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TRANSCRIPTOMIC ANALYSIS OF NO TRICHOME MUTANT AND IDENTIFICATION OF NEW TRICHOME RELATED GENES IN *ARABIDOPSIS*

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Abstract

Trichome is a typical single cell structure formed by the development of epidermal cells through cell fate determination, cell specialization and morphogenesis. Trichome not only protects plants from being injured by drought, high salinity, heavy metals, ultraviolet rays, and pests, but also secretes many kinds of metabolites. A mutant with almost no trichomes (*ntm*) on its leaves, stems, and inflorescence was identified. qPCR results showed that the expressions of *GL1* and *GL2* were significantly decreased, but the expression of *GA1*, *GL3*, *TTG1*, *EGL3* and *SAD2* was enhanced in *ntm*. RNA-seq was performed to explore the reasons for the difference between *ntm* and WT to help elucidate the mechanism of trichome development. The results showed that there were 275 differentially expressed genes (DEGs), including 75 up-regulated and 200 down-regulated genes. GO analysis showed that DEGs were mainly enriched in lipid synthesis, plant cell wall, and redox. KEEG analysis showed that DEGs were mainly enriched in three pathways: cytochrome P450 (*ko00199*), synthesis of cutin, wax and suberine (*ko00073*), and plant hormone signal transduction (*ko04075*). The findings of this study will help to clone the sequence of *ntm* and further understand the development of trichome.

Key words: *Arabidopsis thaliana*, Trichome, No trichomes mutant, Transcriptome.

Introduction

Trichome is a specialized structure growing on the surface of plant tissues, including leaves, stems, flowers, seeds, and other organs. As the outermost barrier between plants and the environment, trichome can not only resist various biological and abiotic stresses, but also synthesize, store, and secrete many metabolites (Tiwari, 2016; Matías-Hernández *et al*., 2017; Champagne *et al*., 2017; Kariyat *et al*., 2018). The trichomes of some plants have the ability to absorb and store heavy metals and secrete salt to improve the ability of plants to resist heavy metals and salt. Cotton fiber is a kind of trichome deriving from the epidermal cells of ovule and an important raw material for the textile industry.

In *Arabidopsis*, about one cell in every three or four epidermal cells develops into trichome, which is regulated by the activation-inhibition mechanism. Epidermal cells express trichome promoting factors, which activate the expression of the suppressor. The suppressor's expression levels determine whether the cells eventually develop into trichomes or not. In this pathway, there are many positive regulatory factors, such as *GL1*, *WER*, *MYB23*, *GL3*, *EGL3* and *TTG1* (Oppenheimer *et al*., 1991; Payne *et al*., 2000; Humphries *et al*., 2005; Balkunde *et al*., 2010; Alahakoon *et al*., 2016), and negative regulatory factors, such as *CPC*, *TRY*, *ETC1*, *ETC2*, and *TCL1* (Schellmann *et al*., 2002; Tominaga *et al*., 2007; Ioannidi *et al*., 2015). GL1 and TTG1 can interact with GL3/EGL3 to form a complex, then activate the expression of *GL2* and *TTG2*, and finally promote the differentiation of epidermal cells into trichomes. Negative regulatory factors can shuttle between cells and compete with GL1 to bind GL3/EGL3, so the complex can not promote the expression of downstream activators *GL2* and *TTG2*. As a result, epidermal cells can not differentiate into trichomes. The *GL2*, *TTG2*, *SIM* and *RBR1* activated by the complex can inhibit the expression of some cell division related genes, such as *CYCD3;1* and *CYCB1;2*, so the cells stop dividing and enter the internal replication process and finally differentiate into trichome (Morohashi *et al*., 2009).

Recently, many new factors have been identified, which directly or indirectly regulate the key factors of trichome development, and thus affect its development. Acetyl transferase General Control Non-repressed protein5 (GCN5) regulates the expression level of *CPC*, *GL1*, *GL2* and *GL3* by influencing the H3K9/14 acetylation of them and affects the initiation of trichomes (Wang *et al*., 2019). The COP9 signaling complex subunit CSN5a enhances the expression of *MYB75* and inhibits the expression of *GL2*, regulating the production of trichome (Wei *et al*., 2018). Transcription factor TCP4 directly activates the expression of *GIS*, *TCL1* and *TCL2*, which inhibits the initiation and branching of trichome (Vadde *et al*., 2017; Vadde *et al*., 2019). *SAD2* coding β motif-containing input transporter is related to the transport of nuclear proteins, and the initiation of trichome on *sad2* is severely inhibited (Verslues *et al*., 2006; Zheng *et al*., 2020). Demethylase JMJ29 directly binds to *GL3* and demethylates its H3K9me2 to improve the expression of *GL3* and increase the density of trichome(Hung *et al*., 2020). Zinc finger protein plays an important role in regulating the development of trichome, such as GIS, GIS2, GIS3, ZFP5, ZFP6 and ZFP8 (Zhou *et al*., 2011; Sun *et al*., 2015; Kim *et al*., 2018).

Because of the complexity of trichome development and the diversity of biological functions, more genes

related to trichome development need to be identified and studied. In this study, a mutant without trichome was investigated and named no trichome mutant (*ntm*). It had almost no trichome on its leaves, inflorescences, and stems, and this trait was controlled by a single recessive gene. In addition, there was no difference in plant height, fertility and growth between *ntm* and wild-type seedlings (WT). In order to further study why the initiation of trichome on the surface of *ntm* was completely inhibited, this study analyzed the transcriptome of *ntm* and WT, which lays a foundation for cloning *ntm* and revealing its mechanism for regulating trichome development.

Materials and Methods

Plant materials: The seeds of *Arabidopsis thaliana* (Col-0) were washed with 10% NaClO solution for 10 minutes, washed with sterilized water 5 times. Seeds were resuspended with 0.2% agar and sown on ½ MS medium, and then treated under 4℃ for 2 days. The seeds were cultured at 22^{o}C in a culture room for two weeks. Ten-day-old seedlings were transferred to soil. Two weeks later, every fifth plant was harvested as a repeat for mRNA sequencing, and 3 repeats were set for each sample. To investigate the genetic pattern, *ntm* used as female parent was crossed with WT. Seedlings of F1 were self-crossed and offspring of F2 were generated, and then the separation ratio was counted.

qRT-PCR: The total RNA of *ntm* and WT was extracted using TRIzol according to the manufacturer's directions, and then the total RNA was detected by gel electrophoresis. The M-MLV kit was used to generate the first strand of cDNA according to the manufacturer's instruction. The real-time PCR was performed using gene specific primers and *ACTIN* as internal control (Table 1). The PCR parameters was shown as follows 94℃ for 1 minute, 95℃ for 15 seconds, 60℃ for 20 seconds, 72℃ for 30 seconds and 45 cycles. Three repeats were set for each experiment, and the results were analyzed by the $2^{-\triangle CT}$ method.

RNA extraction, library construction and sequencing: Total RNA was extracted using a plant RNA extraction kit, mRNA was fragmented, reverse transcribed and spliced. The library was tested by gel electrophoresis and Agilent Bioanalyzer. Real-time PCR was used for quantitative detection of the library. After passing the library inspection, different libraries were pooled according to the requirements for concentration, and then the library was sequenced using Illumina novaseq 6000.

Data processing: Fastp software was used to filter raw data; remove primers, adaptors, sequences less than 50 bp, reads with a certain proportion of N bases (5 bp by default) and low-quality bases with mass value less than 20; calculate the average mass value of bases with 4 bases. The filtered data was aligned to the rRNA database using Bowtie2 software, and then the rRNA sequence were removed from the sequencing data. Hisat2 was used to compare the similarity between clean data and the reference genome, obtain the location information of reads on the reference genome and the characterization of samples, and generate BAM files. RseQC software was used to evaluate the distribution and quality of transcriptome data, such as saturation, RNA degradation and redundant sequence.

GO and KEGG enrichment analysis of DEGs: After quality control, the sequences were compared with the reference genome using HISAT2. Stringtie was used to detect the expression of known genes, new genes, and transcripts. Differential expression was analyzed using Deseq2 or edgeR. DEGs require genes or transcripts whose sum of mapping reads ≥ 10 in two samples. The expression fold change was calculated. If | log2 (fold change) | > 1 was met, it was defined as a DEG. The p value was corrected by FDR to obtain the Q value, which meets the requirements of P value ≤ 0.05 and Q value ≤0.05.

GO and KEGG enrichment analysis of DEGs were performed using R package Cluster profiler software. Enrichment analysis was carried out for all DEGs, both up-regulated and down-regulated. According to the results of GO enrichment analysis, DEGs were classified into cellular component (CC), molecular function (MF) and biological process (BP). The 10 GO functions with the most significant enrichment (p-value) were selected from the three categories of BP, CC, and MF respectively and displayed in figures. If there were less than 10 GO functions, they were all listed. In this report, the R software package cluster Profiler was used for KEGG enrichment analysis.

Table 1. Sequence of primers for RT-qPCR amplification used in this study.

Primer name	Primer sequence(5'-3')	Primer name	Primer sequence(5'-3')
qPCR-*GL1*F	CAATGGAACCGCATCGTCAG	qPCR-*GA1*F	TGAAGAACAATGACCTAACTAACT
qPCR-*GL1*R	TGATGAACAATGACGGTGGA	qPCR-*GA1*R	CTCTCCGACAATGCTAACTC
qPCR-*GL2*F	CTCAGTGGCAATCCAGACAG	qPCR-*SAD2*F	CACCCAAGTTCCAACCTTACTC
qPCR-*GL2*R	ACTCTACTCCATCAGGTATG	qPCR-*SAD2*R	TACTTATGGCACGCAAACAACC
qPCR-*GL3*F	CGCAGGAGAAAGAACATCAG	qPCR-*ACTIN*F	ATCCTTGTATGCTAGCGGTCGA
qPCR-*GL3*R	CGAGGATTGAACCGAATGAG	qPCR-*ACTIN*R	ATCCAACCGGAGGATAGCATG
qPCR-*TTG1*F	TATTCGTTCGCCGACTATGCC	*GL1*-F	TCTAGAATGAGAATAAGGAGAAGAG
qPCR-*TTG1*R	ATTGGGTCCAGCAACAGTAGG	*GL1*-R	GGTACCAAGGCAGTACTCAATATC
qPCR-*EGL3*F	TCGGTTATGCTGGTCTAACG		
qPCR-*EGL3*R	CCATGCAACCCTTTGAAGTG		

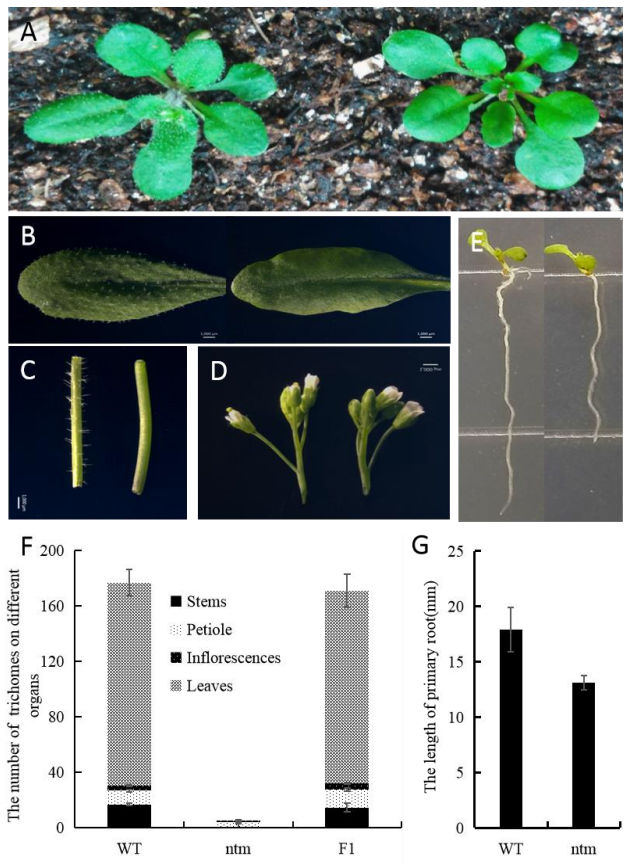

Fig. 1. Phenotype of WT and *ntm*
A. Three-week-old seedling growing in soil; B. Rosette leaf, C. Stem; D. Inflorescence; E. Four-day-old seedling after germination; F. The statistical analysis of trichomes of WT and *ntm*, n≥12; G. The statistical analysis of primary roots of four-week-old seedlings, n≥20.

Results

Phenotypic identification of *ntm*: There was no significant difference in plant height, leaf size and flower size between *ntm* and WT plants, but the trichomes on the surfaces of all organs of *ntm* could not be observed (Figs. 1A-D). However, the *ntm* primary root was shorter than the WTs (Figs. 1E and G). As shown in the figure 1, the main stem of WT has 17 trichomes per cm, while the stem of *ntm* has no trichome (Figs. 1C and F). The petiole of the mutant *ntm* had a small amount of trichomes, which was significantly less than for WT. The number of trichome on rosette leaves and inflorescence of WT was dense, while there were no trichomes on the leaves and inflorescence of *ntm* at all.

Genetic analysis of *ntm*: In order to clarify the inheritance pattern of *ntm*, F1 was generated by crossing Col-0 to *ntm*. The results showed that the number of trichome on leaves, stems and flowers of F1 generation was the same as that of WT (Fig. 1F). The F2 generation was separated, with 26 lines without trichome and 94 lines with normal trichome. It was checked by chi square and the separation ratio was nearly 3:1. The results showed that the trait of *ntm* was controlled by a single recessive gene.

To further study the reason for trichome defects, we detected the expression of several genes related to trichome development by qPCR. The results showed that the expression of *GL1* and *GL2* genes in *ntm* was lower than that in WT. Compared with the WT, the expression of *GL1* and *GL2* in *ntm* decreased by 5 to 6 times. The expression level of *GA1*, *GL3*, *TTG1*, *EGL3* and *SAD2* in *ntm* was higher than that in WT (Fig. 2). Previous studies have shown that mutations of *GL1*, *TTG1*, *SAD2*, *GA1* or double mutations of *GL3* and *EGL3* can lead to trichome loss. We cloned *GL1* from *ntm* and WT respectively followed by sequencing. The results showed that there was no difference in the sequences of *GL1* between *ntm* and WT.

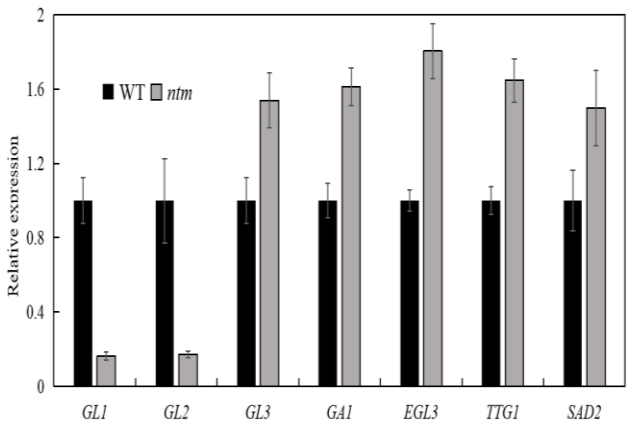

Fig. 2. Expression pattern of trichome-related genes in WT and *ntm*.

Quality analysis of recorded data: To study the trichome defects of *ntm* further, the transcriptomes of *ntm* and WT were analyzed by RNA SEQ. The original reading length was > 38572232, the maximum 50878430, and the original fragment q30 was between 0.91-0.93. The original base 5321935800 and q30 obtained by *ntm*-2 sequencing were 4908033343. The original base and q30 obtained by sequencing of other samples were greater than this value. After removing the low-quality data, sequencing connectors and rRNA sequences that affect the data quality and subsequent analysis, the filtered data reads were at least 33624646, q30 was 0.937098, the net base number was 4995900722, q30 was 4681646576, and other sample data were better than this sample. More than 93% of the fragments in each sample were located on the genome, and more than 72% of the single genes were located on the genome (Table 2).

Screening and analysis of DEGs: Analysis of transcriptome showed that there were 275 DEGs between *ntm* and WT, of which 75 were up-regulated with a LogFC value between 4.2-1.0 and 200 were down-regulated genes with LogFC value between 13-1.0. Among the up-regulated genes, there were 6 genes with LogFC > 2, such as *At1G53480*, *At3G01345*, *At1G12805*, *At3G06620*, *At3G09870*, and *At4G33560*. Among the down-regulated genes, 85 genes had a LogFC value > 2, among which 10 genes had a LogFC value > 10, such as *At3G07820*, *At5G39880*, *At5G45880*, *At2G47030*, *At3G01230*, *At2G22055*, *At5G60615*, *At5G45890*, *At3G05610* and

At1G55560. Compared with the WT, the expression of multiple genes changed in *ntm*, the number of down-regulated genes was significantly more than that of up-regulated genes, and the change fold of the expression level of down-regulated genes was much greater than that of up-regulated genes (Fig. 3 and Table 3).

GO analysis of DEGs: GO analysis of 275 differential genes showed that 182 differential genes were enriched in 137 entries. As shown in the figure, the down regulation of DEGs in biological processes is mainly related to fatty acid synthesis, metabolism and cellular anaerobic reaction. Among the cell component related genes, the down-regulated DEGs are mainly involved cell membrane, endoplasmic reticulum, lipid, mediator complex and axon. Down-regulated DEGs related to molecular function mainly involve oxidoreductase, lipase, dehydrogenase, amino acid ligase and sodium transporter (Fig. 4A). Among the up-regulated DEGs, the genes related to biological processes are mainly involved in plant flower organ development, vernalization, low temperature response, terpene catalysis, ABA catalysis and metabolism. Genes related to cell components mainly involve a variety of organelles, including vacuole, endoplasmic reticulum, peroxisome, microsome, ribosome, chloroplast, plastid and nucleolus. Genes related to molecular function are mainly involved in glycosyl transfer, nitrate transport, etc (Figs. 4B and C). The results showed great differences in the biological process of gene enrichment between up-regulated and down-regulated expression.

Table 2. Statistics of sequencing data by RNA-Seq.

Sample	Raw reads	Q30 of raw reads	Raw bases	Q30 of raw bases	Clean bases	Q30 of clean bases	Clean reads	Q30 of clean read	Mapping ratio	Uniquely mapping ratio
WT_1	50878430	0.920289	7631764500	7023431277	7160348046	6688309131	47822644	0.934076	93.48%	72.96%
WT_2	38572232	0.917892	5785834800	5310769654	5388918171	5036679418	36332324	0.934636	96.29%	89.04%
WT_3	40717476	0.926732	6107621400	5660126907	5712367798	5376399948	38369882	0.941186	95.40%	81.00%
ntm_1	39672354	0.92511	5950853100	5505192773	5570854336	5234247257	37507848	0.939577	96.51%	88.58%
ntm_2	35479572	0.922227	5321935800	4908033343	4995900722	4681646576	33624646	0.937098	95.67%	87.24%
ntm_3	38819836	0.927215	5822975400	5399150730	5549736009	5208640467	37366160	0.938538	96.63%	89.37%

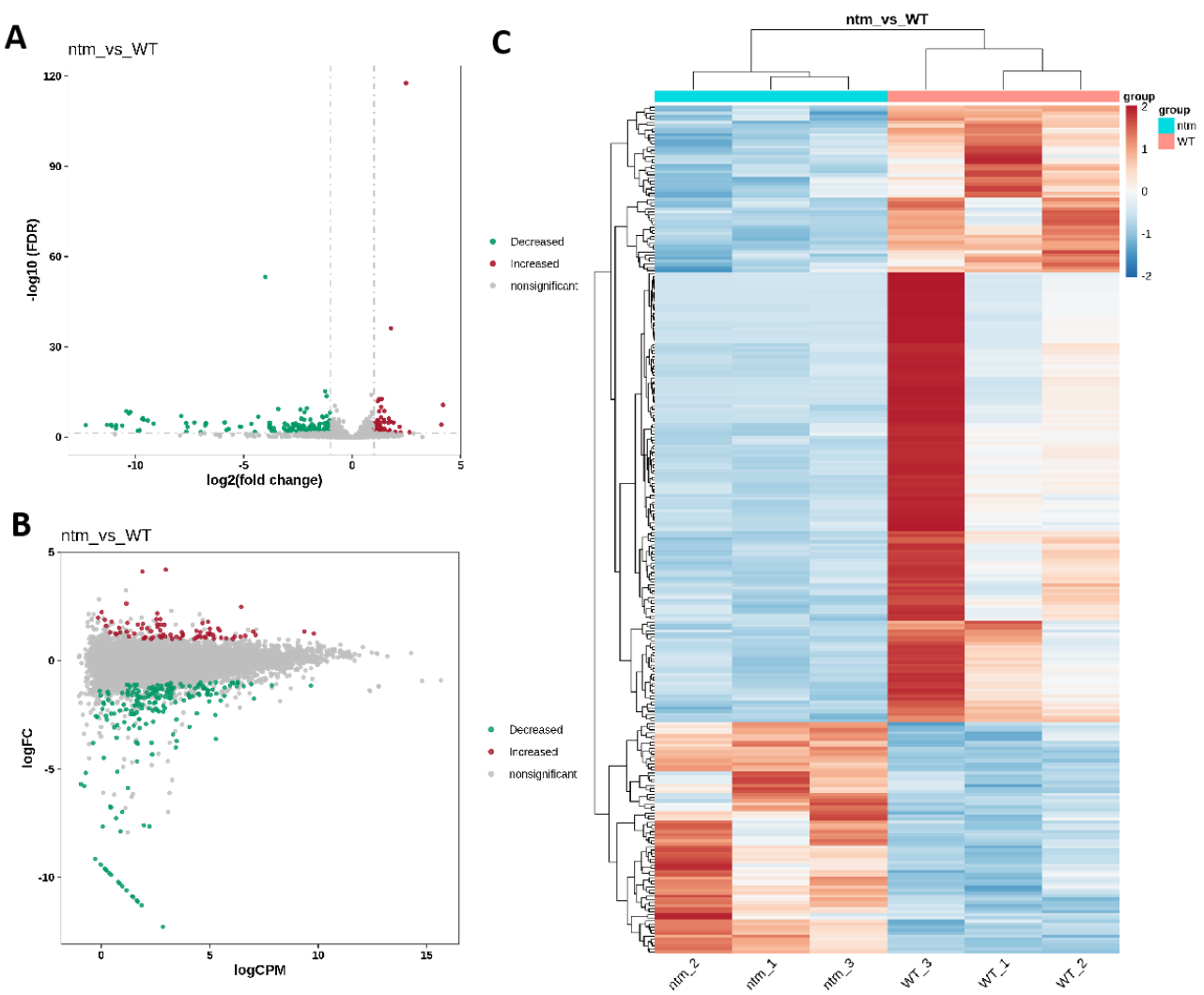

Fig. 3. Analysis of DEGs in transcriptome of WT and *ntm*
A, Volcano plot of DEGs; B, M-A plot of DEGs; C, Heat map of DEGs

Table 3. Part of DEGs of *ntm* versus WT.

Gene_id	LogFC	LfcSE	Pvalue	FDR	Level	Description
AT1G53480	4.196466851	0.54320802	1.12E-14	2.15E-11	Increased	Mto 1 responding down 1
AT3G01345	4.109763372	0.806214762	3.44E-07	7.13E-05	Increased	Expressed protein
AT1G12805	2.635169678	0.775918788	6.83E-04	0.027315089	Increased	Nucleotide binding protein
AT3G06620	2.48260101	0.105389955	1.08E-122	2.08E-118	Increased	PAS domain-containing tyrosine kinase family protein
AT3G09870	2.234766625	0.670418212	8.58E-04	0.0320831	Increased	SAUR-like auxin-responsive protein family/F8A24.8 protein
AT4G33560	2.182322071	0.464274078	2.60E-06	3.82E-04	Increased	WOUND-INDUCED POLYPEPTIDE 5
AT3G07820	-12.28590488	2.452658522	5.47E-07	1.03E-04	Decreased	Pectin lyase-like superfamily protein
AT5G39880	-11.29841463	2.240209331	4.57E-07	9.08E-05	Decreased	Transmembrane protein
AT5G45880	-11.12309854	2.190896496	3.84E-07	7.86E-05	Decreased	Pollen Ole e 1 allergen and extensin family protein
AT2G47030	-11.07769796	2.289118276	1.30E-06	2.11E-04	Decreased	Pectinesterase 4
AT3G01230	-10.89010508	2.512491868	1.46E-05	0.001459263	Decreased	Splicing regulatory glutamine/lysine-rich-like protein
AT2G22055	-10.88032474	2.177053327	5.80E-07	1.07E-04	Decreased	Protein RALF-like 15
AT5G60615	-10.60271217	2.158345044	9.00E-07	1.55E-04	Decreased	Putative defensin-like protein 274
AT5G45890	-10.41299307	1.488218454	2.62E-12	2.68E-09	Decreased	Senescence-specific cysteine protease SAG12
AT3G05610	-10.29313321	1.526412591	1.55E-11	1.24E-08	Decreased	Probable pectinesterase/pectinesterase inhibitor 21
AT1G55560	-10.22487255	1.476155854	4.31E-12	4.15E-09	Decreased	SKU5 similar 14/Sks14
AT3G54800	-9.886400988	2.582435291	1.29E-04	0.008257573	Decreased	Pleckstrin homology and lipid-binding START domains-containing protein
AT3G26110	-9.80407378	2.479435725	7.68E-05	0.00556516	Decreased	Anther-specific protein agp1-like protein
AT3G62710	-9.683044477	1.60498504	1.61E-09	8.61E-07	Decreased	Beta-D-glucan exohydrolase-like protein
AT1G05580	-9.652469368	1.583316427	1.09E-09	6.34E-07	Decreased	Cation/H(+) antiporter 23, chloroplastic
AT5G48140	-9.601492359	1.626275155	3.55E-09	1.67E-06	Decreased	Pectin lyase-like superfamily protein
AT1G01980	-9.416873096	1.629287416	7.48E-09	3.13E-06	Decreased	Berberine bridge enzyme-like 1
AT3G20580	-9.155975211	1.747856091	1.62E-07	3.72E-05	Decreased	COBRA-like protein 10
AT1G24520	-7.884125963	1.22873255	1.39E-10	9.95E-08	Decreased	Homolog of Brassica campestris pollen protein 1/BCP1
AT2G24450	-7.658649239	1.428538594	8.27E-08	2.07E-05	Decreased	Fasciclin-like arabinogalactan protein 3/FLA3
AT3G49540	-7.65267139	2.125062288	3.17E-04	0.015895227	Decreased	hypothetical protein
AT3G01240	-7.598404426	1.631258407	3.19E-06	4.36E-04	Decreased	Splicing regulatory glutamine/lysine-rich-like protein
AT5G47000	-7.27635364	1.330991244	4.58E-08	1.40E-05	Decreased	Peroxidase 65
AT5G50030	-6.986941186	2.024504868	5.58E-04	0.023897907	Decreased	Plant invertase/pectin methylesterase inhibitor
AT4G03290	-6.781269566	1.365957693	6.89E-07	1.23E-04	Decreased	Calmodulin-like protein 6
AT2G36020	-6.740326411	1.26431228	9.76E-08	2.41E-05	Decreased	HVA22-like protein

Note: gene_ID: DEGs; LogFC: represents the ratio of the expression value between the two samples. After taking the logarithm based on 2, it is log2FC. By default, the absolute value of Log2FC is greater than 1 as the screening standard of differential genes. LogCPM: Log 2 (CPM) is taken as the base logarithm of CPM (count per million), which is standardized. pvalue: Statistical significance level. FDR: false discovery rate, which is obtained by correcting the difference significance p-value. Level: increased means up-regulation and decreased means down-regulation

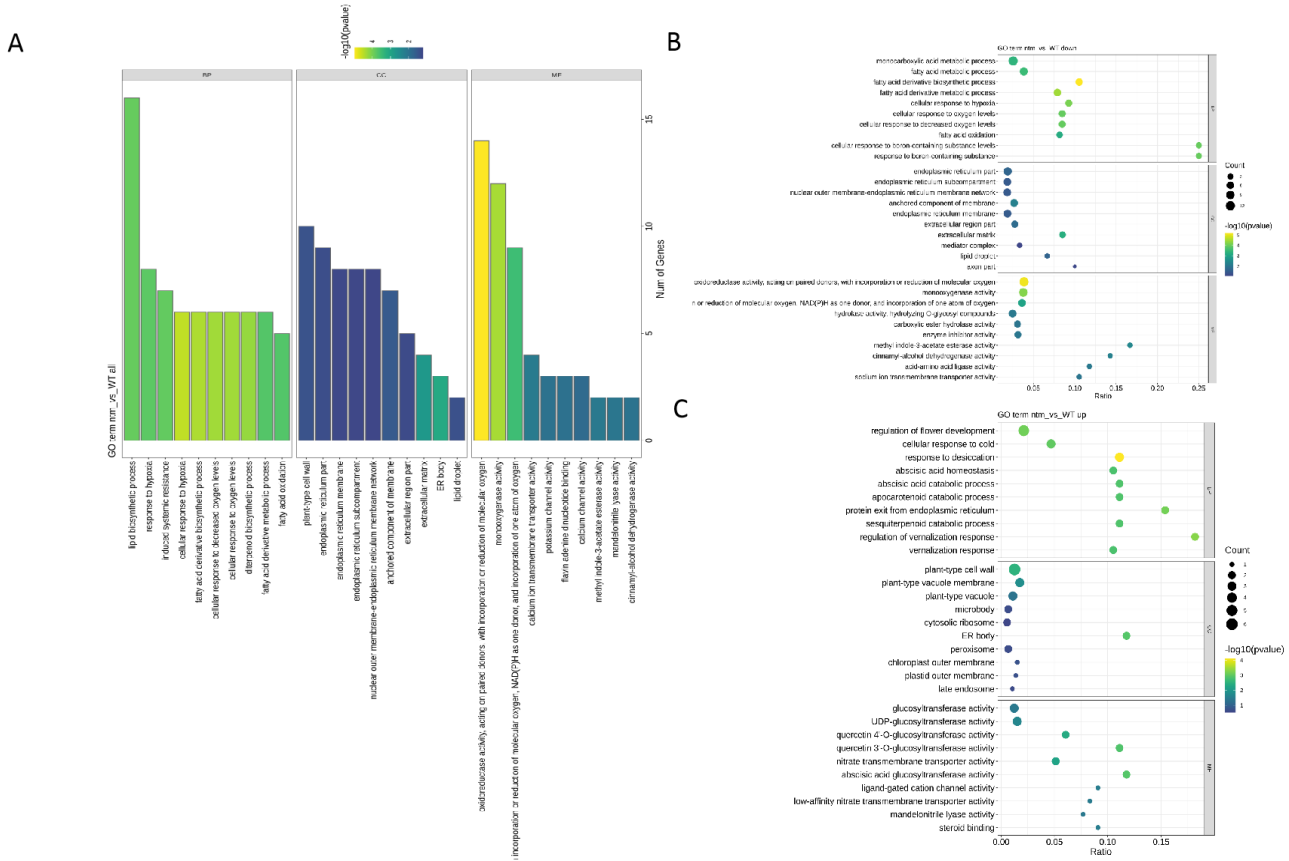

Fig. 4. GO functional annotation of DEGs
A, GO functional annotation of DEGs; B, GO enrichment of down-regulated DEGs; C: GO enrichment of up-regulated DEGs.

KEGG analysis of DEGs: The main biochemical metabolic pathway and signal transduction pathway in which DEGs are mainly involved can be determined through enrichment analysis of pathway. According to the results of the KEGG analysis, DEGs in *ntm* are mainly enriched in cytochrome P450, plant hormone signal transduction, pentose and glucuronic acid conversion, and biosynthesis of various substances, such as cutin, wax, phenylpropane, diterpenes, isoflavones, steroids, and other biological pathways. Among the biological pathways of differential gene enrichment, there are at most 13 differential genes related to cytochrome P450, 10 related to keratin and wax synthesis, and 11 related to plant hormone signal transduction. Down-regulated genes were mainly enriched in four pathways: cytochrome P450 (11), cutin wax synthesis (9), phenylpropanoid synthesis (7) and pentose and glucuronic acid conversion (7) (Fig. 5A). There are a few up-regulated genes, mainly concentrated in phytohormone signal transduction (4) and cytochrome P450 (2). Only one gene in other pathways is enriched (Fig. 5B).

Discussion

The development of trichome is a complex process, controlled by many positive and negative factors, and their expression patterns are not consistently the same. In *sad2*, the expressions of *GL1*, *MYB23*, *GL2* and *TTG1* decreased, but the expressions of *GL3* and *EGL3* increased (Gao *et al*., 2008). Additionally, the transcription factor TPR negatively regulates *MYB23*, *GL1*, *TTG1*, *GL3*, *EGL3*, *GIS*, *ZFP8* and *GIS2*, but can not affect *ZFP5*, *ZFP6* and *GIS3* (Kim *et al*., 2018). In *ttg1-13*, the expression of positive regulatory factors *GL2*, *SPL8*, *MYC2* and *BLT* decreased, but the expression of negative regulatory factors *ETC1* and *CPC* decreased (Wei *et al*., 2019). There are many factors controlling the initiation of trichome, but only single mutations of *GL1*, *TTG1*, *GA1* and *SAD2* and double mutations of *GL3* and *EGL3* can lead to the complete absence of trichome. In this study, the expression levels of *GA1*, *SAD2*, *TTG1*, *GL3* and *EGL3* were slightly up-regulated in *ntm*, while *GL1* and *GL2* were significantly down-regulated (Fig. 2). The deletion of *GA1* affects the seed germination of *Arabidopsis thaliana*, resulting in dwarf plants inconsistent with the *ntm* phenotype. At the same time, the coding sequence of *GL1* shows no difference between *ntm* and WT. Therefore, it is speculated that *NTM* may be a new gene controlling trichome initiation in *Arabidopsis*, which may inhibit trichome initiation by inhibiting *GL1* expression.

Known as a mini factory of natural plant products, trichome can synthesize, store and secrete organic acids, phenols, polysaccharides, alkaloids, proteins, terpenoids and other metabolites. Cytochrome P450 is a kind of monooxygenase widely existing in animals, plants, and microorganisms. In plants, cytochrome P450 mainly catalyzes the synthesis and metabolization of plant hormones, alkaloids, terpenoids, sterols, signal molecules, pigments, fatty acids, flavonoids and isoflavones, and

often binds to membranes of endoplasmic reticulum, mitochondria, plastids, and golgi. KEGG analysis showed that thirteen DEGs encoding cytochrome P450 were found in *ntm*, making it the most obvious enrichment pathway. At the same time, multiple genes involved in biosynthesis of metabolites, such as cutin, wax, phenylpropane, diterpenes, isoflavones and steroids were differentially expressed in *ntm*, including 10 genes involved in wax and cutin synthesis (Fig. 5). The mutation of *TTG1*, a key gene in trichome development, affects the development of trichome and the accumulation of flavonoids (Wei *et al*., 2019). *GL2* can also affect the synthesis of anthocyanins and procyanidins (Ioannidi *et al*., 2016). Transcription factors *TCP14* and *TCP15* can regulate the branches of trichome, as well as the expression of keratin and wax synthesis related-genes, such as *CYP86A4*, *GPAT6*, *CUS2*, *SHN1*, and *SHN2* (Camoirano *et al*., 2020). GO analysis shows that multiple DEGs are involved in cell components such as cell membrane, vacuole, endoplasmic reticulum, peroxisome, chloroplast, and plastid, which are closely related to material synthesis, metabolism, and transportation in plants (Fig. 4). In conclusion, the development of trichome is related to the synthesis and secretion of metabolites in plants. The change of key genes related to trichome development also affects the expression of some metabolites related genes, and, finally, interferes with the synthesis and metabolism of these compounds. Then, it is worth considering whether the yield of Artemisinin can be improved by changing the number of trichome on *Artemisia annua*, and how to balance the relationship between metabolite content and growth.

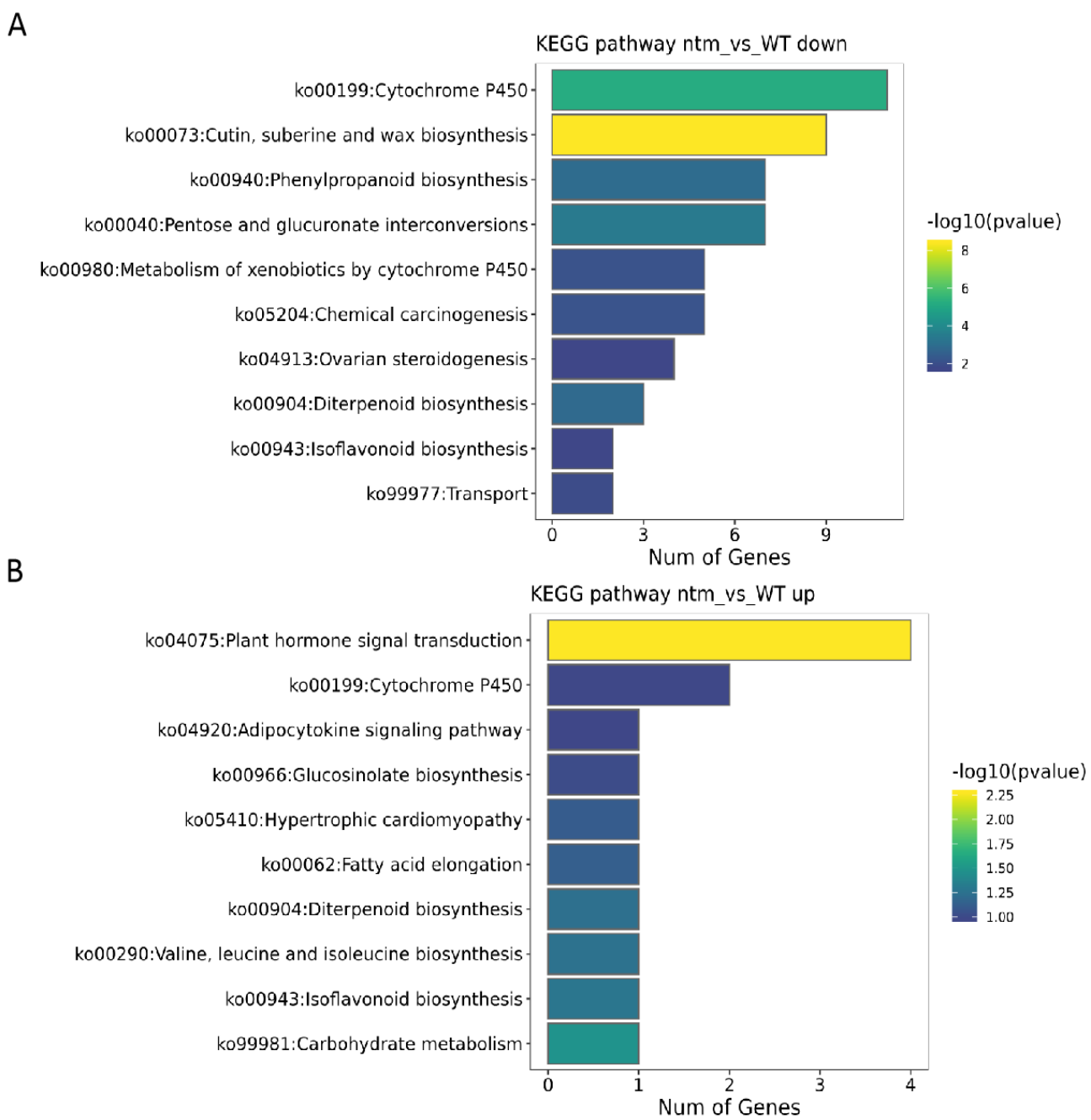

Fig. 5. KEGG enrich analysis of DEGs
A, Bar chart of KEGG enrichment analysis of down-regulated DEGs; Bar chart of KEGG enrichment analysis of up- regulated DEGs .

Plant hormones play a key role in the development of trichome. Recent studies have shown that JAZs can regulate the initiation of trichome by interaction with the C-terminal of *GL3/EGL3/TT8* and affecting its stability (Qi *et al*., 2011). Meanwhile, JA and GA can induce the expression of *GL1* and *GL3*, and GA can also enhance the stability of GL1 protein. DELLAs and JAZs competed to bind EGL3 and regulated the development of trichome (Tian *et al*., 2016). DELLA protein RGA can interact with GL1 and EGL3, the key factors of trichome development, inhibit its transcriptional activity, and affect the development of trichome. The expression of *ZFP5*, *GL1*, *GL3*, *TTG1* and *TRY*, important factors in trichome formation, is regulated by gibberellin. Our results show that two gibberellin related genes, *At1G74670* (*GASA6*) and *At1G75750* (*GASA1*), are differentially expressed in *ntm*. *GASA* is a kind of GA-regulated small peptides with conserved N-terminal and C-terminal and variable intermediate peptides. *GhGASA1* is negatively regulated the response of cotton to salt stress by regulating the expression gibberellic acid and polyamine related to decrease the content of them (Gu *et al*., 2021). Overexpression of *GASA6* leads to early flowering of *Arabidopsis*, and can also promote cell elongation, thereby regulating seed germination (Qu *et al*., 2016; Zhong *et al*., 2021). Therefore, GA induced cell elongation plays an important role in the initiation of trichome. Compared with JA and GA, there are few studies on the regulation of trichome development by auxin. In tomato, auxin treatment can increase the expression of *SlARF4*, and then enhance the expression level of downstream *SlTHM1* and *SlMYB52*, which leads to the increase of the expression of *SlCYCB2* and increases the density of trichome (Yuan *et al*., 2012). In this study, 13 genes involved in phytohormone signal transduction were differentially expressed, of which 9 genes were related to auxin, including 4 SAUR genes, such as *AT5G50760 (SAUR55)*, *AT3G09870 (SAUR48)*, *AT4G34810 (SAUR5)*, *AT5G18010 (SAUR19)*, *AT5G13320 (PBS3/GH3.12)*, *AT4G32280 (IAA29)*, *AT4G37390 (GH3.2/YDK1)*, and *AT2G17500 (PILS5*). SAURs, such as *SAUR19* and *SAUR41*, are growth factors that can regulate cell expansion and growth (Spartz *et al*., 2012; Kong *et al*., 2013). By this means, plants can respond to the environment, including the elongation of hypocotyls, roots and stems. Meanwhile, *PILS5*, *GH3.2/YDK1* and *IAA29* are important factors in auxin signal transduction and regulate cell elongation (Takase *et al*., 2004; Sun *et al*., 2020). To sum up, the trichome of *Arabidopsis* belongs to the single-cell structure, and cell extension is very important for trichome formation after its initiation. Because the development of trichome and cotton fiber are regulated by similar mechanisms, it is meaningful to identify much more genes that participate in cell elongation and trichome development. That will help us to improve the yield and length of cotton fiber.

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References

Alahakoon, U.I., A. Taheri, N.K. Nayidu, D. Epp, M. Yu, IParkin, D. Hegedus, P. Bonham-Smith and M.Y. Gruber. 2016. Hairy canola (*Brasssica napus*) revisited: down-regulating TTG1 in an AtGL3-enhanced hairy leaf background improves growth, leaf trichome coverage, and metabolite gene expression diversity. *BMC Plant Biol.*, 16: 12.

Balkunde, R., M. Pesch and M. Hülskamp. 2010. Trichome patterning in *Arabidopsis thaliana* from genetic to molecular models. *Curr. Top. Dev. Biol.*, 91: 299-321.

Camoirano, A., A.L. Arce, F.D. Ariel, A.L. Alem, D.H. Gonzalez and I.L. Viola. 2020. Class I TCP transcription factors regulate trichome branching and cuticle development in Arabidopsis. *J. Exp. Bot.*, 71(18): 5438-5453.

Champagne, A. and M.A. Boutry. 2017. A comprehensive proteome map of glandular trichomes of hop (*Humulus lupulus* L.) female cones: identification of biosynthetic pathways of the major terpenoid-related compounds and possible transport proteins. *Proteomics*, 17(8): DOI: 10.1002/pmic.201600411.

Gao, Y., X. Gong, W. Cao, J. Zhao, L. Fu, X. Wang, K.S. Schumaker and Y. Guo. 2008. SAD2 in *Arabidopsis* Functions in Trichome Initiation through Mediating GL3 Function and Regulating *GL1*, *TTG1* and *GL2* Expression, 50(7): 906-917.

Gu, Q., H. Ke, C. Liu, X. Lv, Z. Sun, Z. Liu, W. Rong, J. Yang, Y. Zhang, L. Wu, G. Zhang, X. Wang and Ma Z. 2021. A stable QTL qSalt-A04-1 contributes to salt tolerance in the cotton seed germination stage. *Theor. Appl. Genet.*, 134(8): 2399-2410.

Humphries, J.A., A.R. Walker, J.N. Timmis and S.J. Orford. 2005. Two WD-repeat genes from cotton are functional homologues of the *Arabidopsis thaliana* TRANSPARENT TESTA GLABRA1 (TTG1) gene. *Plant Mol. Biol.*, 57(1): 67-81.

Hung, F.Y., J.H. Chen, Y.R. Feng, Y.C. Lai, S. Yang and K. Wu. 2020. *Arabidopsis* JMJ29 is involved in trichome development by regulating the core trichome initiation gene GLABRA3. *Plant J.*, 103(5): 1735-1743.

Ioannidi, E., S. Rigas, D. Tsitsekian, G. Daras, A. Alatzas, A. Makris, G. Tanou, A. Argiriou, D. Alexandrou, T. Wada and R. Tominaga-Wada. 2015. CAPRICE family genes control flowering time through both promoting and repressing CONSTANS and FLOWERING LOCUS T expression. *Plant Sci.*, 241: 260-265.

Kariyat, R.R., S.B. Hardison, A.B. Ryan, A.G. Stephenson, C.M. De Moraes and M.C. Mescher. 2018. Leaf trichomes affect caterpillar feeding in an instar-specific manner. *Comm. Integ. Biol.*, 11(3): 1-6.

Kim, S.Y., S. Hyoung, W.M So and J.S. Shin. 2018. The novel transcription factor TRP interacts with ZFP5, a trichome initiation-related transcription factor, and negatively regulates trichome initiation through gibberellic acid signaling. *Plant Mol. Biol.*, 96(3): 315-326.

Kong, Y., Y. Zhu, C. Gao, W. She, W. Lin, Y. Chen, N. Han, H. Bian, M. Zhu and J. Wang. 2013. Tissue-specific expression of SMALL AUXIN UP RNA41 differentially regulates cell expansion and root meristem patterning in *Arabidopsis*. *Plant Cell Physiol.*, 54(4): 609-621.

Matías-Hernández, L., W. Jiang, K. Yang, K. Tang, P.E. Brodelius and S. Pelaz. 2017. AaMYB1 and its rthologue AtMYB61 affect terpene metabolism and trichome development in *Artemisia annua* and *Arabidopsis thaliana*. *Plant J.*, 90(3): 520-534.

Morohashi, K. and E. Grotewold. 2009. A systems approach reveals regulatory circuitry for arabidopsis trichome initiation by the GL3 and GL1 Selectors. *PLoS Genet.*, 5(2): e1000396.

Oppenheimer, D.G, P.L. Herman, S. Sivakumaran, J. Esch and M.D. Marks.1991. A myb gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. *Cell*, 67(3): 483-493.

Payne, C.T., F. Zhang and A.M. Lloyd. 2000. *GL3* encodes a bHLH protein that regulates trichome development in *Arabidopsis* through interaction with GL1 and TTG1. *Genet.*, 156(3): 1349-1362.

Qi, T., S. Song, Q. Ren, D. Wu, H. Huang, Y. Chen, M. Fan, W. Peng, C. Ren and D. Xie. 2011. The jasmonate- ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. *Plant Cell*, 23(5): 1795-1814.

Qu, J., S.G. Kang, C.Hah and J.C. Jang. 2016. Molecular and cellular characterization of GA-Stimulated Transcripts *GASA4* and *GASA6* in *Arabidopsis thaliana*. *Plant Sci.*, 246: 1-10.

Schellmann, S., A. Schnittger, V. Kirik, T. Wada, K. Okada, A. Beermann, J. Thumfahrt, G. Jürgens and M. Hülskamp. 2002. TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in *Arabidopsis*. *The EMBO J.*, 21(19): 5036-5046.

Spartz, A.K., S.H. Lee, J.P. Wenger, N. Gonzalez, H. Itoh, D. Inzé, W.A. Peer, A.S. Murphy, P.J. Overvoorde andW.M. Gray. 2012. The *SAUR19* subfamily of SMALL AUXIN UP RNA genes promote cell expansion. *Plant J.*, 70(6): 978-90.

Sun, L., A. Zhang, Z. Zhou, Y. Zhao, A. Yan, S. Bao, H.Yu and Y. Gan. 2015. GLABROUS INFLORESCENCE STEMS3 (GIS3) regulates trichome initiation and development in *Arabidopsis*. *New Phytol.*, 206(1): 220-230.

Sun, L., E. Feraru, M.I. Feraru, S. Waidmann, W. Wang, G. Passaia, Z.Y. Wang, K. Wabnik and J. Kleine-Vehn. 2020. PIN-LIKES Coordinate Brassinosteroid Signaling with Nuclear Auxin Input in *Arabidopsis thaliana*. *Curr. Biol.*, 30(9): 1579-1588.

Takase, T., M. Nakazawa, A. Ishikawa, M. Kawashima, T. Ichikawa, N. Takahashi, H. Shimada, K. Manabe and M. Matsui. 2004. YDK1-D, an auxin-responsive GH3 mutant that is involved in hypocotyl and root elongation. *Plant J.*, 37(4): 471-83.

Tian, H., T. Qi, Y. Li, C. Wang, C. Ren, S Song and H. Huang. 2016. Regulation of the WD-repeat/bHLH/MYB complex by gibberellin and jasmonate. *Plant Signal. Behav.*, 11(8): e1204061.

Tiwari, P. 2016. Recent advances and challenges in trichome research and essential oil biosynthesis in *Mentha arvensis* L. *Ind. Crops Prod.*, 82: 141-148.

Tominaga, R., M. Iwata, K. Okada and T. Wada. 2007. Functional analysis of the epidermal-specific MYB genes CAPRICE and WEREWOLF in *Arabidopsis*. *Plant Cell*, 19(7): 2264-2277.

Vadde, B.V.L., K.R. Challa and U. Nath. 2017. The TCP4 transcription factor regulates trichome cell differentiation by directly activating GLABROUS INFLORESCENCE STEMS in *Arabidopsis thaliana*. *Plant J.*, 93(2): 259-269.

Vadde, B.V.L., K.R. Challa, P. Sunkara, A.S. Hegde and U. Nath. 2019. The TCP4 transcription factor directly activates trichomeless1 and 2 and suppresses trichome initiation. *Plant Physiol.*, 181(4): 1587-1599.

Verslues, P.E., Y. Guo, C.H. Dong, W. Ma and J.K. Zhu. 2006. Mutation of SAD2, an importin β-domain protein in *Arabidopsis*, alters abscisic acid sensitivity. *Plant J.*, 47(5): 776-787.

Wang, T., Q. Jia, W. Wang, S. Hussain, S. Ahmed, Adnan, D.X. Zhou, Z. Ni and S. Wang. 2019. GCN5 modulates trichome initiation in *Arabidopsis* by manipulating histone acetylation of core trichome initiation regulator genes. *Plant Cell Rep.*, 38(6): 755-765.

Wei, S., L. Xiang, M.Y. Gruber, B.A. Feyissa, A. Lisa and H. Abdelali. 2018. COP9 signalosome subunit 5A affects pheny- lpropanoid metabolism, trichome formation and transcription of key genes of a regulatory tri-protein complex in *Arabidopsis*. *BMC Plant Biol.*, 18(1): 134.

Wei, Z., Y. Cheng, C. Zhou, D. Li, X. Gao, S. Zhang and M. Chen. 2019. Genome-wide identification of direct targets of the TTG1-bHLH-MYB Complex in regulating trichome formation and flavonoid accumulation in *Arabidopsis Thaliana*. *Int. J. Mol. Sci.*, 20(20): 5014.

Yuan, Y., X. Xu, Y. Luo, Z. Gong, X. Hu, M. Wu, Y. Liu, F. Yan, X. Zhang, W. Zhang, Y. Tang, B. Feng, Z. Li, C.Z. Jiang and W. Deng. 2012. R2R3 MYB-dependent auxin signalling regulates trichome formation, and increased trichome density confers spider mite tolerance on tomato. *Plant Biotechnol. J.*, 19(1): 138-152.

Zheng, Y., Q. Zhan, T. Shi, J. Liu, K. Zhao and Y. Gao. 2020. The nuclear transporter SAD2 plays a role in calcium- and H_2O_2-mediated cell death in *Arabidopsis*. *Plant J.*, 101(2): 324-333.

Zhong, C., B. Patra, Y. Tang, X.Li, L. Yuan and X. Wang. 2021. A transcriptional hub integrating gibberellin-brassinosteroid signals to promote seed germination in *Arabidopsis*. *J. Exp. Bot.*, 72(13): 4708-4720.

Zhou, Z., L. An, L. Sun, S. Zhu, W. Xi, P. Broun, H. Yu and Y. Gan. 2011. Zinc finger protein5 is required for the control of trichome initiation by acting upstream of zinc finger protein8 in *Arabidopsis*. *Plant Physiol.*, 157(2): 673-682.

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