acylsugars from the Solanaceae

family acting negatively on these orders (Weinhold and Baldwin 2011; Firdaus et al. 2013; Vosman et al. 2018; Wang et al. 2022). Our results showed that *Get02/Wo* plants hampered the infestation of whiteflies (less oviposition than the control MT) and caterpillars (individuals were smaller in the presence of acylsugars than when fed control leaves) (Fig. 4). Regarding the disease resistance evaluation, the *Get02/Wo* plants appear to show improved tolerance against SLS. We noticed an incubation delay of 2 d and an elevated resistance level during the 2 crucial days of exponential fungal growth. This can be a good starting point in commercial cultivation since it would provide farmers more time to take measures to contain this disease by using either compounds approved for organic cropping (e.g., copper) or synthetic fungicides (e.g., azoxystrobin, difenoconazole, and dithiocarbamate). Furthermore, these results can also be used to improve integrated pest control, population control of natural enemies, and entomopathogenic fungi.

The pest evaluation panel showed that the trichome/acylsugar profile combination in *Get02/Wo* plants offers a lower resistance level against insects and fungi than in the parental *S. galapagense*. Since these plants produce the same total amount of acylsugars while sporting double the density of type-IV trichome compared to the parental wild species (Fig. 2), the bottleneck is likely to be the specific composition of the acylsugar mélange that the plants are accumulating. Acyl chains with a length range of C6 to C9 are commonly associated with caterpillar's acylsugar-based resistance (Van Dam and Hare 1998; Luu et al. 2017; Feng et al. 2021; Wang et al. 2022). Recently, the S3:15 and S3:21 types were predicted to provide whitefly resistance (Kortbeek et al. 2021). Additionally, Puterka et al. (2003) established that octanoate and decanoate fatty acid chains had the highest broad-spectrum insecticidal activity. Based on our results, *S. galapagense* produces acylsugars using acyl chains with 2, 4, 5, 10, and 12 carbons, so it does not accumulate octanoate acyl chains (Fig. 3). *Get02/Wo* has the same production of S3:15 as the wild species. Therefore, we hypothesize that introgressing the genetic components responsible for longer acyl chains (10 or 12 carbons) might be the key to further enhancing the insect resistance of *Get02/Wo* plants.

Concluding remarks and perspectives

In conclusion, we have engineered a tomato genotype combining a specific mutation affecting trichome differentiation fate and a natural genetic variation likely affecting heterochronic alterations in the juvenile/adult phase. *Get02/Wo* exhibits enhanced resistance to insects and a fungal disease, overcoming the polygenic nature generally associated with pest resistance. Since *Get02/Wo* had yield and fruit Brix levels comparable to those observed in MT (see Fig. 5), this allelic combination is a compelling candidate for further evaluation in commercial tomato cultivars and hybrids within prebreeding programs. Next, we aim to refine the acylsugar composition further to bolster its protective properties and achieve resistance levels on par with *S. galapagense* and other wild accessions. We postulate that increasing the accumulation of long acyl chains containing acylsugars could accomplish this goal. Consequently, our future endeavors will focus on genes related to branched-chain amino acid metabolism, which provide acyl chain precursors and trichome-specific fatty acid synthases that catalyze acyl chain elongation to yield *n*-decanoate (10C) and *n*-dodecanoate (12C) acyl chains (Slocombe et al. 2008; Mandal et al. 2020; Ji et al. 2023). Moreover, the forthcoming discovery of *Get02* genetic identity will significantly enhance our comprehension of the developmental pathways regulating the differentiation of each type of digitate trichomes.

Materials and methods

Plant material

Seeds of the tomato (*S. lycopersicum*) varieties and the natural variations in the cv. MT background were from the University of São Paulo (Piracicaba, Brazil) repository (www.esalq.usp.br/tomato). The introgression process of natural variation into the MT cultivar was previously described (Pino et al. 2010; Lombardi-Crestana et al. 2012; Vendemiatti et al. 2022). Plants were cultivated in a growth chamber with a temperature of 28 °C, 13-h photoperiod, 60% ambient relative humidity, and 150 to 250 µmol photons m^{-1} s^{-2} PAR irradiance.

Double variant line *Get02/Wo*

First, we segregated each of the introgressed *S. galapagense*'s chromosomal segments (Vendemiatti et al. 2022) into distinct sublines of MT-*Get* using CAPS markers to enable evaluating the impact of each locus on the trait "density of type-IV trichomes on the fifth leaf." For that, a segregating population (BC_7F_2) of MT-*Get* containing 315 plants was screened for the presence of type-IV trichomes on leaves developed during the mature stage of the plant. Selected selfed plants were screened repeatedly until isolines with a single chromosomal segment from the wild progenitor were obtained. With that, each derivative subline from MT-*Get* contains only 1 homozygous fragment from the wild progenitor, *S. galapagense* (LA1401). Supplementary Table S3 lists the primers used in this work. One of the lineages generated by this process is MT-*Get02*, described here. To produce the double variant line, *Get02/Wo*, MT-*Get02* emasculated flowers received MT-Wo^m pollen, and the F_1 plants were self-fertilized to produce the F_2 generation, which was screened for type-IV trichome quantification (Supplementary Fig. S1).

Trichome morphometric analysis

Trichome quantification was carried out using the methodology described by Vendemiatti et al. (2017, 2022). In summary, clear nail polish fixed leaflet samples in glass slides. A Styrofoam block supported the slide so we could photograph the trichomes in the lateral view, allowing for precise classification and quantification of each type. A Leica

Stereoscope EZ4W (Wetzlar, Germany) set to 55× magnification was used to photograph the trichomes. Trichome densities were calculated as trichome counts per unit leaf area.

PACE-based SNP genotyping of Wo^m mutation

Since developing a CAPS marker for the Wo^m allele was difficult, we decided to employ PACE. A SNP of interest was selected to develop the PCR allelic competitive extension (PACE) marker, and allele-specific primers were designed with a common reverse primer (Integrated DNA Technologies). The PACE PCR components included 5 µL of PACE Genotyping Master Mix (2×) (3CR Bioscience, Essex, UK) containing FAM, HEX, and ROX fluorophores, 0.11 µL of primer assay mix (72×), 2 µL of template DNA, and 3 µL of molecular biology grade water. The PCR conditions were adopted in 3 stages as follows: (i) 1 cycle at 94°C for 15 min, (ii) 10 cycles each including template denaturation (94 °C, 20 s) and annealing/extension with a drop of 0.8 °C /cycle (65 to 57 °C , 60 s), (iii) 30 cycles each comprising denaturation at 94 °C for 20 s and annealing/extension at 57°C, for 60 s (3CR Bioscience, UK). Further, 3 to 9 cycles of final denaturation and annealing/extension were performed to increase the amplification and generate dense, well-separated clusters. Genotypes were clustered using StepOne plus auto caller (Applied Biosystems). The primer sequences used in this analysis are described in Supplementary Table S3.

Profiling of acylsugar types

Acylsugars were separated and detected using LC-MS in negative ion mode following extraction from leaf dips. Individual types are labeled according to their annotated structure. For example, S3:14(4,5,5) indicates an acylsugar with a sucrose core and 3 acyl chains totaling 14 carbons of lengths 4, 5, and 5 carbons in each acyl chain. Acylsugars were extracted from a single fully expanded leaf from 5-week-old plants, representing the adult phase in MT (Vendemiatti et al. 2017). A leaf was placed into 1 mL of extraction solvent (3:3:2 acetonitrile: isopropanol:water + 0.1% [*v*/*v*] formic acid + 1 µM telmisartan internal standard) and gently mixed for 3 min. AS were analyzed in 2 ways: quantification and annotation of AS were performed using liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), and acyl chains were identified and quantified using GC-MS. Acylsugar extracts (4 µL) were run on an Ascentis Express C18 UHPLC column (100 mm × 2.1 mm, 2.7 µm) coupled to a Bruker Impact LC-QTOF-MS. The following chromatography conditions were used: column temperature 30 °C, flow rate 0.3 mL/min, Solvent A 10 mM ammonium formate pH 2.8, Solvent B 100% acetonitrile, gradient: 0-min 95% Solvent A, 3-min 45% Solvent A, 13-min 0% Solvent A, 16-min 0% Solvent A, 16.01-min 95% Solvent A, and 20-min 95% Solvent A. Acylsugars were annotated based on fragmentation patterns in electrospray ionization modes. The following conditions were used: dry gas 10 L/min, dry temperature 200 °C, nebulizer gas 40 psi, and capillary voltage 2.8 kV. The mass range collected was *m*/*z* 50 to 1,500, and lock mass correction using sodium formate adducts $[M–H]^-$ as the reference was applied during data collection. AS were manually annotated based on accurate mass and fragmentation patterns and quantified based on peak areas for extracted ion chromatograms of the $[M–HCOO]^-$ adduct corrected for internal standard peak area and tissue dry weight (Schenck et al. 2022).

As previously described, the acylsugar acyl chain analysis was performed on pooled acylsugar extracts using an Agilent 6890 GC-MS (Fan et al. 2020). Acyl chains were determined through library matches of the mass spectra of the corresponding ethyl esters using the NIST Library version 14. The relative abundances were calculated by integrating the related peak area based on the total ion chromatogram over the total acyl chain peak area.

Pest resistance evaluation

B. tabaci assay

An assay using clip cages was performed using *S. galapagense*, MT, MT-*Wo*, MT-*Get*, MT-*Get02*, and *Get02*/*Wo*. Fifteen adult, healthy, female, and young whiteflies were placed in a clip cage on each plant. The plants were 5 wk old, and the clip cages were placed on the third or fourth leaf from the top. After 24 h, the whiteflies were removed from the clip cages using CO_2 sedation, and the eggs were counted.

We also performed a no-choice bioassay using adult female *B. tabaci* whiteflies placed in leaf discs (ø 12 mm) of *S. lycopersicum* cv. MT, MT-*Get*, MT-*Woolly*, and *Get02*/*Wo* (Supplementary Fig. S5). The leaf discs were placed adaxial side up, with the main vein inserted into 0.6% agar on the edge of vented Petri dishes. Ten female whiteflies were put on each Petri dish, and they were sealed and placed in an artificial climate box with controlled conditions (27 °C, 16-h photoperiod, 70% relative humidity) for 5 d. After 5 d, the number of whiteflies alive was quantified, and the number of eggs deposited on both foliar surfaces was counted.

M. sexta assay

Four-week-old MT-*Wo* and *Get02*/*Wo* plants were placed in a growth chamber (28 °C, 11-h photoperiod). Two newly emerged, healthy *M. sexta* caterpillars were placed on each plant. Two plants of the same genotype were placed together in an insect cage. The caterpillars were monitored daily to check for survival and to replace the plant they were feeding on with a new one if needed. Seven days after the start of the experiment, each caterpillar was weighed.

S. lycopersici assay

In this assay, we employed a single spore isolate of *S. lycopersici*, sourced from naturally infected tomato plants in Long Island (New York). The fungal isolation entailed the pathogen extraction from infected tomato leaf tissues and then cultivation on V8 agar within 10-cm Petri dishes. After this, the plates were incubated at a controlled temperature of 23 °C. After an incubation period of 10 to 12 d, conidia were meticulously harvested from the Petri dishes by

inundating them with sterile distilled water. The inoculum concentration was adjusted to 5 × 105 conidia mL^{-1} using a hemocytometer. To ensure uniform spore deposition on tomato leaves during inoculation, a minute volume (approximately 10 μL) of Triton X-100 (2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol) was introduced into the inoculum suspension. This suspension was subsequently transferred to Nalgene aerosol spray bottles (Thermo Fisher Scientific) for application. Artificial inoculation was carried out on 9-wk-old tomato plants. These plants were positioned within a controlled humidity chamber, facilitating the maintenance of a relative humidity exceeding 95%, accomplished through a humidification system. Evaluating plant responses to the fungus involved a comprehensive examination of each inoculated plant for SLS symptoms. This evaluation encompassed quantifying lesions (dark spots) on the fifth oldest leaflets and determining the percentage of infected leaflets per plant. The resulting data were subsequently transformed into a *Septoria* tolerance score, graded on a scale ranging from 1 to 5, with the highest score of 5 signifying the highest resistance level.

Plant phenotyping

The diameter and length of the main stem and the number of leaves until the first inflorescence were measured on 60-d-old plants that grew in 350-mL pots. Five plants were evaluated for each genotype. At this time, all the fruits were harvested for weight measurements. From these fruits, 10 units were used for Brix determination using a refractometer with automatic temperature compensation.

Statistical analyses

The statistical comparisons were performed using Student's *t*-test or Dunnett's test with GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla, CA, USA).

Accession numbers

Sequence data from this article can be found in the Sol Genomics Network (http://genomics.net). The accession numbers are listed in Supplementary Table S3.

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Author contributions

E.V., L.E.P.P., and V.A.B. conceived and designed the experiment, and wrote the manuscript; E.V. produced the tomato lines and performed the experiments; I.O.H.-D.L. conducted the disease assay; R.S., R.T., and P.B. conducted the herbivory assays; C.L.-O. and U.K.R. conducted the PACE analysis; T.T.-S. and C.A.S. conducted the acylsugar profiling. G.S.P. helped to collect and analyze the data; P.B. and C.A.S. helped to revise the manuscript, providing valuable suggestions. All authors read and approved the manuscript.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Acylsugar acyl chain composition from the different genotypes.

Supplementary Figure S2. Trichome quantification in the *Get02/Wo* recombinant population.

Supplementary Figure S3. Chromatograms showing qualitative differences in acylsugar profiles from the different genotypes.

Supplementary Figure S4. Preliminary *B. tabaci* no-choice bioassay.

Supplementary Figure S5. Comparative phenotypical analysis of the genotypes used in this work.

Supplementary Figure S6. Complementary trichome characterization.

Supplementary Figure S7. Rhodamine B test.

Supplementary Table S1. Description of the genotypes used in this work.

Supplementary Table S2. Updated *S. lycopersicum* genome coordinates of the QTLs on Chromosome 2 associated with type-IV trichomes.

Supplementary Table S3. Oligonucleotide sequences used in this work.

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Conflict of interest statement. None declared.

Data availability

We have provided all data, and materials will be made available upon request by community members.

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