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Natural genetic variation in the *HAIRS ABSENT (H)* gene increases type-VI glandular trichomes in both wild and domesticated tomatoes

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ABSTRACT

Glandular trichomes produce and exude secondary metabolites, conferring insect resistance in many crop species. Whereas some of its wild relatives are insect-resistant, tomato (*Solanum lycopersicum*) is not. Identifying the genetic changes that altered trichome development and biochemistry during tomato domestication would contribute to breeding for insect resistance. A mutation in the *HAIRS ABSENT* (*H*) gene, which encodes a C2H2 zinc finger protein (ZFP8), leads to reduced trichome density. Several geographic accessions of *S. pimpinellifolium*, the wild ancestor of domesticated tomato, have glabrous organs that resemble the phenotype caused by *h*. Here, we investigated allelic diversity for *H* in tomato and *S. pimpinellifolium* accessions and their associated trichome phenotypes. We also evaluated how the developmental stage can affect trichome development in glabrous and non-glabrous plants. We found that glabrous accessions of *S. pimpinellifolium* have different *ZFP8* nucleotide sequence changes, associated with altered trichome development and density. We also found that while the glabrous appearance of *h* mutants is caused by a lower density of long trichomes, the density of type-VI glandular trichomes is increased, particularly in the adult stages of plant development. These insights on the genetic control of trichome development may contribute to breeding for insect resistance in tomatoes and other crops.

1. Introduction

Crop domestication led to reduced resistance to pathogens and pests (Harlan, 1992). Tomatoes (*Solanum lycopersicum*), for instance, are more vulnerable to pests and diseases than their wild relatives, requiring a high volume of agrochemicals (Machado and dos Santos, 2017). Wild relatives of tomato have glandular trichomes (Simmons and Gurr, 2005), which produce and exude secondary compounds with insecticide activity (Ben-Israel et al., 2009; Sallaud et al., 2009; Yu et al., 2010; Bleeker et al., 2011; Gonzales-Vigil et al., 2012). Tomato and its wild relatives have eight different types of trichomes, of which four (types I, IV, VI and VII) are glandular and four (types II, III, V and VIII) are non-glandular (Glas et al., 2012; Luckwill, 1943). Type-IV trichomes and, to a lesser extent, type-I trichomes, which are rare, are sources of acylsugars. These compounds can poison, bind or tag insects to be detected by their predators (Weinhold and Baldwin, 2011). Tomatoes can produce acylsugars (Schilmiller et al., 2015), however, type-IV trichomes are only found in the juvenile phase of development (Vendemiatti et al., 2017). Type-VI trichomes are sources of natural insecticides based on chloroplast-derived sesquiterpenes and methyl

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ketones (Williams et al., 1980; Fridman et al., 2005; Ben-Israel et al., 2009; Besser et al., 2009; Sallaud et al., 2009; Yu and Pichersky, 2014). Type-VI trichomes in tomato have a different morphology and a lower density than in insect-resistant wild species (Besser et al., 2009; Bergau et al., 2015). However, little is known about how type-VI trichome development and density are controlled.

Domestication may have caused the loss of insect resistance by altering either glandular trichome formation or the secondary metabolites (acylsugars, sesquiterpenes and methyl ketones) biosynthesis pathways in tomato (Bleeker et al., 2012; Chen et al., 2015; Gao et al., 2019; Rakha et al., 2015). However, the picture appears to be more complex, as many geographic accessions of the wild ancestor of tomato, *S. pimpinellifolium*, lack insect resistance altogether. The pathways for sesquiterpene and methyl ketone biosynthesis have evolved independently in wild relatives of tomato, like *S. habrochaites*, and not in the lineage leading to domesticated tomatoes (Ben-Israel et al., 2009; Sallaud et al., 2009; Yu et al., 2010; Bleeker et al., 2011; Gonzales-Vigil et al., 2012). The genes involved in sesquiterpene and methyl ketone biosynthesis in tomato wild relatives are well known (Sallaud et al., 2009; Yu et al., 2010). Thus, it would be possible to transfer these biosynthetic pathways to cultivated tomato via wide crosses with wild relatives followed by selection (Therezan et al., 2021). However, the genetic control of trichome density remais elusive. Some information has been gained by studying mutants in domesticated tomato; for instance, *Woolly* (*Wo*), a *HOMEODOMAIN-LEUCINE ZIPPER IV* (*HD-ZIP IV*) gene, and *hairless* (*hl*), a *SPECIFICALLY RAC1-ASSOCIATED (SRA1)* gene (Yang et al., 2011; Kang et al., 2016). Notably,the tomato *hairs absent* (*h*) mutation produces a phenotype that resembles the glabrous organs of some wild relatives (Fig. 1).

The *H* gene of tomato encodes a C2H2 zinc finger protein similar to *ZFP8* from Arabidopsis, whose loss of function impairs type-I trichome formation (Chang et al., 2018). Recently another transcription factor, encoded by the *bHLH95* gene, was identified as a negative regulator of trichome formation in tomato (Chen et al., 2020). Both loci are located very close to one another on chromosome 10 (Chang et al., 2018), making resolution of their respective functions on trichome development highly challenging. To further compound the complexity, trichome density and relative abundance of different trichome types are strongly dependent on organ and developmental stage (Chien and Sussex, 1996; Vendemiatti et al., 2017). Thus, a thorough characterization of the genes controlling trichome development necessitates a careful analysis of different organs over the life cycle of the plant.

Here, we investigated the role of both genes, *H* (*ZFP8*) and *bHLH95*, in the glabrous phenotype of the tomato wild ancestral species *S. pimpinellifolium*. We also evaluated how developmental stage affects trichome density and type in glabrous and non-glabrous plants. We explored the phenotypic variation found in different geographic accessions of *S. pimpinellifolium* and tomato cv. Micro-Tom (MT) plants harboring the *h* mutation. Glabrous *S. pimpinellifolium* accessions, as well as *h* mutants, show changes in *ZFP8* nucleotide sequence that can impair protein function and modify trichome development and density. Lastly, we showed that a prominent phenotype in glabrous accessions is an increase in trichome type-VI density, particularly in adult stages of plant development.

2. Material and methods

2.1. Plant material and experimental setup

Seeds of the tomato wild relatives were donated by the Tomato Genetics Resource Center (TGRC, Davis, University of California). Seeds of the *S. pennellii* introgression lines (ILs) were kindly donated by Prof. Dani Zamir (The Hebrew University of Jerusalem, Israel). The Near Isogenic Line (NIL) harboring the *hairs absent* (*h*) mutation was generated by introgression into tomato cv. Micro-Tom (MT) as described (Carvalho et al., 2011). Seeds of MT were donated by Prof. Avram Levy (Weizmann Institute of Science, Israel) in 1998 and kept as a true-to-type cultivar through self-pollination. All the steps in the introgression process were performed in the Laboratory of Hormonal Control of Plant Development (HCPD), Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), University of São Paulo, SP, Brazil.

Plants were grown in a greenhouse at ESALQ, Piracicaba, SP, Brazil (546 m asl, 22° 42′ 30″ S; 47° 38' 00" W). Seeds were germinated in 350 mL pots containing a 1:1 mixture of commercial potting mix Basaplant® (Base Agro, Artur Nogueira, SP, Brazil) and expanded vermiculite supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} dolomite limestone ($MgCO_3 + CaCO_3$). Upon appearance of the first true leaf, seedlings were transplanted to 350 mL (for MT and *h*) or 10 L (for indeterminate growth

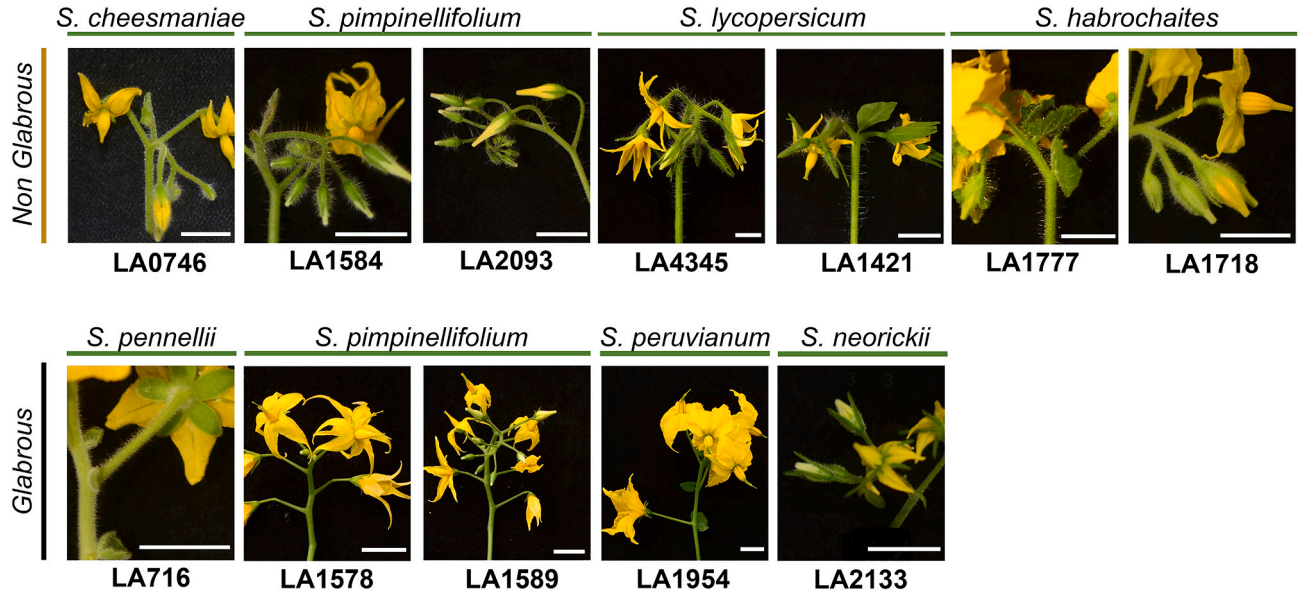

Fig. 1. Natural genetic variation for long trichome density in inflorescences. Representative images of accessions of tomato and its wild relatives showing glabrous and non-glabrous inflorescences. Glabrous accessions: Solanum pennellii LA716; S. peruvianum LA1954; S. neorickii LA2133; S. pimpinellifolium LA1578; S. pimpinellifolium LA1589. Non glabrous accessions: S. pimpinellifolium LA1584; S. pimpinellifolium LA2093; S. cheesmaniae LA0746; S. lycopersicum LA4345 and LA1421; S. habrochaites LA1777 and LA1718. Scale bar = 1 cm.

plants) pots containing the soil mix described above, except for NPK supplementation, which was increased to 8 g L^{-1}.

2.2. Trichome analysis

Trichome identification and counting were performed on stems and peduncles. On the stem, analyses were performed 15 and 48 days after germination (DAG). Three or four individual per genotype were sampled, and 10 or 8 images, respectively, were analyzed per plant (n = 30). Photographs were taken using a Leica S8AP0 (Wetzlar, Germany) magnifying glass set to 80 × magnification, coupled to a Leica DFC295 camera (Wetzlar, Germany). Trichome counts were performed on the images and the density was calculated in relation to the image size.

We classified trichome types I and II under a single category because in more advanced stages of development, type I glandular trichomes may lose the gland leading to similarity with type II trichomes. All other trichome types were identified based on their morphological characteristics (Luckwill, 1943; Glas et al., 2012).

2.3. Sequencing and sequence analysis of H and bHLH95

Sanger-sequencing of the *H* and *bHLH95* genes was performed for *S. pennellii* (LA716), IL10-2, *S. pimpinellifolium* (accessions LA1578, LA1584, LA1589 and LA2093), Micro-Tom (MT) and the *hairs absent* near isogenic line (*h*). Genomic DNA was extracted from the young leaves according to Fulton et al. (1995). The genes were amplified with specific primers using PlatinumTM Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The PCR product was purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR product was then inserted into the pGEM®-T-Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* Top-10 competent cells (Thermo Fisher Scientific, Waltham, MA, USA). Through blue/white selection (using X-Gal and IPTG), colonies harboring the genes of interest were selected. Plasmids were isolated with GenElute™ Plasmid miniprep kit (Sigma-Aldrich), and then sent for sequencing (CENA, USP). Tomato sequence data for the *H* and *bHLH95* genes in different accessions (Table 1) were obtained from the Solanaceae Genomics network (SGN) between May 2019 and May 2021 (solgenomics.net). The sequences were applied as input to build a *H* and *bHLH95* consensus domain profile with the MEME software (meme-suite.org).

2.4. Gene expression analysis

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Invitrogen). To remove genomic DNA, the total RNA was treated with RNase-free DNase (RQ1 Promega, Madison, WI, USA). Quantity and quality of total RNA were assessed by ND-1000 Nanodrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1.5% agarose gel. DNase I-treated RNA (1.0 μg) was reverse-transcribed to generate first-strand cDNA using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) platform. Two technical and biological replicates were performed for each sample. The threshold cycle (CT) was determined automatically by the instrument, and fold changes for each gene were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Relative expression was normalized using the actin and ubiquitin genes.

Table 1
Description of accessions used for *in silico* analysis of *H* and *bHLH95* genes.

Species	Accession	Phenotype
S. lycopersicum cv. Heinz	LA4345	Non glabrous
S. lycopersicum	LA1421	Non glabrous
S. galapagense	LA1044	Non glabrous
S. cheesmaniae	LA0483	Glabrous
S. pennellii	LA716	Glabrous
S. pimpinellifolium	LA1578	Glabrous
S. pimpinellifolium	LA1584	Non glabrous
S. chmielewskii	LA2695	Glabrous
S. chmielewskii	LA2663	Glabrous
S. neorickii	LA2133	Glabrous
S. peruvianum	LA1954	Glabrous
S. habrochaites	LA1777	Non glabrous
S. habrochaites	LA1718	Non glabrous

2.5. Alignment and sequence analysis

Multiple alignment was performed using ClustalW (www.genome.jp/tools-bin/clustalw). The sequences were analyzed using MEGA (www.megasoftware.net) and GeneDoc software (Nicholas and Nicholas, 1997).

3. Results

3.1. Natural allelic variation in the H gene

We first characterized the presence or absence of long trichomes in a panel of 13 selected accessions of tomato and its wild relatives. We found that *S. pennellii* LA716, *S. peruvianum* LA1954, *S. neorickii* LA2133, *S. pimpinellifolium* LA1578 and LA1589 are all glabrous, while *S. pimpinellifolium* LA1584 and LA2093, *S. cheesmaniae* LA0746, *S. lycopersicum* LA4345 and LA1421, *S. habrochaites* LA1777 and LA1718 are non-glabrous (Fig. 1).

To search for variation in nucleotide and amino acid sequence of *HAIRS ABSENT* (*ZFP8*) and *bHLH95*, we performed *in silico* analyses in the *Solanum* accessions described above (Figs. S1 and S2). The analysis of the nucleotide sequences of *ZFP8* showed several single nucleotide polymorphisms (SNPs) along the coding sequence, with a few of them within the zinc finger domain (Fig. S1). An amino acid sequence alignment showed different changes between the evaluated accessions; most were inconclusive regarding the function of the protein and their effect in promoting the non-glabrous or glabrous phenotype (Fig. S1). However, in *S. pimpinellifolium* LA1578 the substitution of a cytosine (C) to adenine (A) in nucleotide sequence generates a premature stop codon in the protein sequence, leading to a predicted non-functional protein (Fig. S1). The amino acid sequence of bHLH95 was highly conserved among all the evaluated accessions (Fig. S2).

3.2. The glabrous phenotype of S. pimpinellifolium accessions masks the increased density of type-VI glandular trichomes

The macroscopic phenotypic variation (presence of trichomes visible to the naked eye, Fig. 2) in four accessions (LA1578, LA1584, LA1589 and LA2093) accessions of *S. pimpinellifolium* of diverse geographical origin (Fig. 2), led us to evaluate the stem trichome density at 15 and 48 days after germination (DAG). In tomatoes, these time points are representative of the juvenile and adult phases of plant development (Vendemiatti et al., 2017). At both time points accessions LA1584 and LA2093 showed non-glabrous phenotype, whereas LA1578 and LA1589 had glabrous phenotype (Figs. 3 and 4). At 15 DAG, accession LA1584 showed higher density of type-IV and -VI trichomes compared to type I/II trichomes (Fig. 3). However, the glabrous accessions showed a prevalence of type-VI trichomes in relation to the other types of trichomes (Fig. 3). Type I/II trichomes were more abundant in accession LA2093, whereas higher density of type-IV trichomes was found in LA1589 (Fig. 3d). Glabrous accessions had type-VI trichomes in greater density (LA1578 and LA1589) (Fig. 3d).

The evaluations performed on the stem at 48 DAG showed that the higher density of type-VI trichomes was consistent for all accessions (Fig. 4). At this stage of development, accession LA1584 showed more

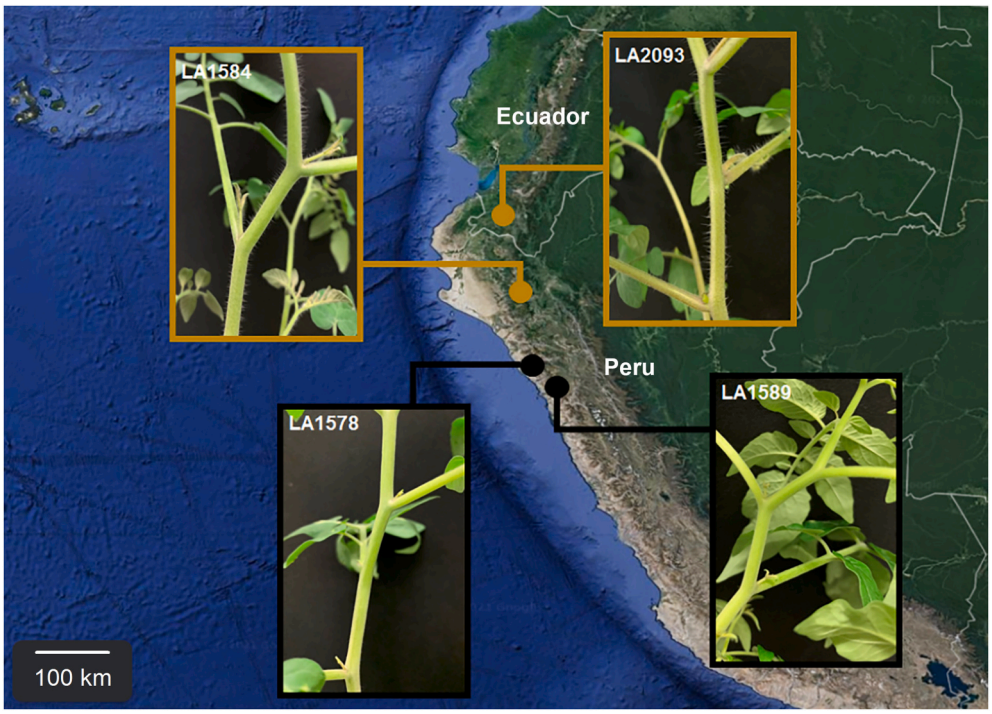

Fig. 2. Accessions of *Solanum pimpinellifolium* vary in long trichome phenotype in stems. LA1584 and 2093 are non-glabrous, LA 1578 and LA1589 are glabrous. Scale bar = 1 cm.

type I/II trichomes density (Fig. 4d). No variation for type-IV glandular trichome density was found between the accessions (Fig. 4d). At 15 DAG, accessions with a glabrous phenotype (LA1578 and LA1589) showed a higher density of type-VI glandular trichomes (Fig. 4d).

No difference in the density of type I/II trichomes between developmental stages was found in LA1578 or LA1589. LA1584, however, had a seven-fold higher density of type I/II trichomes ($p < 0.001$) at 48 DAG compared to 15 DAG. Likewise, LA2093 showed 50% ($p = 0.025$) more type I/II trichomes at 48 DAG than 15 DAG. At 48 DAG, no type-IV trichomes were observed in the accessions of *S. pimpinellifolium* analyzed, with the exception of LA1584 (Fig. 4d). Type-VI trichome density increased significantly in LA2093 (+83%; $p < 0.001$), LA1578 (+54%; $p < 0.001$), LA1589 (+20%; $p < 0.001$) at 48 DAG, compared to 15 DAG. Although LA2093 showed a greater increase comparing the two evaluated stages, LA1578 showed a higher density of type-VI trichomes at 48 DAG (Fig. 4d).

In Arabidopsis, which has only non-glandular trichomes, *ZFP8* gene expression is associated with the development of trichomes mainly in the inflorescence (Gan et al., 2007). Thus, we analyzed the phenotype of flower buds and peduncles in *S. pimpinellifolium* (Fig. S3). In LA1584 and LA2093, flower buds showed long trichomes, which were absent in the LA1578 and LA1589 glabrous accessions (Fig. S3). In the peduncle of all accessions, type-VI trichomes showed higher density in relation to other trichomes (Fig. S3). However, we observed variation in the density of type-VI trichomes between accessions (Fig. S3). Glabrous accessions, LA1578 and LA1589, showed a higher density of type-VI trichomes compared to non-glabrous accessions LA1584 and LA2093 (Fig. S3). Both LA1578 and LA589 had between 6 × and 8.5 × higher type-VI trichome density than LA1584 and LA2093.

3.3. Sequencing and gene expression analyses of ZFP8 and bHLH95

To identify variation in the *ZFP8* and *bHLH95* genes, associated with the *h* phenotype, we analyzed the nucleotide sequence and expression levels of both genes. The non-glabrous accessions of *S. pimpinellifolium* (LA1584 and LA2093) showed high similarity in *ZFP8* sequence (Fig. 5a). The glabrous accession LA1578 showed a premature stop codon at amino acid position 46 caused by a C→A nucleotide substitution, which we had detected previously in our *in silico* analysis (Fig. S1) in the reference sequence available online (solgenomics.net) (Fig. 5a). LA1589 showed a deletion in G287 of the nucleotide sequence, resulting in a frameshift and compromising the protein sequence (Fig. 5a). The amino acid sequence of bHLH95 was highly conserved among the *S. pimpinellifolium* accessions evaluated and no alteration was found in the bHLH domain (Fig. 4). The expression of *ZFP8* in glabrous and non-glabrous *S. pimpinellifolium* accessions showed no significant difference (Fig. 5b). Higher *bHLH95* expression was found in LA1584 compared to LA1589 (Fig. 5c).

3.4. The hairs absent (h) mutation leads to increased density of type-VI glandular trichomes

The *h* mutant showed a reduction in the density of long trichomes visible to the naked eye (Fig. 6a and b). Analysis of trichomes on the stem revealed that the *h* phenotype is caused by the reduction in type-I/II trichomes and accompanied by an increase in type-VI glandular trichomes (Fig. 6c). Additionally, we observed that *h* showed an increase in type-III trichomes in the stem compared to MT (Fig. 6c). Similar results were found in the peduncle of *h* mutant. However, in the floral peduncle of *h* there was also a significant reduction in the density of type-III trichomes, compared to MT (Fig. S5). Principal component analysis (PCA) indicates a negative relationship between the density of both type-I/II and –III trichomes and type-VI trichomes (Fig. S6). Thus, the data suggest that the *h* phenotype is associated with a reduction in long trichomes and an increase in the density of type-VI glandular trichomes.

Sequencing of the *h* allele introgressed in MT showed SNPs at various positions and a T-insertion in position 379. Such modifications affected the amino acid sequence of the *ZFP8* protein in *h* compared to the wild type protein in MT (Fig. 7a). We did not observe variation in *ZFP8* expression in hypocotyl, leaf and reproductive meristem of *h* compared to MT (Fig. 7b, c, e). However, *ZFP8* expression was decreased in the stem of *h* plants (Fig. 7d). In contrast, the expression of *bHLH95*

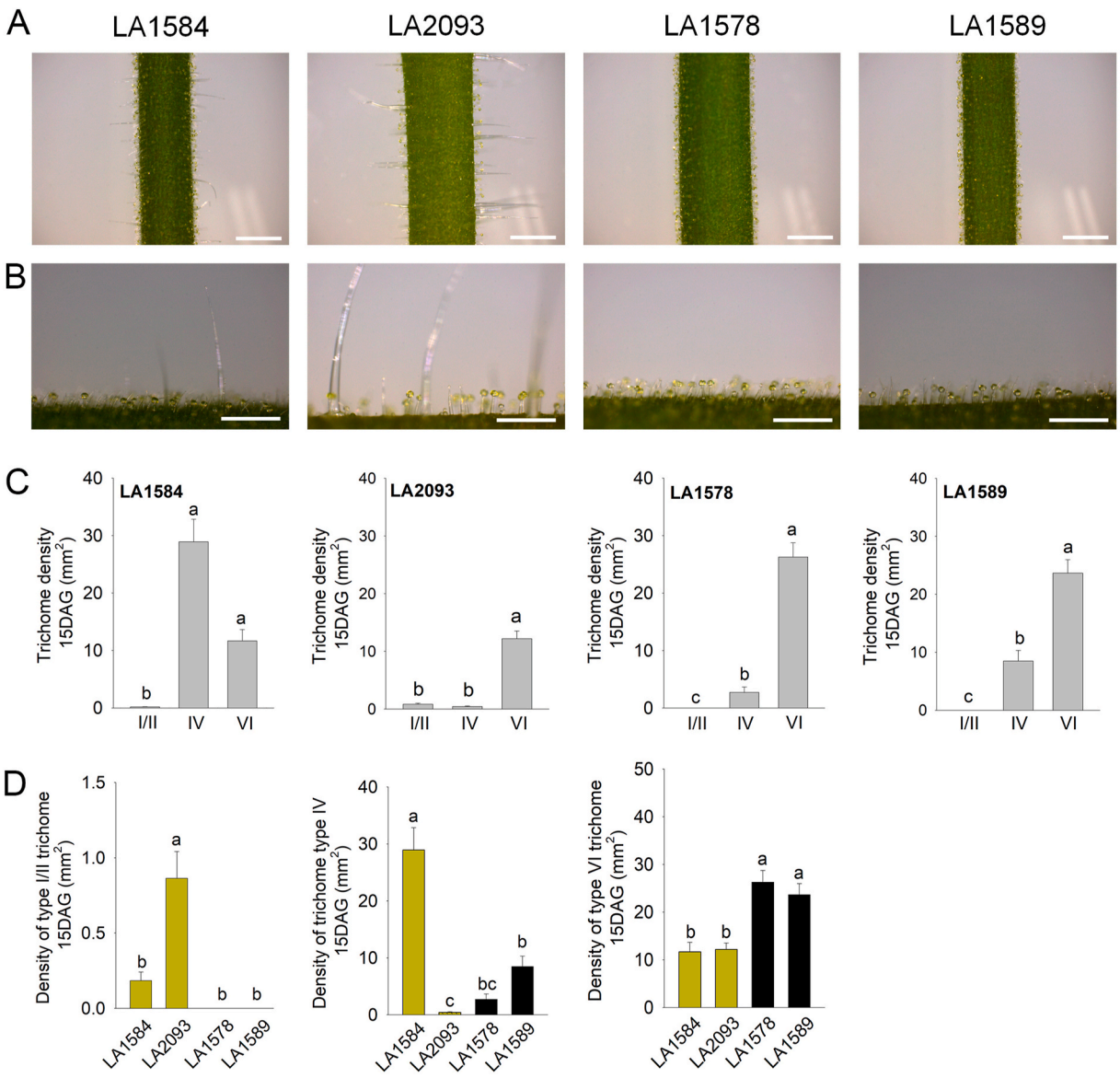

Fig. 3. Type-IV and -VI trichomes predominate in stems of *Solanum pimpinellifolium* 15 days after germination (DAG). (a) Representative stems of 15-days old *S. pimpinellifolium* accessions LA1584, LA2093, LA1578 and LA1589. (b) Detailed view of stem trichome density. (c) Stem trichome density. (d) Density of type-I/II, -IV and -VI trichomes. Arrows show types of trichomes. Scale bars = 1 mm (a) and 500 μm (b). Data are mean (n = 30) ± s.e.m. Different letters indicate significant differences by Tukey's test at 5% probability.

increased in *h* stem compared to MT (Fig. 7f).

3.5. Hypocotyls of h plants show long trichomes

Unlike the phenotype observed in the stem, the plants with the *h* allele showed long trichomes on the hypocotyl, like non-glabrous MT plants (Fig. S7). All trichomes observed in hypocotyls were glandular, especially type-I trichomes. But we also detected the presence of type-IV and -VI trichomes in those samples (Fig. S7).

4. Discussion

Trichomes are potentially valuable for crop breeding, as they provide protection against biotic and abiotic stresses. A major hurdle to exploit trichomes in agriculture is the insufficient knowledge about the genes that control trichome types and metabolic pathways (Chalvin et al., 2020). Since variation in trichome type and density is common in natural populations (Glas et al., 2012), genomic analyses in these populations may aid in the identification of genetic pathways involved in trichome development. Here, we show that the tomato *hairs absent* (*h*) mutation, phenotypically recognizable by the reduction of density of long trichomes (types I, II and III) (Chang et al., 2018; Vendemiatti et al., 2017), is a natural genetic variant between different species and between different accessions of the same species of the tomato clade.

Loss of function in the *h* gene, which encodes a zinc finger protein (ZFP), has previously been associated with reduction of type-I and -VI glandular trichomes (Chang et al., 2018). Here we show that while the *h* mutation does indeed reduce long trichome (types I, II, III) density, it simultaneously leads to higher type-VI glandular trichomes density. Both glandular trichome types I and VI are sources of secondary metabolites (acyl sugars in type-I trichome and terpenoids, flavonoids and methyl ketones in type-VI trichomes) that have a fundamental role in biotic stress responses (Sallaud et al., 2009; Yu et al., 2010; Schilmiller et al., 2015). A reduction in glandular trichome density and an alteration in their secondary metabolites profile has occurred in cultivated tomatoes as a by-product of domestication and breeding (Bleeker et al.,

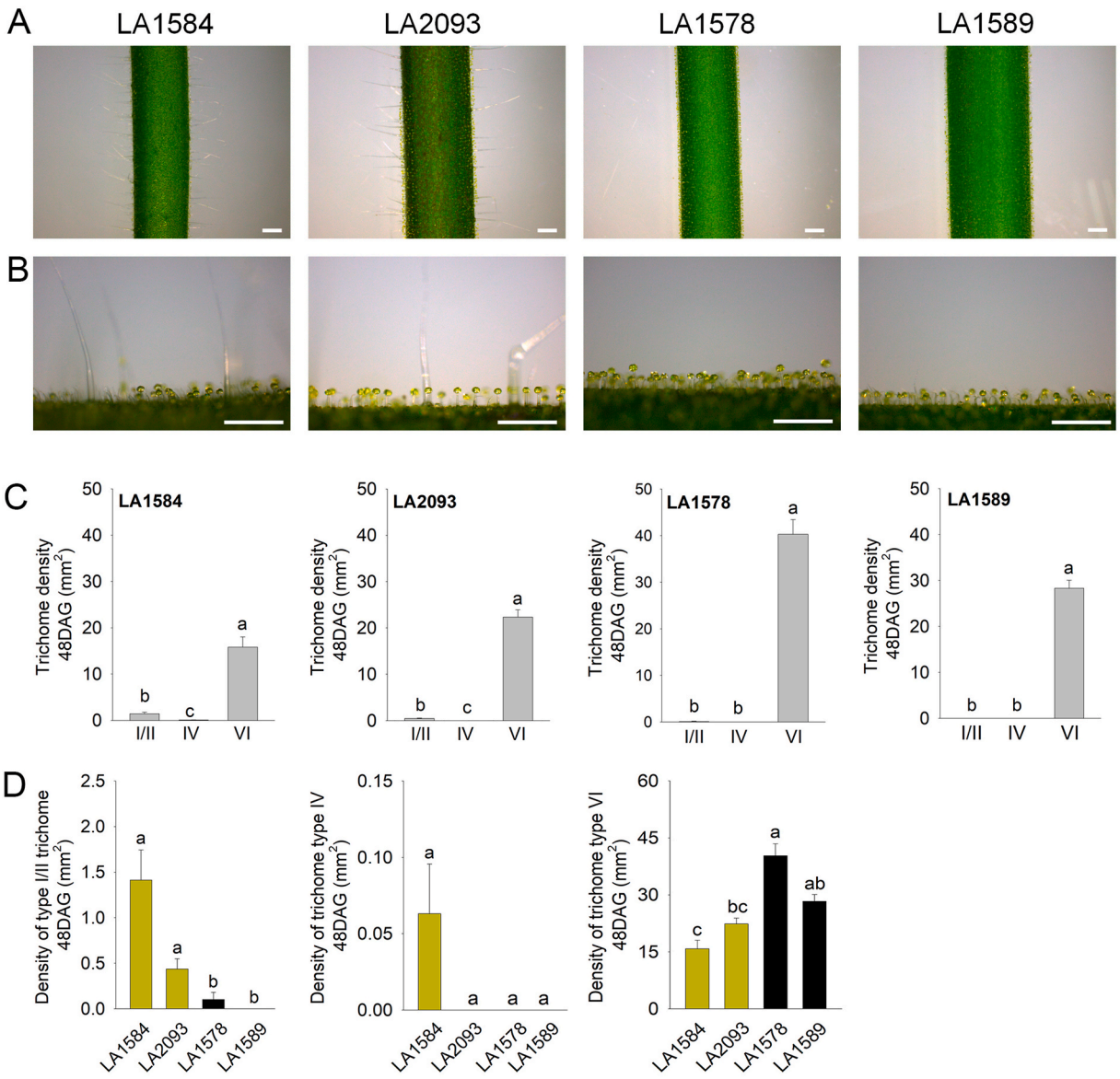

Fig. 4. Type-VI glandular trichomes predominate in stems of *Solanum pimpinellifolium* 48 days after germination (DAG) and the glabrous accessions present a higher density. (a) Representative stems of 15-days old *S. pimpinellifolium* accessions LA1584, LA2093, LA1578 and LA1589. (b) Detailed view of stem trichome. (c) Stem trichome density. (d) Density of type-I/II, -IV and -VI trichomes. Arrows show types of trichomes. Scale bars = 1 mm (a) and 500 μm (b). Data are mean (n = 30) ±s. e.m. Different letters indicate significant differences by Tukey's test at 5% probability.

2012). Presumably, the energetic cost of trichome and metabolite production would be reduced, while the reduction in biotic stress protection is compensated by increased pesticide application and more controlled growth conditions provided by agronomic management. However, this raises the question of why the loss of glandular trichomes would persist as a polymorphism in natural populations, where humans would not provide the compensatory effect. This question prompted us to revisit the phenotypic effects of the *h* mutation in *S. pimpinellifolium*, where functional and non-functional allelic variants of *h* coexist in different accessions of diverse geographical origins.

The glabrous *S. pimpinellifolium* accessions (LA1578 and LA1589) showed a premature stop codon in the *h* gene and an increase in type-VI trichomes in stems. Although discrepant with the results described by Chang et al. (2018), the increase of type-VI trichomes has been previously reported for leaves of *h* in tomato (Vendemiatti et al., 2017), and here we extend this observation for stems of the *h* mutant in natural accessions. The disagreement between our results and those of Chang et al. may be related to the stage of development at which the evaluations were conducted, since the development of different types of trichomes is strongly dependent on plant age and progression from juvenility to adult stages (Chen et al., 2018; Vendemiatti et al., 2017). We found that in the *S. pennellii* introgression lines (ILs) studied by Chen et al. (2018), the juvenile phase is marked by a predominance of type-IV trichomes in the stems. Thus, the glabrous phenotype of IL10-2 is initially associated with a reduction of type-I trichomes, an easily recognizable phenotype (Fig. S8). However, during stem development, the reduction in trichome type-IV density, the appearance of type-V trichomes and the increase in type-VI trichomes may also be associated with the *h* phenotype in IL10-2 (Fig. S8).

One of the difficulties of assessing glandular trichome type and density is the apparent redundancy of genetic pathways that control their development. Alteration in the density of type-VI trichomes has been related to disturbances in trichome metabolic pathways (Kang et al., 2014; Paetzold et al., 2010), which indicate an indirect control of trichome development. Particularly for type-VI trichomes, only a few genes directly related to their development have been identified.

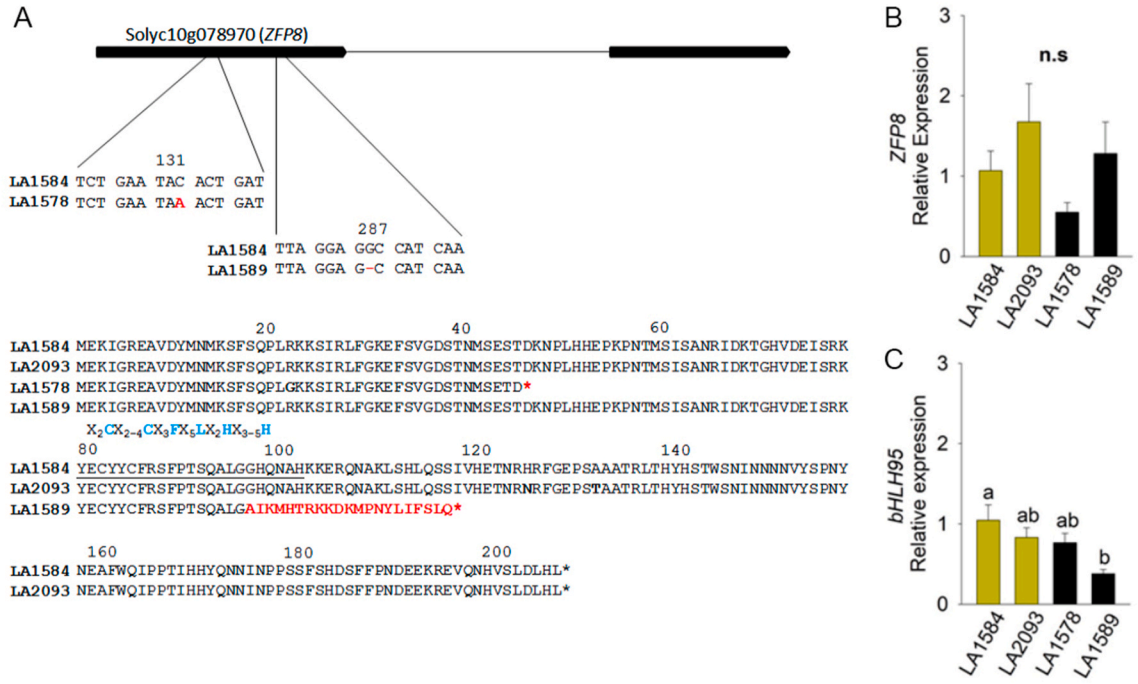

Fig. 5. A stop codon in the Zinc Finger Protein 8 (ZFP8) may be related to the glabrous phenotype in *S. pimpinellifolium*. (a) Amino acids alignment of ZFP8 of *S. pimpinellifolium* accesions. (b) *ZFP8* relative expression in stems. (c) *bHLH95* relative expression in stems. Note that LA1578 shows cytosine (C)-to-adenine (A) substitution that generates a premature stop codon, indicated by the red asterisk in the amino acid sequence. LA1589 contains a guanine (G) deletion that generates a frameshift in the amino acid sequence, indicated by red capital letters and an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

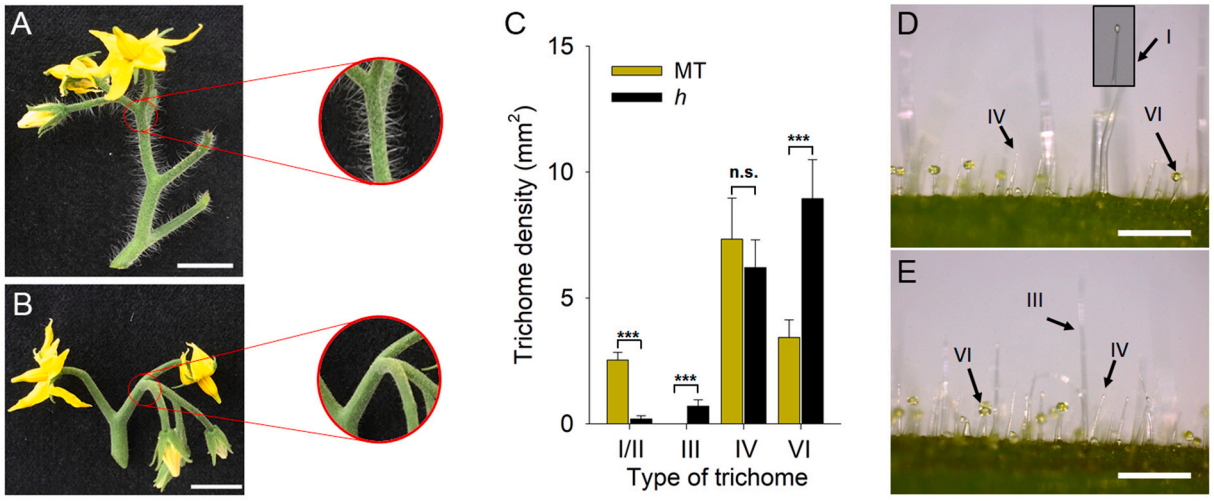

Fig. 6. The tomato cv. Micro-Tom (MT) near isogenic line harboring the *hairs absent* (*h*) mutation shows an increase in type-VI glandular trichomes and a reduction in long trichomes in the stem compared to the MT control. Representative images of inflorescences of (a) MT and (b) *h* mutant. (c) Density of different trichome types in the stem of MT and *h*. Representative images of stems of (d) MT and (e) *h*. Asterisk indicates significant difference by Student's *t*-test (***P < 0.001). n.s. = non-significant. Glandular trichome type I is shown in the inset. Arrows show types of trichomes. Scale bars = 500 μm.

Recently, it has been shown that *SlMYC1*, a basic helix–loop–helix (bHLH) transcription factor family member, positively regulates the initiation of type-VI glandular trichomes and is essential in the later steps of type-VI glandular trichome morphogenesis in tomato (Xu et al., 2018). Knocking down *SlMYC1* led to the production of smaller type-VI glandular trichomes at lower densities, and knocking out the gene impaired their formation (Xu et al., 2018). SlMYC1 is involved in the biosynthesis of terpenoids in type-VI glandular trichomes (Xu et al., 2018), highlighting the important role of SlMYC1 in two different but complementary processes in glandular trichome of tomatoes.

The development of glandular trichome types I and VI is under common regulatory control by *WOOLLY* (*WO*), an HD-ZIP transcription factor, and other genes such as *HAIRLESS* (*HL*), *HAIRS ABSENT* (*H*) and *INQUIETA* (*INI*) (Chang et al., 2018; Jeong et al., 2017; Kang et al., 2016, 2010; Yang et al., 2011a, 2011b). This supports our hypothesis that the *h* mutant phenotype is caused by alterations in both trichome types, type-I and type-VI, and additionally other long trichomes (types II and III). However, even in plants with loss of *H* function, we observed a high number of type-I trichomes in the hypocotyl (Fig. S7), which indicates that other genes besides *ZFP8* and/or other pathways are involved in

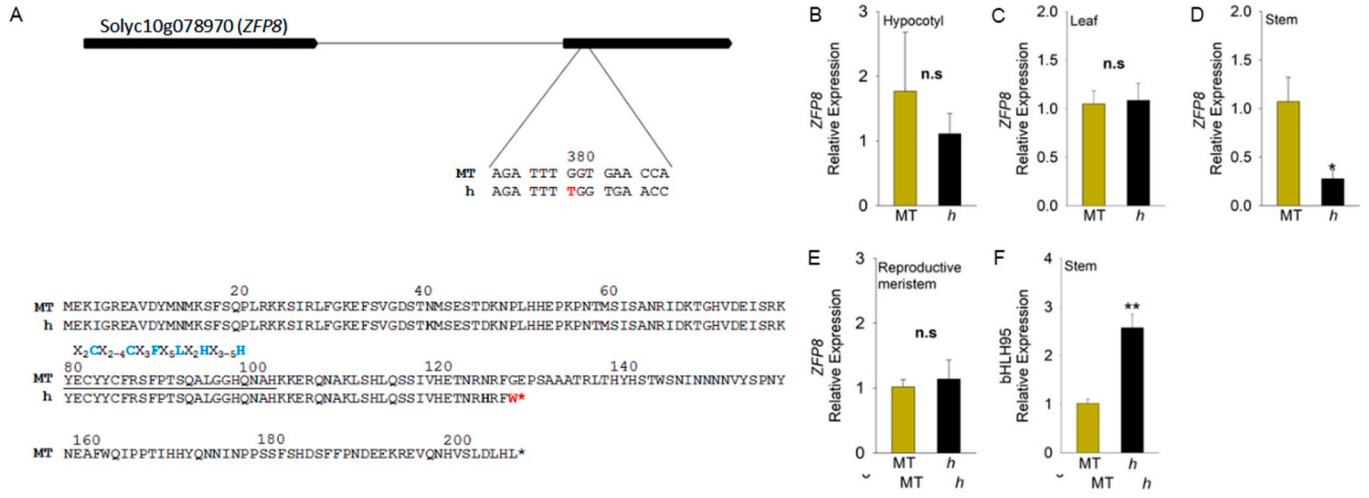

Fig. 7. The *hairs absent (h)* allele introgressed into Micro-Tom (MT) has a premature codon stop on ZFP8 protein. (a) Amino acids alignment of ZFP8 of MT and *h*. Relative expression of *ZFP8* in hypocotyl (b), leaf (c), stem (d) and reproductive meristem (e). Relative expression of *bHLH95* in stem (f). Asterisk indicates significant difference by Student's *t*-test (**P < 0.01; *P < 0.05). n.s. = non-significant.

type-I trichome development. A recent study has shown that differences in type-I trichome density on stems and leaves could be associated with particular expression patterns of *H* and *H2* (a novel *ZFP* also involved in type-I trichome development) (Chun et al., 2021). In general, *H2* is mainly expressed in leaves while *H* is highly expressed in the stem (Chang et al., 2018; Chun et al., 2021). Interestingly, both genes, *H* and *H2*, are located in close vicinity on tomato chromosome *10*.

In addition, bHLH95, a negative regulator of trichome development in tomato, was also mapped to the same region of chromosome *10* as *H* and *H2* (Chen et al., 2020). Overexpression of bHLH95 reduced the density of stem trichomes, especially types I and V, but no changes were observed in the density of type-VI glandular trichomes (Chen et al., 2020). Since bHLH95 controls type-I and -V density in tomato, it is possible that variation in the expression of this gene, combined with the functionality or expression of *H* regulates the transition between trichome types across developmental stages in tomato. In fact, we observed that in MT-*h* increased expression of *bHLH95* occurs simultaneously with reduced expression of *ZFP8* (Fig. 7). Thus, the *h* mutation could alter protein function with no effect on gene expression (the mutation is in the coding sequence). Post-transcriptional control could play a key role in the regulation of trichome development. This is particularly true in the case of *H,* which appears to be part of a wider, complex network of factors controlling trichome development (Hua et al., 2021, 2022; Li et al., 2021; Zheng et al., 2022). Taken together, the results presented here, and our reanalysis of previously published work, suggest that sequence and regulatory variation in a cluster of three contiguous loci on chromosome *10* may have been instrumental in determining the altered balance of type-I and type -VI trichome in tomato domestication. This notion is reinforced by the three genes being contained within a domestication sweep with decreased nucleotide sequence variation between the tomato ancestral species *S. pimpinellifolium* and the cherry tomatoes *S. lycopersicum* var. *cerasiforme* (Fig. 8) (Lin et al., 2014).

5. Conclusion

At least three transcription factors modulate gene expression patterns and fine-tune the relative abundance of trichome types along the

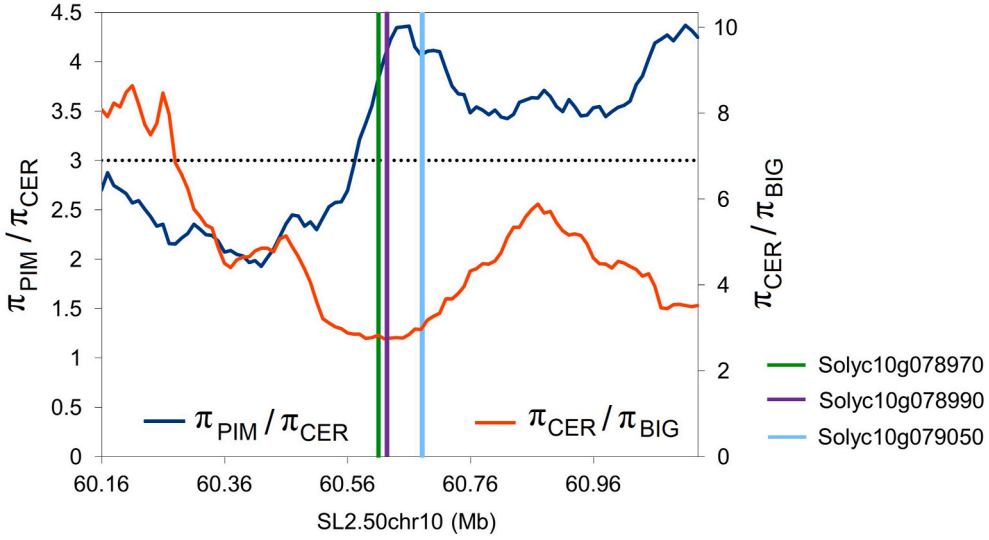

Fig. 8. Three genes controlling trichome development are located on a domestication sweep in chromosome 10. Nucleotide diversity (π) of the ancestral wild species *S. pimpinellifolium* (PIM), *S. lycopersicum* var. *cerasiforme* (CER) and big-fruited cultivars (BIG) of domesticated tomato. The horizontal lines indicates genome-wide top 5% cutoff ratio for domestication sweeps.

developmental progression of the tomato plant. Both sequence and regulatory variation may have contributed to the trichome profile changes between wild and domesticated tomatoes. Further work should be undertaken to dissect the relative contribution of each gene to specific phenotypic outcomes and how their interaction specifies different trichome types and densities. The existence of a wide repository of natural genetic variation over a defined geographical area, along with abundant genome and transcriptome information, make the tomato an ideal system to assess the impact of domestication and breeding on trichome development.

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CRediT authorship contribution statement

Karla Gasparini: Formal analysis. **Tetsu Sakamoto:** Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2022.153859.

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