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ORIGINAL PAPER

Transcriptome profiling of *trichome-less* reveals genes associated with multicellular trichome development in *Cucumis sativus*

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Abstract Trichomes on plants, similar to fine hairs on animal and human bodies, play important roles in plant survival and development. They also represent a useful model for the study of cell differentiation. Although the regulatory gene network of unicellular trichome development in *Arabidopsis thaliana* has been well studied, the genes that regulate multicellular trichome development remain unclear. We confirmed that *Cucumis sativus* (cucumber) trichomes are multicellular and unbranched, but identified a spontaneous mutant, *trichome-less* (*tril*), which presented a completely glabrous phenotype. We compared the transcriptome profilings of the *tril* mutant and wild type using the Illumina HiSeq 2000 sequencing technology. A total of 991 genes exhibited differential expression: 518 were up-regulated and 473 were down-regulated. We further identified 62 differentially expressed genes that encoded crucial transcription factors and were subdivided into seven categories: homeodomain, MADS, MYB, and WRKY domains, ethylene-responsive, zinc finger, and other transcription factor genes. We further analyzed the tissue-expression profiles of two candidate genes, *GLABRA2-like* and *ATHB51-like*, using qRT-PCR and found that these two genes were specifically expressed in the epidermis and trichomes, respectively. These results and the *tril* mutant provide useful tools to study the molecular networks associated with multicellular trichome development.

Keywords *Cucumis sativus* · Multicellular trichomes · Transcriptome · Differential expression · Transcription factors

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Introduction

Plant trichomes are specialized organs originating from the epidermal cells. They may be uni- or multicellular, glandular or glandless, and branched or unbranched (Werker 2000). Trichomes provide an excellent model system for studying cell differentiation and cell morphogenesis at the single-cell level (Hülskamp 2004; Szymanski et al. 2000). Trichomes are also involved in many developmental processes including deterrence of insects, herbivores, and microbes, maintenance of leaf temperature, reflectance of visible and UV light, and transpiration regulation (Bennett and Wallsgrove 1994; Wagner et al. 2004). They may also be involved in water absorption, secretion of heavy metals, and pollen collection (Choi et al. 2001; Küpper et al. 2000; Wagner et al. 2004; Werker 2000).

Unicellular trichome differentiation in *Arabidopsis thaliana* is regulated by a series of competing transcription factors that either up- or down-regulate relevant activities. The down-regulating activity inhibits trichome differentiation and is passed through neighboring cells (Larkin et al. 1997; Ohashi et al. 2002; Schnittger et al. 1999). The four crucial

positive transcription factors are GL1 (GLABRA1), an R2R3 MYB protein (Kirik et al. 2005), GL3 (GLABRA3) and EGL3 (ENHANCER OF GLABRA3), which are both basic helix–loop–helix proteins (Payne et al. 2000), TTG1 (TRANSPARENT TESTA GLABRA1), a WD40-repeat protein (Walker et al. 1999), and a MYB–bHLH–WD40 complex that activates GL2 (GLABRA2), a homeodomain protein that initiates trichome differentiation (Pesch and Hülskamp 2009). Proteins in the small R3 single-repeat MYB family involved in the down-regulation of unicellular trichome differentiation include TRY (TRIPTYCHON), CPC (CAPRICE) and ETC (ENHANCER OF TRY AND CPC). These proteins compete with the R2R3 MYB protein GL1 and bind to bHLH proteins including GL3 or EGL3, inhibiting their activity (Kirik et al. 2004; Wester et al. 2009).

Cucumis sativus (cucumber) is an annual species that is commercially important worldwide. *C. sativus* has diverse sex types and is a useful model plant for sex determination studies (Tanurdzic and Banks 2004). Trichomes are commonly found on most organs of wild-type *C. sativus* plants, and fruit spines (trichomes on the fruits) are valued and commercially important (Fig. 1a, b). We report here a spontaneous mutant, CGN19839, which appeared completely glabrous (Fig. 1c, d); we named this mutant "*trichome-less* (*tril*)". The regulatory mechanisms of multicellular trichome development have not been extensively studied. Here, we confirmed that *C. sativus* trichomes are multicellular and unbranched. We compared the transcriptome changes between the *tril* mutant and wild type with the Illumina HiSeq 2000 sequencing technology. From our results, we identified a series of candidate genes encoding

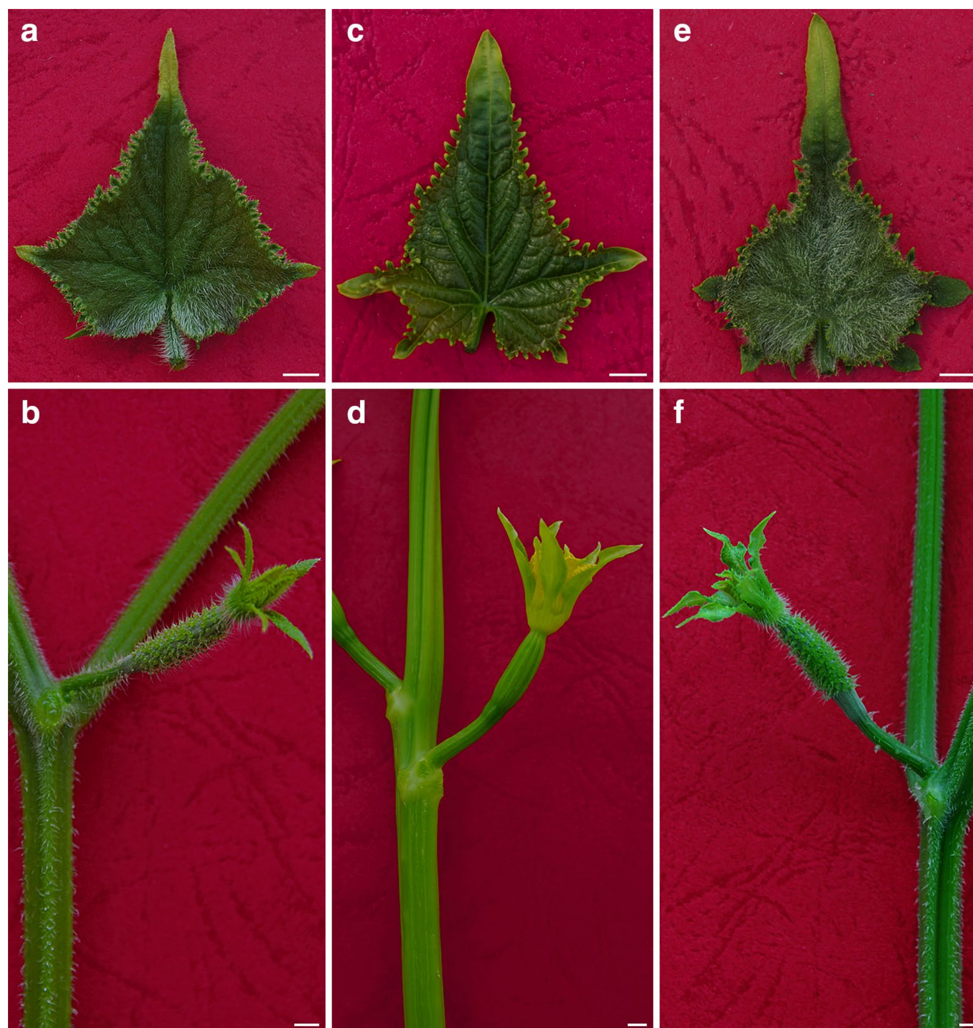

Fig. 1 Phenotypes of leaves, branches, flowers, fruits, and stems of various *C. sativus* lines. **a**, **b** Wild type. **c**, **d** The *tril* mutant. The *tril* mutant is characterized by the absence of leaf, branch, flower, fruit, and stem trichomes. **e**, **f** F_1 progeny from a cross between *tril* and wild type. *Scale bars* represent 5 mm

crucial transcription factors that can be associated with multicellular trichome development.

Materials and methods

Plant materials

C. sativus North China inbred line 06-1 was used as the wild-type background. The spontaneous mutant CGN19839 was used as the *tril* mutant. The F_1 descendants and F_2 segregating population were generated from a cross between 06-1 and CGN19839. All *C. sativus* plants were grown in a greenhouse under a natural photoperiod.

Scanning electron microscopy analysis

Three centimeter-long juvenile leaf samples were fixed in aqueous formaldehyde–acetic acid–ethanol (FAA) containing 50 % (v/v) ethanol, 5 % (v/v) acetic acid, and 3.7 % (v/v) formaldehyde at 4 °C for 24 h, dehydrated through a seven-step ethanol series (50–100 %, v/v) and critical point dried in a Leica EM CPD030 desiccator. The dried specimens were sputter-coated with gold–palladium and observed under JSM-6360LV and JEM-2010HT scanning electron microscopes.

Illumina HiSeq 2000 transcriptome sequencing

Poly-A RNA was isolated with a TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and fragmented into 100-bp inserts to create cDNA libraries. Quality control used Pico green fluorescence spectrophotometry and an Agilent 2100 bioanalyzer.

Gene annotation, expression, classification, and enrichment analyses

The spliced reads were mapped using TopHat (http://ccb.jhu.edu/software/tophat/), and transcripts were assembled with Cufflinks (http://cufflinks.cbcb.umd.edu/). Gene assemblies were annotated with BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) against the *C. sativus* database. The reference *C. sativus* genome was ftp://ftp.ncbi.nlm.nih.gov/genomes/Cucumis_sativus/.

For each gene, the expression level was calculated from the baseMean value, which was the sequencing depth for each transcript normalized to the library size. The HTSeq (http://www-huber.embl.de/users/anders/HTSeq/) and DESeq (http://www-huber.embl.de/users/anders/DESeq/) programs were used to measure differential gene expression. The functional categories of genes were established with the eggNOG (evolutionary genealogy of genes: Non-supervised orthologous groups) (http://eggnog.embl.de/). GO (Gene ontology) and KEGG (Kyoto encyclopedia of genes and genomes) enrichment analyses were performed via http://www.geneontology.org/ and http://www.genome.jp/kegg/. Analyses were conducted as described by Powell et al. (2012), Ashburner et al. (2000), and Kanehisa et al. (2004), respectively.

Extraction of nucleic acids and qRT-PCR

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and first-strand cDNA was prepared according to PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan) protocol. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted using a SYBR *Premix Ex Taq* II Kit (TaKaRa). *CsActin3* was used as the reference gene to correct gene expression values. The primers used in this study are listed in Supplementary Dataset S1.

Accession numbers

Transcriptome raw data can be found in the NCBI BioProject database under accession numbers: SAMN03276490 (WT) and SAMN03382548 (*tril*).

Results

Tril acts as a single dominant nuclear gene

A cross between the *tril* mutant and wild type generated F_1 descendants that all had a wild-type trichome phenotype (Fig. 1e, f). The F_2 segregating population contained 79 of a total of 296 F_2 plants that exhibited the mutant phenotype. This closely fit an expected 3:1 segregation ratio (217 wild type, 79 mutant type, $\chi^2 = 0.450 < \chi^2_{0.05,1} = 3.84$), indicating that the mutation was recessive and *Trichomeless* (*Tril*) acts as a single dominant nuclear gene.

Multicellular trichome structure controlled by *Tril*

Scanning electron microscopy imaging showed that three distinct types of cells constitute a *C. sativus* trichome: (a) a non-glandular pyramid-shaped apical cell, (b) a two- to four-celled cylindrical-shaped stalk, and (c) a flat pie-shaped base cell, in contact with the epidermis (Fig. 2a, b). The *tril* mutant, however, exhibited a completely glabrous morphology, and only epidermal cells including stomata and encircling guard cells were visible (Fig. 2c, d) indicating that *Tril* functions in trichome cell fate determination.

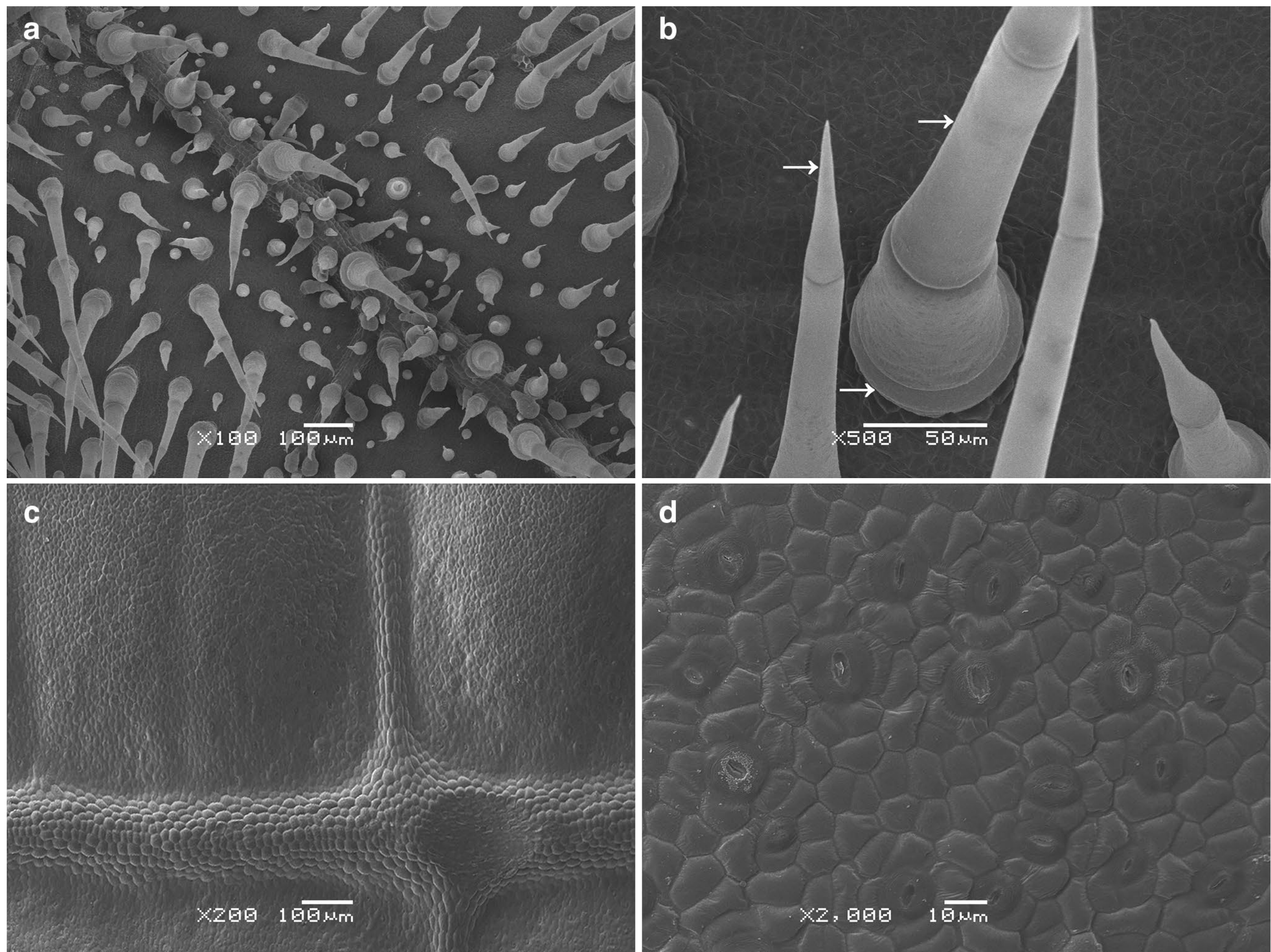

Fig. 2 Scanning electron microscopy images of wild-type and *tril* leaves. **a**, **b** Trichomes on the wild-type leaf. *Arrows* indicate the apical cell (*middle*), stalk cell (*upper*), and base cell (*lower*). **c**, **d** The *tril* leaf

Read mapping and gene annotation

The transcriptomes of 21-day-old leaves (trichomes attached with leaf epidermis) from both the *tril* mutant and wild type (each sample was mixed with three individuals) were sequenced on the Illumina HiSeq 2000 platform. The sequences of the cDNA libraries generated 40.41 and 105.40 million high-quality reads (reads obtained after data quality filtering from raw reads), respectively, with an average read length of 100 bp. A total of 36.74 (90.90 %) and 98.57 (93.52 %) million reads were mapped to the *C. sativus* genome, including 34.17 (93.01 %) and 92.04 (93.38 %) million unique reads and 2.57 (6.99 %) and 6.53 (6.62 %) million multiple reads (reads can be mapped to various genes), among which 35.54 (81.77 %) and 95.44 (82.91 %) million were mapped to genes, 34.99 (98.43 %) and 92.87 (97.31 %) million were mapped to exons, and 7.92 (18.23 %) and 19.68 (17.09 %) million were mapped to intergenic regions, respectively (Supplementary Dataset S2). All high-quality reads were assembled by the Cufflinks program and annotated via BLAST searches against the *C. sativus* database. As a result, 17,148 genes were predicted with annotations for each sample (Supplementary Dataset S3 and S4).

Functional category analysis by eggNOG

The functions of orthologous genes were classified with eggNOG. A total of 12,885 (75.14 %) genes were categorized into 25 groups (Fig. 3). "Function unknown" and "General function prediction only" represented the largest clusters in *C. sativus* species, containing 3528 (27.38 %) and 2041 (15.84 %) genes, respectively. These were followed by "Signal transduction mechanisms" (934 genes, 7.25 %), "Posttranslational modification, protein turnover, chaperones" (906 genes, 7.03 %), and "Transcription" (690 genes, 5.36 %) clusters that contained slightly fewer genes. "Extracellular structures" (10 genes, 0.08 %) and "Cell

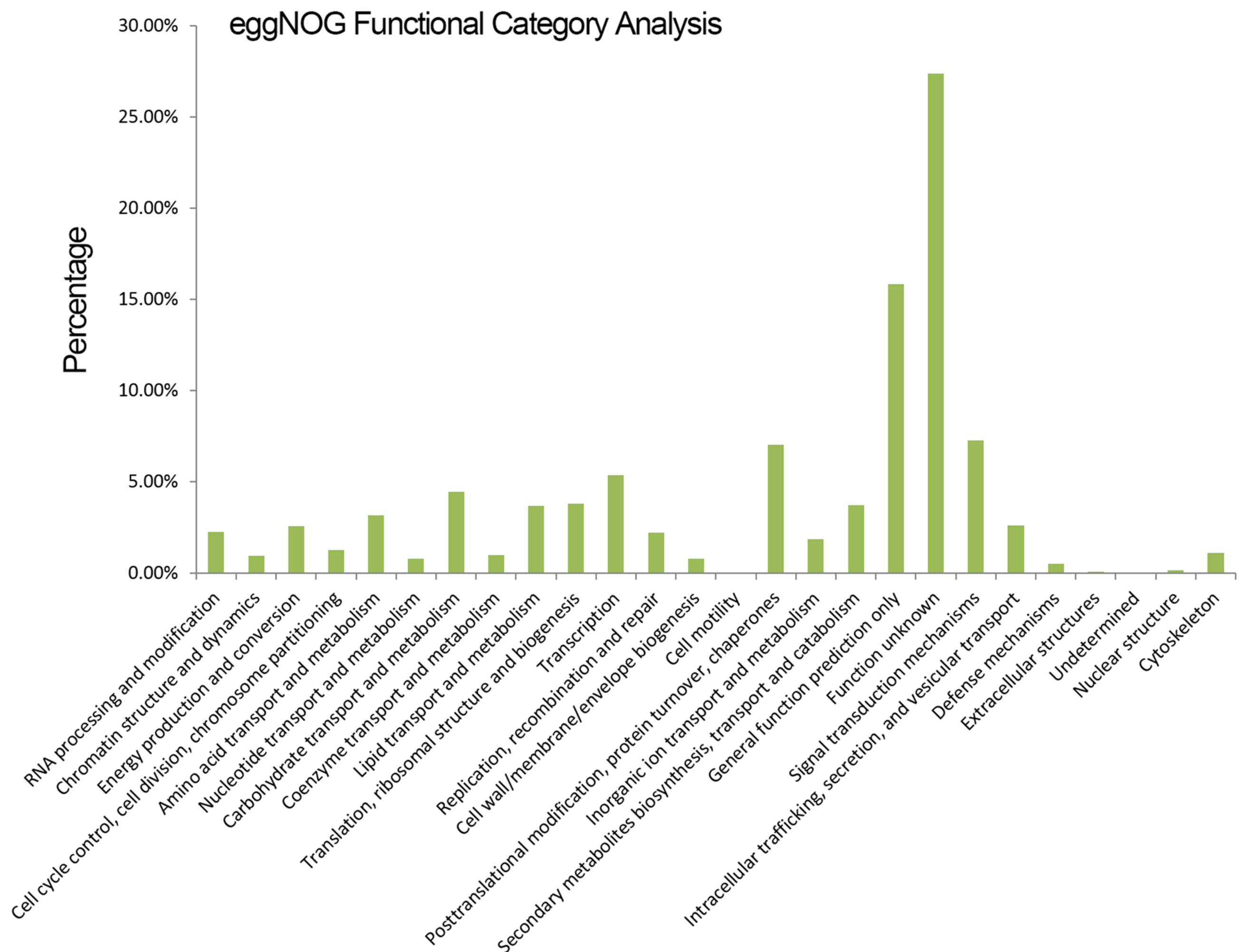

Fig. 3 eggNOG functional category analysis. A total of 12,885 (75.14 %) genes were categorized into 25 eggNOG. The details of eggNOG functional category analysis can be found in Supplementary Dataset S5

motility" (6 genes, 0.05 %) clusters had the least orthologous genes (Supplementary Dataset S5).

Differential expression and enrichment analyses

Gene expression levels were calculated by baseMean values. Differential gene expression was defined with the following statistical parameters: $P < 0.05$ with a fold change >2 or <−2. Following this analysis, a total of 991 genes were identified that exhibited differential expression, including 518 up-regulated genes and 473 down-regulated genes (Fig. 4, Supplementary Dataset S6).

The biological functions of these genes were determined by GO enrichment analysis. Among all 53 GO terms, "Thylakoid" ($P = 2.04\text{E}{-}30$; 71 up-regulated genes, 1 down-regulated), "Plastid" ($P = 5.32\text{E}{-}15$; 120 up-regulated genes, 4 down-regulated), and "Extracellular region" ($P = 8.09\text{E}{-}08$; 25 up-regulated genes, 10 down-regulated) were the top three significantly enriched clusters. "Sequence-specific DNA binding transcription factor activity" ($P = 2.25\text{E}{-}05$; 24 up-regulated genes, 34 down-regulated), "Response to biotic stimulus" ($P = 2.12\text{E}{-}04$; 24 up-regulated genes, 12 down-regulated), and "Response to external stimulus" ($P = 5.99\text{E}{-}04$; 27 up-regulated genes, 17 down-regulated) clusters were also significantly enriched (Fig. 5, Supplementary Dataset S7).

KEGG enrichment analysis was carried out to determine if trichome-related genes were involved in specific pathways. A total of 257 differentially expressed genes were classified into the 24 KEGG categories. The most enriched category was "Energy metabolism" ($P = 3.22\text{E}{-}12$; 46 differentially expressed genes), followed by "Biosynthesis of other secondary metabolites" ($P = 3.11\text{E}{-}03$; 14 differentially expressed genes) category (Fig. 6, Supplementary Dataset S8–S10).

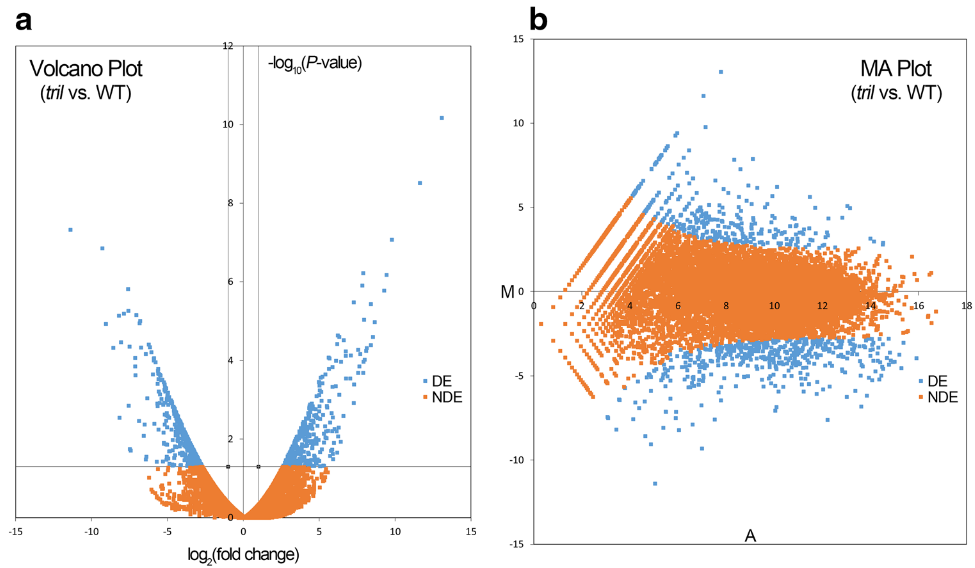

Fig. 4 Differential gene expression analysis. **a** Volcano plot of $\log_2$(fold change) versus $-\log_{10}(P\text{-value})$; the *horizontal line* represents $P = 0.05$. **b** MA plot of $\log_2$(fold change) versus baseMean fold change. *Genes in blue* are differentially expressed, and *genes in orange* show no significant difference in expression. The details of differential expression analysis can be found in Supplementary Dataset S6 (colour figure online)

Fig. 5 GO enrichment analysis of differentially expressed genes. The *red line* represents $P = 0.05$. The details of GO enrichment analysis can be found in Supplementary Dataset S7 (colour figure online)

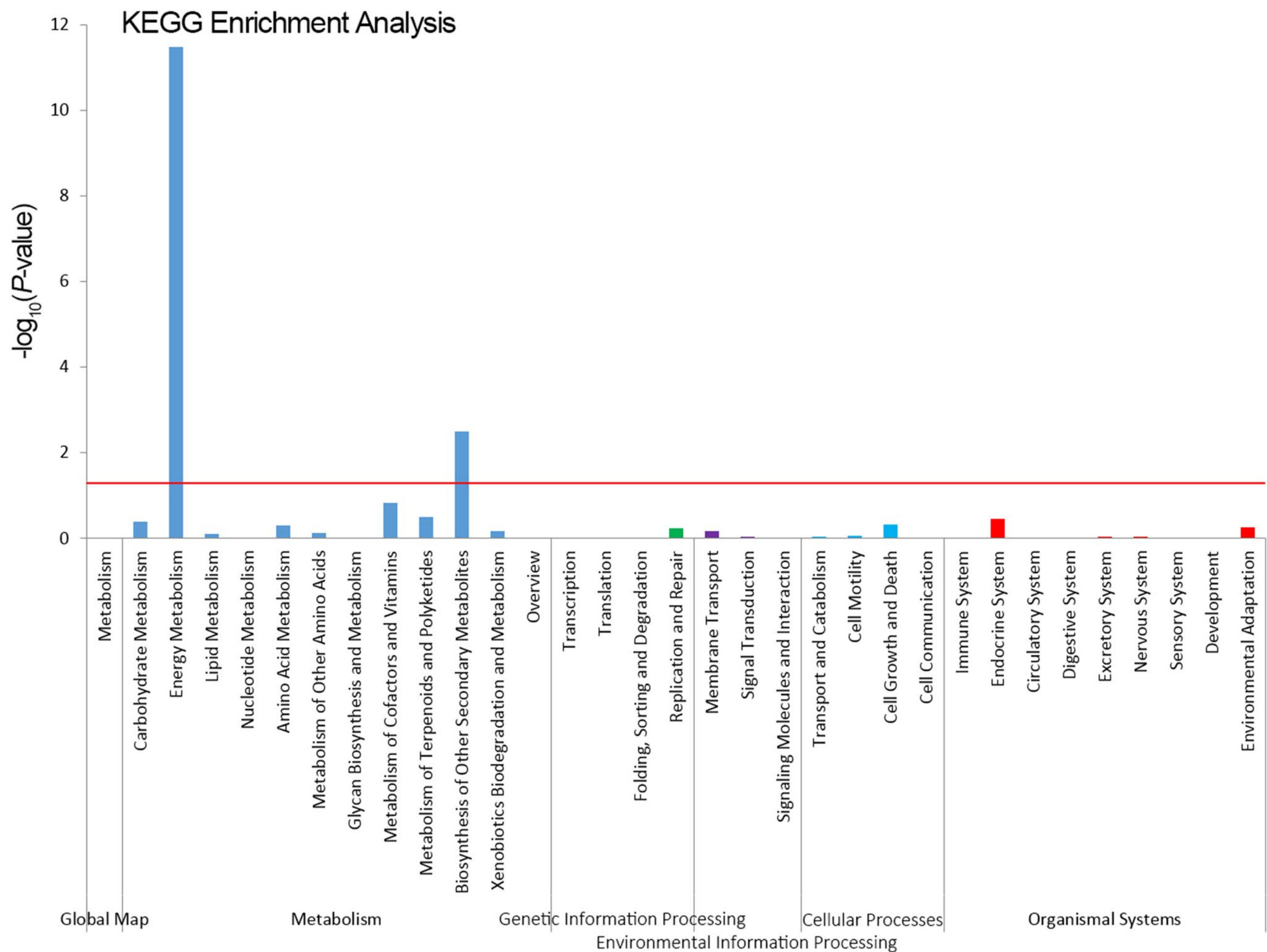

Fig. 6 KEGG enrichment analysis of differentially expressed genes. The *red line* represents $P = 0.05$. The details of KEGG enrichment analysis can be found in Supplementary Dataset S8–S10 (colour figure online)

Candidate genes associated with multicellular trichome development

A total of 62 differentially expressed genes were screened out. These genes encoded crucial transcription factors and were identified as candidates associated with multicellular trichome development. Twenty-two genes were not expressed at all (baseMean = 0) in the *tril* mutant, but were highly expressed in the wild-type background. The 62 genes were subdivided into seven categories according to the protein structure: homeodomain, MADS, MYB, and WRKY domains, ethylene-responsive, zinc finger, and other transcription factor genes (Table 1). For example, we identified a "*Homeodomain-leucine zipper protein ATHB51-like*" gene. The ATHB51 protein combines with LEAFY and together act as meristem regulators that induce *CAULIFLOWER* expression; the *ATHB51* gene also controls leaf morphogenesis, floral meristem determinacy, and bract formation in *A. thaliana* (Saddic et al. 2006). We also found a "*Homeodomain-leucine zipper protein GLABRA2-like*" gene. The *GLABRA2* gene regulates unicellular trichome and root hair development in *A. thaliana* (Pesch and Hülskamp 2009). Additionally, the 62 differentially expressed genes included a "*Floral homeotic protein APETALA1-like*" gene. The APETALA1 transcription factor acts as a MADS-domain protein and floral meristem regulator, and also interacts with LEAFY to regulate the flowering time genes *SVP* (*SHORT VEGETATIVE PHASE*) and *AGL24* (*AGAMOUS-LIKE24*) (Gregis et al. 2006, 2008; Pastore et al. 2011). Finally, a "*Transcription factor RAX2-like*" gene was identified. The *RAX2* (*REGULATOR OF AXILLARY MERISTEMS2*) gene belongs to the class R2R3 MYB family and regulates axillary meristem formation (Müller et al. 2006; Stracke et al. 2001).

Table 1 Candidate genes associated with multicellular trichome development

Gene ID	Gene description	baseMean (*tril*)	baseMean (WT)	Fold change	*P*-value
Homeodomain transcription factor genes					
101219997	*Homeobox protein knotted-1-like 2-like*	2.3758069	1470.294023	618.8609113	1.67E−06
101203403	*Homeobox protein knotted-1-like 1-like*	0	930.4452458	Infinity	1.94E−06
101213104	*Homeobox protein knotted-1-like 2-like*	0	875.1901356	Infinity	3.37E−06
101221983	*Homeobox-leucine zipper protein ATHB51-like*	0	518.2548263	Infinity	8.64E−05
101211566	*Homeobox protein SBH1-like*	0	389.3262359	Infinity	0.00011314
101221341	*Homeobox-leucine zipper protein GLABRA2-like*	0	422.3522788	Infinity	0.000124597
101216890	*Homeobox-leucine zipper protein ATHB21-like*	40.3887173	787.5440988	19.49911142	0.002982403
101210523	*Homeobox protein knotted-1-like 6-like*	19.0064552	366.4620524	19.2809258	0.011100434
MADS-domain transcription factor genes					
101220998	*MADS-box protein CMB1-like*	0	2273.716027	Infinity	4.41E−08
101212022	*MADS-box transcription factor 6-like*	0	836.4480469	Infinity	2.72E−06
101222239	*Floral homeotic protein PMADS 2-like*	0	817.3945606	Infinity	4.75E−06
101204197	*MADS-box transcription factor 8-like*	0	639.5620222	Infinity	1.11E−05
101224510	*Floral homeotic protein GLOBOSA-like*	0	620.5085359	Infinity	1.64E−05
101216798	*Agamous-like MADS-box protein AGL9 homolog*	0	577.9557499	Infinity	1.71E−05
101219414	*Floral homeotic protein APETALA1-like*	0	382.9750739	Infinity	7.85E−05
101211542	*MADS-box protein SOC1-like*	2.3758069	444.5813461	187.1285693	0.000205319
101223132	*MADS-box protein SVP-like*	137.7968002	4440.732531	32.22667381	0.000433855
101209501	*MADS-box protein SOC1-like*	30.8854897	654.8048112	21.20104999	0.004283364
101217987	*Agamous-like MADS-box protein AGL1-like*	4.7516138	206.4127678	43.44056073	0.006333491
101203152	*MADS-box protein SVP-like*	9.503227599	181.0081195	19.04701509	0.035138901
WRKY-domain transcription factor genes					
101219926	*WRKY transcription factor 75-like*	0	151.7927739	Infinity	5.45E−03
101212174	*WRKY transcription factor 51-like*	546.435587	31.75581044	0.058114463	6.79E−03
101207416	*WRKY transcription factor 51-like*	209.0710072	12.70232417	0.060756029	2.20E−02
101223628	*WRKY transcription factor 54-like*	1180.776029	134.6446362	0.114030631	3.31E−02
101203902	*WRKY transcription factor 65-like*	0	113.6858014	Infinity	3.46E−02
101211406	*WRKY transcription factor 40-like*	641.467863	76.21394504	0.11881179	4.35E−02
MYB-domain transcription factor genes					
101212616	*Transcription factor MYB76-like*	14.2548414	652.8994626	45.80194506	1.55E−03
101219416	*Transcription factor MYB86-like*	223.3258486	3.810697252	0.017063395	4.00E−03
101232352	*MYB-related protein B-like*	16.6306483	240.0739269	14.43563249	3.10E−02
101213982	*Transcription factor MYB3-like*	377.7532971	35.56650769	0.094152739	3.63E−02
Ethylene-responsive transcription factor genes					
101206564	*Ethylene-responsive transcription factor 1B-like*	1294.81476	12.06720797	0.00931964	4.60E−05
101207090	*Ethylene-responsive transcription factor WIN1-like*	0	395.677398	Infinity	2.96E−04
101209535	*Ethylene-responsive transcription factor ESR1-like*	0	291.5183398	Infinity	5.74E−04
101210326	*Ethylene-responsive transcription factor 1B-like*	712.74207	19.05348626	0.026732653	0.001181079
101215374	*Ethylene-responsive transcription factor CRF4-like*	477.5371869	14.6076728	0.030589603	4.95E−03
101212143	*AP2-like ethylene-responsive transcription factor ANT-like*	35.6371035	622.4138845	17.46533313	0.007322323
101220325	*Ethylene-responsive transcription factor 1B-like*	242.3323038	6.351162087	0.026208483	0.012273089
101204131	*Ethylene-responsive transcription factor RAP2-7-like*	7319.861058	735.4645697	0.100475209	1.95E−02
101227287	*Ethylene-responsive transcription factor 1A-like*	1653.561602	191.1699788	0.115611041	3.04E−02
101214089	*Ethylene-responsive transcription factor ERF104-like*	2988.76508	375.3536793	0.125588218	3.64E−02
101206124	*Ethylene-responsive transcription factor ERF114-like*	166.306483	9.526743131	0.057284256	4.52E−02

Table 1 continued

Gene ID	Gene description	baseMean (*tril*)	baseMean (WT)	Fold change	*P*-value
Zinc finger transcription factor genes					
101203630	*Zinc finger protein CONSTANS-LIKE 16-like*	2822.458597	130.833939	0.0463546	0.002987849
101219152	*Zinc finger protein CONSTANS-LIKE 2-like*	180.5613244	1.905348626	0.010552363	8.21E−03
101250621	*Zinc finger protein JACKDAW-like*	26.1338759	449.0271596	17.181805	1.05E−02
101219003	*Zinc finger protein CONSTANS-LIKE 6-like*	1653.561602	167.6706791	0.101399717	2.57E−02
101211656	*Zinc finger protein MAGPIE-like*	66.5225932	662.4262057	9.957913152	2.98E−02
Other transcription factor genes					
101205960	*PHD finger protein MALE STERILITY1-like*	0	663.6964381	Infinity	2.74E−05
101212392	*Transcription factor ABORTED MICROSPORES-like*	9.503227599	1011.105004	106.395958	7.46E−05
101229883	*Transcription factor bHLH041-like*	239.9564969	1.270232417	0.005293595	0.001408326
101215198	*Transcription factor UNE10-like*	4186.171758	189.8997464	0.045363582	0.002537778
101218813	*Transcription factor bHLH96-like*	85.52904839	1725.610739	20.17572709	0.002556556
101216625	*Transcription factor RAX2-like*	0	183.5485843	Infinity	3.61E−03
101204923	*Transcription factor HBP-1b(c1)-like*	0	168.9409115	Infinity	0.005979806
101206328	*AP2/ERF and B3 domain-containing transcription factor RAV1-like*	2663.279535	181.6432357	0.068202843	7.75E−03
101206841	*Transcription factor TCP4-like*	29871.02015	2428.684382	0.081305706	1.05E−02
101212507	*Nuclear transcription factor Y subunit A-9-like*	2.3758069	147.3469604	62.0197544	1.23E−02
101209353	*Transcription factor BEE3-like*	0	118.766731	Infinity	1.27E−02
101214530	*Transcription factor FAMA-like*	921.8130771	65.4169695	0.070965547	1.52E−02
101223651	*Transcription factor TCP4-like*	18730.8616	1911.699788	0.102061498	0.019264879
101218318	*NAC transcription factor 25-like*	0	127.0232417	Infinity	2.02E−02
101205864	*Transcription factor AIG1-like*	427.645242	29.2153456	0.068316779	0.02399868
101204885	*NAC transcription factor 25-like*	21.3822621	276.2755508	12.92078217	3.14E−02

Tissue-specific qRT-PCR validation of *GLABRA2-like* and *ATHB51-like* identified from the transcriptomic sequencing

Homeodomain-leucine zipper genes are unique to plants and participate in a wide variety of biological roles including trichome development (Ariel et al. 2007). Previous studies have shown that the class IV homeodomain-leucine zipper subfamily members, such as the *A. thaliana GLABRA2* gene and its homologs, e.g., the *Gossypium hirsutum* (cotton) *GaHOX1* gene and the *Solanum lycopersicum* (tomato) *Wo* (*Woolly*) gene, all regulate trichome development (Guan et al. 2008; Pesch and Hülskamp 2009; Yang et al. 2011); and the class I homeodomain-leucine zipper subfamily members have widely diverse roles that are specific to different tissues and organs in different species (Ariel et al. 2007), such as the *A. thaliana ATHB51* gene regulates floral meristem determinacy, bract formation, and leaf morphology (Saddic et al. 2006), and its homolog, e.g., the *Pisum sativum* (pea) *Tl* (*Tendril-less*) gene, regulates tendril formation (Hofer et al. 2009). These results implied that homologs in the class I homeodomain-leucine zipper subfamily have acquired distinct functions over evolution. In the present study, both the *GLABRA2* homolog, *GLABRA2-like*, and the *ATHB51* homolog, *ATHB51-like*, were not expressed at all in the *tril* mutant, but were highly expressed in the wild-type background, indicating that *GLABRA2-like* and *ATHB51-like* might be linked to multicellular trichome development in *C. sativus*.

We conducted qRT-PCR for *GLABRA2-like* and *ATHB51-like* using leaf samples (with trichomes attached to leaves; leaf trichomes could not be stripped perfectly from leaves) at the same developmental stage as those in the transcriptomic sequencing. There was good agreement between the two methods for the expression of these genes; neither *GLABRA2-like* nor *ATHB51-like* was expressed in the *tril* mutant, but both were highly expressed in the wild-type background (Fig. 7a, b; Table 2).

Second, tissue-specific qRT-PCR with total RNA extracted from detached trichomes and trichome-stripped epidermis of various tissues including branches, fruits, and stems was conducted to detect the spatial expression patterns of *GLABRA2-like* and *ATHB51-like*. The results showed that the expression level of *GLABRA2-like* in the epidermis was significantly higher than in trichomes in

these tissues (Fig. 7a). Conversely, *ATHB51-like* had a significantly higher expression level in trichomes compared with the epidermis (Fig. 7b). Neither of them was expressed in these tissues in the *tril* mutant (Fig. 7a, b). Given that trichomes develop from epidermal cells, these results indicated that both *GLABRA2-like* and *ATHB51-like* function in a tissue-specific manner in epidermal and trichome tissues, respectively.

Third, tissue-specific qRT-PCR with trichome-less tissues including cotyledons and roots was conducted. The results showed that neither *GLABRA2-like* nor *ATHB51-like* was expressed in the *tril* mutant, but both exhibited low-level expression in the wild-type background (Fig. 7a, b). In particular, *ATHB51-like* was barely expressed in the root tissues, which was in accordance with the previous results showing that *ATHB51-like* was expressed specifically in trichomes (Fig. 7b).

Thus, the wild-type and *tril* mutant transcriptomes represent a useful reference for further studies on multicellular trichome development.

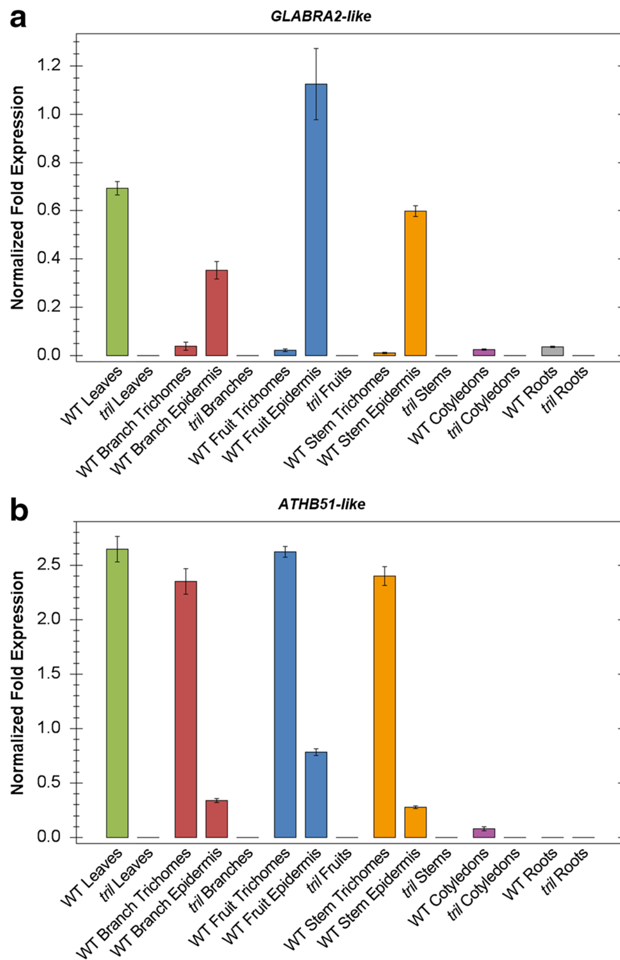

Fig. 7 Tissue-specific qRT-PCR validation of *GLABRA2-like* and *ATHB51-like* identified from the transcriptomic sequencing. The tissues examined included WT (wild-type) leaves (trichomes plus epidermis), *tril* leaves, WT branch trichomes, WT branch epidermis, *tril* branches, WT fruit trichomes, WT fruit epidermis, *tril* fruits, WT stem trichomes, WT stem epidermis, *tril* stems, WT cotyledons, *tril* cotyledons, WT roots, and *tril* roots. *CsActin3* was used as the reference gene to correct gene expression values. *Error bars* represent the standard deviation of three biological replicates

Discussion

To date, there have been few reports of regulatory genes that control multicellular trichome development in plants. The regulatory mechanisms of multicellular trichome development differ from that in species such as *A. thaliana* and *G. hirsutum*. It appears that both *A. thaliana* and *G. hirsutum* use similar genes to control unicellular trichome development. For example, the *G. hirsutum GaMYB2* gene controls cotton fiber development and is homologous to the *A. thaliana GL1* gene. The *GaMYB2* gene successfully rescued the trichome phenotype of the *A. thaliana gl1* mutant (Wang et al. 2004). Similarly, the *G. hirsutum GaHOX1* gene, which is homologous to the *A. thaliana GL2* gene, rescued the trichome phenotype of the *A. thaliana gl2* mutant (Guan et al. 2008).

However, plants with multicellular trichomes function with a different set of genes. For example, the *Antirrhinum majus MIXTA* gene, a MYB-like gene, regulates floral papillae development. This gene could not rescue the trichome phenotype of the *A. thaliana gl1* mutant. However, this gene regulates trichome differentiation in *Nicotiana tabacum* (tobacco) (Payne et al. 1999). The *S. lycopersicum Wo* gene is a class IV homeodomain-leucine zipper gene. This gene is homologous to the *A. thaliana GL2* and *PDF2* (*PROTODERMAL FACTOR2*) genes, but *Wo* has an additional role in embryo development and the homozygous mutant is embryo lethal (Yang et al. 2011). Thus, the research literature suggests that the developmental

Table 2 qRT-PCR validation of *GLABRA2-like* and *ATHB51-like* identified from transcriptomic sequencing

Gene ID	Gene description	*P*-value (transcriptome)	*P*-value (qRT-PCR)
101221341	*Homeobox-leucine zipper protein GLABRA2-like*	0.000124597	1.44E−05
101221983	*Homeobox-leucine zipper protein ATHB51-like*	8.64E−05	6.94E−06

processes of unicellular and multicellular trichomes are controlled by different regulatory genes. We concentrated on a new set of 62 transcription factor genes identified in our transcriptome analysis.

The genes identified as being associated with trichome development all appear to encode transcription factors. In this study, a total of 62 genes identified as candidates encoding crucial transcription factors associated with multicellular trichome development could be subdivided into seven categories: homeodomain, MADS, MYB, and WRKY domains, ethylene-responsive, zinc finger, and other transcription factor genes. In *A. thaliana*, responses to environmental conditions and developmental regulation of floral meristems, vascular systems, and lateral organs all involve homeodomain-leucine zipper transcription factor genes (Ariel et al. 2007; Baima et al. 2001; Henriksson et al. 2005; Otsuga et al. 2001; Williams et al. 2005). These transcription factor genes are unique to flowering plants and are involved in a range of activities; e.g., the *GLABRA2* gene regulates unicellular trichome and root hair development in *A. thaliana* (Pesch and Hülskamp 2009). We identified a "*Homeodomain-leucine zipper protein GLABRA2-like*" gene which was not expressed in the *tril* mutant, but expressed strongly in the wild-type epidermis tissues. The *A. thaliana ATHB51* gene regulates several different processes including floral meristem determinacy, bract formation, and leaf morphology (Saddic et al. 2006). The *P. sativum Tl* gene is homologous to the *A. thaliana ATHB51* gene and regulates leaf tendril formation (Hofer et al. 2009). However, we found that a "*Homeodomain-leucine zipper protein ATHB51-like*" gene was expressed specifically in the wild-type trichome tissues, but was not expressed in the *tril* mutant. It is likely that the *C. sativus ATHB51-like* gene is associated with multicellular trichome development or another species-specific developmental process.

The MADS-domain transcription factors, initially identified as floral meristem regulators, play important roles especially in flower and fruit development (Smaczniak et al. 2012). It has been reported that this family of proteins or genes can interact with or be regulated by homeodomain or MYB-domain transcription factors. For example, the *A. thaliana* APETALA1 protein, a MADS-domain transcription factor, interacts with LEAFY to regulate floral meristem and sepal development (Gregis et al. 2006; William et al. 2004); in addition, *APETALA1* is also regulated by LMI2 (LATE MERISTEM IDENTITY2), which belongs to the R2R3 MYB family (Pastore et al. 2011). In our analysis, an *APETALA1-like* gene and an R2R3 MYB *RAX2-like* gene exhibited significantly differential expression between the *tril* mutant and wild type.

In addition to transcriptional regulators, trichome development may also be regulated directly by phytohormones; e.g., salicylic and jasmonic acid decrease and increase the number of trichomes on leaves, respectively, in *A. thaliana* (Traw and Bergelson 2003). Ethylene gas can stimulate epidermal cell division in *C. sativus*, resulting in aberrant guard cell and trichome formation (Kazama et al. 2004). Cytokinin and gibberellin signals, which regulate inflorescence trichome initiation in *A. thaliana*, are integrated by *ZFP6* (*Zinc Finger Protein 6*), a new zinc finger transcription factor gene (Zhou et al. 2013). A series of ethylene-responsive and zinc finger genes were also identified in our transcriptome analysis. These genes may be associated with phytohormone-related regulation of multicellular trichome development in *C. sativus*.

In conclusion, the loss of *Tril* function led to the identification of a group of 62 candidate genes that appear to be associated with multicellular trichome development in *C. sativus*. Our approach and the transcriptome profiling of the *tril* mutant provide useful tools to study the relevant molecular networks of multicellular trichome development in plants.

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Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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