Domain-specific expression of meristematic genes is defined by the LITTLE ZIPPER protein DTM in tomato

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Shoot meristems, which harbor a small population of stem cells, are responsible for generating new above-ground organs in plants. The proliferation and differentiation of these stem cells is regulated by a genetic pathway involving two key meristematic genes: **CLAVATA3 (CLV3)** and **WUSCHEL (WUS)**. However, it is not well understood how **CLV3** and **WUS** expression domains in the shoot meristems are specified and maintained during post-embryogenic development. Here, we show that a tomato mutant with fasciated stems, flowers and fruits, due to impaired stem cell activity, is defective in a **LITTLE ZIPPER** gene denoted as **DEFECTIVE TOMATO MERISTEM (DTM)**. DTM forms a negative feedback loop with class III homeodomain-leucine zipper (HD-ZIP III) transcription factors to confine **CLV3** and **WUS** expression to specific domains of the shoot meristems. Our findings reveal a new layer of complexity in the regulation of plant stem cell homeostasis.

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Shoot apical meristems (SAMs) provide meristematic cells for the formation of plant aerial organs, and maintaining SAM activity is crucial for plants to complete their life cycles and to adapt to changing environments. Within the SAM, stem cell niches are maintained by the CLAVATA-WUSCHEL (CLV-WUS) feedback loop. In this regulatory module, WUS controls stem cell fate by preventing cell differentiation, while CLV3 regulates cell division to restrict SAM size. At the transcription level, CLV3 restricts WUS expression to a limited number of cells, while low WUS activity promotes CLV3 expression. In addition, SHOOTMERISTEM-LESS (STM) prevents the entrance of stem cell differentiation in the SAM. It has been well documented that the coordinated actions of these three meristematic genes in maintaining the SAM are highly dependent on their layer-specific and overlapping expression.

Class III homeodomain-leucine zipper (HD-ZIP III) genes regulate SAM activity in addition to the identities of the adaxial tissues of lateral organs in Arabidopsis thaliana. The A. thaliana loss-of-function HD-ZIP III triple mutant, revoluta phialoosa phavoluta (rev phb phv), only forms pin-like shoots, whereas high expression of HD-ZIP III genes causes enlarged SAMs. Recently, it was shown that HD-ZIP III proteins regulate WUS expression through interaction with A-type ARABIDOPSIS RESPONSE REGULATOR proteins during de novo shoot regeneration. Furthermore, REV directly activates STM expression in the meristematic cells at the leaf axil for axillary meristem formation. Regulation of SAM activity by HD-ZIP III transcription factors can be WUS-dependent or independent, suggesting the involvement of additional modifiers.

HD-ZIP III transcription factors are regulated at the transcriptional, post-transcriptional, and post-translational levels. For example, LITTLE ZIPPER proteins (ZPRs) inhibit HD-ZIP III activities by forming presumably nonfunctional heterodimers with them. Thus, ZPRs control SAM development through suppression of HD-ZIP III activities. The A. thaliana double mutant of ZPR3 and ZPR4, zpr3–2 zpr4–2, produces ectopic shoot meristems, while overexpression of ZPR3 causes early meristem termination.

The genetic program governing the SAM stem cell system is generally conserved across plant species. Changes in meristem size have also been observed in the tomato (Solanum lycopersicum) locule number and fasciated (fas) mutants, which have mutations in the WUS and CLV3 orthologs SIWUS and SICLV3, respectively. However, unlike A. thaliana, tomato is a typical sympodial plant: its shoot development is indeterminate since after primary shoot meristems terminate into inflorescences, sympodial meristems (SYM) are formed at the leaf axil immediately beneath the inflorescence meristems to sustain its growth. The difference in SAM activity between the two species is likely due to diversified regulatory mechanisms, as exemplified by SAM doming being regulated by LATE TERMINATION, which occurs in tomato but is absent in A. thaliana. To identify new genetic components that regulate SAM activity in tomato, we screened for defective meristem mutants in a large ethyl methanesulfonate mutagenized population of the cultivated tomato LA2397 for mutations affecting flower and fruit development. We identified a meristem defect mutant, which we named defective tomato meristems-1 (dtm-1). This mutant was first noted for its enlarged fasciated flowers and increased numbers of floral organ. In Figure 1a–I, dtm-1 has seedless fruits, which we determined was due to female sterility, since the mutation was transmitted to wild type plants by pollinating with samples of dtm-1 pollens. To identify the causal mutation underlying the mutant phenotype, dtm-1 was crossed as the pollen donor to Solanum pimpinellifolium LA1781, an accession of a wild relative of cultivated tomato, to generate a F2 mapping population. Using 214 dtm-1 plants, DTM was mapped to a 1.5 Mb interval (SL2.50cH09:2.0–3.5 Mb) between markers xps1857 and xps215 (Figure 1g). Fine mapping, using an additional 678 dtm-1 plants and newly developed markers, further narrowed down the DTM locus to a 25.3 kb region between the xps1882 and xps1892 markers (Figure 1h). Based on the ITAG2.4 annotation from the sol genomics network database (SGN, https://solgenomics.net/), this region was determined to contain four protein encoding sequences, but no mutation was found in any of them in dtm-1. We then performed ab initio gene prediction with the 25.3 kb genomic sequences using FGENESH (www.softberry.com), and found that Solyc09g009620 was annotated incorrectly, missing a noncoding exon and 45 nucleotides (15 amino acids) at its 5′-end. This was confirmed by rapid amplification of cDNA ends analysis (Supplementary Figure 1). After resequencing the corrected Solyc09g009620 coding sequence, we found that dtm-1 has a single point mutation near the beginning of the second exon: a C to T transversion (C22T) that causes the conserved leucine at position 8 to be changed to phenylalanine (L8F) (Figure 1i).

DTM shares high amino acid sequence similarity with the A. thaliana ZPR4 protein (77% identity, 35/47), a member of a small gene family that is widely present in land plants. The tomato genome encodes four additional ZPR proteins, named DTM-like (DTL, Solyc11g007100), SIZPR1 (Solyc01g091490), SIZPR2A (Solyc08g007570), and SIZPR2B (Solyc08g079690) based on their homology with A. thaliana ZPR proteins (Figure 1j). In a phylogenetic analysis, DTM and its closest homolog DTL (69% identity) grouped with A. thaliana ZPR3 and ZPR4, while the other three tomato ZPR proteins were more similar to A. thaliana ZPR1 and ZPR2 (Figure 1j).

dtm-1 exhibits multiple meristem defects. Although dtm-1 plants looked similar to wild type at the seedling stage, close examination revealed that the mutant had defects in axillary shoot formation. Specifically, axillary organs were formed above the leaf axils, rather than at the leaf axils, as in wild type (Figure 2a–d). Occasionally, leafy organs, instead of axillary buds, were formed on the stems between two consecutive leaves (Figure 2c). Additionally, two lateral shoots were often observed on opposite sides of the stems, and anatomical analysis of cross-sections of the upper shoots revealed that two axillary meristems formed simultaneously (Figure 2e, f). These axillary buds were able to develop into functional shoots with fasciated flowers like those on the primary shoots (Figure 2g–i). However, all branches terminated in single inflorescences, indicating that SYM development on the branches was defective (Figure 2i). Compared with wild type, SYM formation on primary shoots was slightly delayed in the dtm-1
mutant (Fig. 2j, k). These observations suggest that SAM activity is not properly maintained in the dtm-1 mutant.

By dissecting the shoot apices, we found that dtm-1 SAMs were flatter and wider than those of wild type, as revealed by scanning electron microscopy (SEM) (Fig. 2l, m). In addition, the dome surface of dtm-1 SAMs appeared wrinkled, and ectopic trichomes often formed on the epidermis (Fig. 2n-p). As in many other plant species, the SAM gradually develops into a dome structure in tomato30. To better understand the timing of DTM action on SAM development, we analyzed the doming of dtm-1 SAMs. At 3 days after germination (DAG), we observed no difference in SAM morphology between dtm-1 and wild type, except that the SAMs of the mutant were wider (Supplementary Fig. 2). However, doming was limited in dtm-1 and its SAMs remained flat or slightly bulged until the developmental transition to inflorescence meristems.

Interestingly, in the F2 population derived from a cross between dtm-1 and LA1781, we observed extremely elongated flower stalks and a wide range of variations in fruit fasciation (Supplementary Fig. 3). This suggests that the dtm-1 mutation has
pleiotropic effects on flower and fruit development due to undetermined genetic modifiers in the LA1781 background, or to recombination events. To test whether the mutation has different extents of phenotypic penetration in different genetic backgrounds, we also introduced the dtm-1 allele into S. lycopersicum cv. Moneymaker, a cultivar that has been widely used for genetic and molecular studies31, and into the wild species accession LA1781. In the three genetic backgrounds, the dtm-1 mutation had very similar, if not identical, effects on SAM development. All mutant phenotypes observed in the LA2397 background were observed in both the LA1781 and Moneymaker backgrounds (Supplementary Fig. 4).

Phenotypes of null dtm alleles created by CRISPR-Cas9. Since the phenotypic abnormality observed in the dtm-1 mutant is caused by a missense mutation, to gain insight into its loss-of-function effects on SAM development, we generated several null alleles of the DTM gene by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 genome editing, using two single guide sequences located 169 bp apart on the second exon (Fig. 3a). In total, we obtained four lines with 2–176 bp deletions in the protein coding region that likely disrupted DTM functionality, from more than ten primary transformants (Fig. 3b). The four loss-of-function alleles had the same phenotypes and we chose line dtm-cr5, which has a 176 bp deletion (nucleotide 64–239), for further analysis.

Unlike the dtm-1 allele, dtm-cr5 seedlings had abnormal cotyledon numbers, with almost all of the mutants (104 out of 107) having more than two (Fig. 3c–f). Examination of the dtm-cr5 apices revealed that its SAM development was more severely perturbed than in dtm-1. Domed SAM structures were rarely present in the dtm-cr5 seedlings at 3–12 DAG, although multiple SAM-like structures were identified in longitudinal sections of their shoot apices (Fig. 3g, h; Supplementary Fig. 5). Moreover, dtm-cr5 apices were densely covered by trichomes (Fig. 3i). Later in development, the adult dtm-cr5 plants were dwarfed, with extremely fasciated and twisted stems, clustered leaves (Fig. 3j, k), and occasionally flowers formed without petals (Fig. 3l).

Since SlCLV3 is also involved in SAM doming, we tested for genetic interactions between SlCLV3 and DTM by crossing dtm-1 with S. lycopersicum cv. Super Beefsteak, which has a chromosomal conversion that downregulates SlCLV3 expression29. The
double mutant, dtm-1 fas, was indistinguishable from dtm-1 in SAM morphology: both produced flat SAMs with ectopic trichomes on the epidermis (Supplementary Fig. 6). However, SYM development was abolished in dtm-1 fas mutants and side shoots were replaced by inflorescence-like structures (Supplementary Fig. 6), indicating that the two genes may have additive effects on lateral meristem development.

Interactions between DTM and HD-ZIP III proteins. LITTLE ZIPPER proteins function as post-translational suppressors of class III HD-ZIP transcription factors by inhibiting their homodimerization. In tomato, there are six HD-ZIP III members, of which Solyc11g069470 has been named SlREV, based on its sequence similarity to Arabidopsis thaliana class III HD-ZIP transcription factors. We assigned the following names to the remaining tomato HD-ZIP III proteins based on sequence similarity and phylogenetic analysis: SIFHB (Solyc02g069830), SIFHV (Solyc02g069830), SIFH15A (Solyc03g120910), SIFH15B (Solyc12g044410), and SIFH8 (Solyc08g066500) (Supplementary Fig. 7). We tested the interactions between DTM and the tomato
HD-ZIP III proteins by yeast two hybrid and pulldown assays. In yeast, DTM interacted with full-length SlREV, SlPHV, and SlHB8 (Fig. 4a), and with the individual N-terminal regions, which each contains a homeodomain and a leucine zipper domain, of all the tomato HD-ZIP III members except SlHB15A (Supplementary Fig. 8a). Using *Escherichia coli* expressed proteins, cmyc-DTM was

![Diagram](https://example.com/diagram.png)
immunoprecipitated with an HA antibody when incubated with protein extractions containing HA-tagged SlPHV, SlREV, SlHB8, SlHB15A or SlHB15B, but not HA-SlPHB (Fig. 4c). This suggests that DTM may interact with several members of the tomato HD-ZIP III transcription factor family.

The dtm-1 mutant has a missense mutation (L8F) at the interhelical interface in the second heptad of the leucine zipper domain, which presumably affects its interaction with the HD-ZIP III proteins25. Indeed, compared to wild type DTM, the mutant form DTM_L8F and the three truncated proteins created by CRISPR-Cas9 showed weaker and no interaction, respectively, with HD-ZIP III members in yeast (Fig. 4b; Supplementary Fig. 8a). The weakened interaction between DTM_L8F and full-length or the N-terminal regions of HD-ZIP III proteins was further confirmed by pulldown assays (Fig. 4d; Supplementary Fig. 8b, c). To investigate the interactions between DTM and the HD-ZIP III proteins in vivo, we conducted a biomolecular fluorescence complementation assay in tobacco leaves33. DTM or DTM_L8F were separately heterologously expressed in tobacco leaves as translational fusions with the N-terminal half of the firefly luciferase (LUC) reporter protein, together with the C-terminus of LUC fused with different HD-ZIP III proteins. We observed that wild type DTM interacted with all six tomato HD-ZIP III proteins, while its mutant form, DTM_L8F, showed much weaker interactions (Fig. 4e).

Fig. 5 Mutagenesis of the DTM protein. a Interactions between SlREV (fused to the activation domain, AD) and five DTM mutants with single mutations in the conserved Leu or Ile residues in each heptad (fused to the binding domain, BD) in yeast. The five conserved Leu or Ile residues were individually mutated to Ala. b Interactions between SlREV and four DTM double mutants derived from DTM_L8F in yeast. c Binding activities of DTM and its mutant forms to its partner, SlREV. DTM and its mutant forms were fused to cmyc and their binding affinities to SlREV (fused to HA) were assayed by pulldown using an anti-HA antibody. d Electrophoretic mobility shift assay showing dosage-dependent inhibition of DTM on SlREV binding ability to its target sequence. e Inhibitive effect of single and double mutations in the conserved residues of DTM on SlREV binding ability to its target sequence. Both SlREV (1–264 aa) and DTM were synthesized using wheat germ extract, and different concentrations of DTM (d) and/or its mutated forms (e) were tested for their inhibitive effects on SlREV binding to a Cy5-labeled HB9 DNA duplex. 3-AT, 3-amino-1,2,4- triazole; IP, immunoprecipitation; WB, Western blot
between DTM_I15A and SlREV, suggesting that it is less important for the DTM-SlREV interaction. Mutagenesis of DTM_L8F further confirmed the weak effect of the I15A mutation as we still observed an interaction between DTM_L8F (I15A) and SlREV using both the yeast two hybrid and pulldown assays (Fig. 5b, c). DTM_L8F(L22A) interacted with SlREV in pulldown assays, but not in yeast two hybrid assays. No interaction was found between DTM_L8F(L22A), DTM_L8F (L36A) and SlREV. The mutagenesis analysis suggests that the leucine residues in heptad two, four, and six are critical for the interaction between DTM and HD-ZIP III transcription factors.

Previous studies have shown that LITTLE ZIPPER proteins inhibit DNA binding of HD-ZIP III transcription factors\(^\text{25,27}\). We demonstrated that DTM inhibited SlREV binding to the HB9 duplex containing consensus sequence for REV using an electrophoretic mobility shift assay, and further showed that the inhibition by DTM was dosage-dependent (Fig. 5d, e). Moreover, consistent with the weakened interactions between the DTM mutants and SlREV in vitro and in vivo, DTM_L8F and DTM_L8F(L22A) had a weaker interaction between DTM and HD-ZIP III transcription factors.

DTM_L8F further confirmed that DTM regulates SAM development through SlREV, suggesting that it is less important for the DTM-SlREV interaction. Mutagenesis of DTM_L8F further confirmed the weak effect of the I15A mutation as we still observed an interaction between DTM_L8F (I15A) and SlREV using both the yeast two hybrid and pulldown assays (Fig. 5b, c). DTM_L8F(L22A) interacted with SlREV in pulldown assays, but not in yeast two hybrid assays. No interaction was found between DTM_L8F(L22A), DTM_L8F (L36A) and SlREV. The mutagenesis analysis suggests that the leucine residues in heptad two, four, and six are critical for the interaction between DTM and HD-ZIP III transcription factors.

Since DTM inhibited SlREV binding to the HB9 duplex containing consensus sequence for REV using an electrophoretic mobility shift assay, and further showed that the inhibition by DTM was dosage-dependent (Fig. 5d, e). Moreover, consistent with the weakened interactions between the DTM mutants and SlREV in vitro and in vivo, DTM_L8F and DTM_L8F(L22A) had a weaker interaction between DTM and HD-ZIP III transcription factors (Supplementary Fig. 10l). These results suggest that DTL is likely not expressed in the SAM (Supplementary Fig. 10a–g). Consistent with the phenotypic observation, in situ hybridization analysis suggested that DTL is likely not expressed in the SAM (Supplementary Fig. 10a–g). To test the possibility that DTL affects SAM development by interacting with DTM, we analyzed the phenotype of the double dtm-cr5 dtl-cr1 mutant, and observed that it was indistinguishable from dtm-cr5 (Supplementary Fig. 10l–n). These results suggest that DTL is not essential for post-embryogenic meristem development in tomato.

**DTM-SlREV defines expression domains of meristematic genes.** Since DTM expression was detected mainly in the central zone of the SAM that marks the expression domains of SIWUS and SICLV3 (Fig. 7a)\(^\text{29}\), the observed SAM defects in the dtm mutants were likely caused by altered expression of these meristematic genes. We then monitored the expression patterns of the three meristem marker genes SICLV3, SIWUS, and SLSTM in the shoot apices of dtm-1, dtm-cr5, and their corresponding wild types, at 3, 6 and 9 DAG. Expression analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) revealed that SlREV expression was detected mainly in the central zone of the SAM that marks the expression domains of SIWUS and SICLV3 (Fig. 7a, b), and that SlREV expression was downregulated in the mutant leaf primordia, to single leaves without shoot meristems (Fig. 6c). These developmental defects are indicative of premature SAM arrest and leaf phyllotaxis defects of differing severities (Fig. 6a, b). Two mutant alleles, slrev-cr1 and slrev-cr2, exhibited SAM arrest and leaf phyllotaxis defects of differing severities, ranging from relatively normal SAM development, with an opposite leaf arrangement or two leaves (sometimes fused) with slowly initiated leaf primordia, to single leaves without shoot meristems (Fig. 6c–i). These developmental defects are indicative of premature stem cell consumption in the tomato rev mutants, contrasting with the overactive SAM activity in the dtm mutants. To further verify that DTM regulates SAM development through SlREV, we analyzed their genetic interaction by crossing dtm-cr5 with slrev-cr2 (both in the Moneymaker background). The resulting dtm-cr5 slrev-cr2 seedlings were tricots, but looked more similar to slrev-cr2 seedlings (Fig. 6j–m). This suggests that DTM regulates post-embryogenic SAM development in a SlREV-dependent manner, whereas its roles in embryogenesis is SlREV-independent.

Although the closest homolog of DTM, DTL, also interacts with tomato HD-ZIP III proteins, it has a different binding affinity, as revealed by yeast two hybrid and in vitro pulldown assays (Supplementary Fig. 9). Two dtl knockout mutants, created by CRISPR-Cas9 editing, had no obvious phenotypes (Supplementary Fig. 10a–g). Consistent with the phenotypic observation, in situ hybridization analysis suggested that DTL is likely not expressed in the SAM (Supplementary Fig. 10a–g). To test the possibility that DTL affects SAM development by interacting with DTM, we analyzed the phenotype of the double dtm-cr5 dtl-cr1 mutant, and observed that it was indistinguishable from dtm-cr5 (Supplementary Fig. 10l–n). These results suggest that DTL is not essential for post-embryogenic meristem development in tomato.

![Fig. 6](https://example.com/fig6.png)

**Fig. 6** SlREV regulates shoot apical meristem (SAM) maintenance in tomato. a) design of CRISPR-Cas9 editing for the tomato REV gene. The start codon ATG and the gRNA target sequence are indicated. The arrowhead indicates the gRNA target site. b) deduced protein sequences of two slrev alleles created by CRISPR-Cas9. c-i) representative images showing meristem defects of different severity observed in slrev mutants. slrev-cr mutants displayed weak (d) or strong (e) defects in leaf phyllotaxis (indicated by arrowheads). slrev seedlings often produced only one or two true leaves (f, h) with few small leaf-like tissues (g) or barren SAMs (i) (indicated by arrows). Since no allele-specific phenotypic abnormality was observed between slrev-cr1 and slrev-cr2, only those of slrev-cr2 are shown here. Scale bars represent 1 cm (c-f, h) and 100 μm (g, i), respectively. j-l) seedling phenotypes of the dtm slrev double mutant. Scale bars, 1 cm. m) dissected dtm-cr5 slrev-cr2 apices showing SAM defects as revealed by stereomicroscopy. Scale bar, 2 mm.
transcription PCR (qRT-PCR) revealed that SlCLV3 transcript levels were substantially elevated in both the dtm-1 and dtm-cr5 shoot apices at all three time points, except for a marginal difference in expression level, which was detected between dtm-cr5 and its wild type at 3 DAG (Fig. 7c–d; Supplementary Fig. 11a–d). SlWUS expression was also much higher in dtm-cr5 shoot apices at all three time points and in dtm-1 at 6 DAG, while SlSTM expression was apparently only upregulated in the two dtm alleles at 6 DAG.

Similarly, in situ hybridization analysis revealed that SlCLV3 expression was higher and spatially expanded in the dtm-1 and dtm-cr5 shoot apices at 3, 6, and 9 DAG (Fig. 7e; Supplementary...
Fig. 7 DTM defines the SICL3 and SIWUS expression domains. a, b expression of DTM and SIREV in wild type (Moneymaker) shoot apices as revealed by RNA in situ hybridization. c, d expression patterns of the meristematic SICL3, SIWUS and SISTM genes in the shoot apices of dtm-1 (e), dtm-cr5 (d), and their corresponding wild types (LA2397 and Moneymaker) at 6 days after germination (DAG) determined by quantitative reverse transcription (qRT)-PCR. SlEfl4A6 was used as the reference gene to normalize gene expression. Data represent means ± sem, n = 6 biologically independent samples. A Welch’s t-test was applied to compare the differences in means between dtm-1 and wild type. e expression patterns of the SICL3, SIWUS, and SISTM genes in the shoot apices of dtm-1, dtm-cr5 and wild type (Moneymaker) at 6 DAG revealed by RNA in situ hybridization. Scale bars, 100 μm. f expression levels of DTM and the meristematic SICL3, SIWUS, and SISTM genes in slrev-cr2 shoot apices and wild type (Moneymaker) at 6 DAG determined by qRT-PCR. Data represent means ± sem, n = 5 (wild type) or 7 (slrev-cr2) biologically independent samples. Fold changes (FC, mutant vs wild type (WT)) of gene expression levels are given under the graph. A Welch’s t-test was applied to compare the differences in means between slrev-cr2 and wild type. g–r expression of DTM (g–j), SICL3 (k–n), and SIWUS (o–r) in slrev-cr2 (i, j, m, n, q and r) and wild type (Moneymaker, h, l and p) shoot apices revealed by in situ hybridization. Hybridization using sense probes served as negative controls (g, k and o). Scale bar, 100 μm. The experiments were repeated at least twice using different batches of plants with similar results. Expression data collected from the dtm-1 and dtm-cr5 seedlings at 3 and 9 DAG are provided in Supplementary Fig. 10c–d. SISTM expression in slrev-cr2 and wild type apices is available in Supplementary Fig. 10e.

In wild type, SICL3 was expressed in the central zone of the SAM, apart from in the L1 layer. SICL3 expression in the weak dtm-1 allele was expanded but still detected in the central zone, whereas in the dtm-cr5 null allele it was only detected in the outermost two layers of the whole apex, indicating that DTM restricts SICL3 expression in the central zone. SIWUS, which is mainly expressed in the organizer zone, is likely also required for defining the size of the stem cell population in tomato. In the dtm-1 and dtm-cr5 apices, SIWUS expression appeared patchy and expanded. Similarly, SISTM expression was also affected. Unlike in wild type, the SISTM expression in dtm-1 and dtm-cr5 apices was diffuse and no apparent expression domain could be defined.

Since DTM acts on SAM development in a SIREV-dependent manner, and the two genes have overlapping expression domains in the SAM (Fig. 7a, b), we examined the expression of SICL3 and SIWUS in the SAMs of slrev seedling apices at 6 DAG. SICL3 expression, measured by qRT-PCR, was substantially downregulated in slrev-cr2, and while there were indications that the expression of SIWUS was also slightly lower (Fig. 7i). We saw no evidence that SISTM expression was affected by the slrev mutation (Supplementary Fig. 11e). Further, in situ hybridization analyses revealed that SICL3 was expressed in a much deeper region of the SAM, likely in the organizer zone (Fig. 7k–n). The SIWUS expression level correlated with the severity of SAM defects; expression was not detected in the shoot apices without SAMs, but was maintained in the organizer zone with SAM-like structures (Fig. 7o–r). Consistent with previous results identifying ZPR genes as REV targets in A. thaliana, DTM expression was downregulated in slrev-cr2 shoot apices at 6 DAG (Fig. 7i–j). These results suggest that DTM and SIREV form a feedback loop to confine the expression of meristematic genes to specific cell layers in the SAM.

Mutations in DTM impair leaf phyllotaxis. In addition to the defects in stem and axillary bud development, the leaf formation rate was also affected by dtm mutations, since five or more leaves were observed in dtm-cr5 seedlings when the wild type had only three visible leaves (Fig. 3e, f). This likely reflects faster leaf initiation and the less serrated leaves in the dtm-cr5 seedlings appeared to be clustered on a short stem region (Supplementary Fig. 13a, b). After removal of visible leaves, four small leaves or primordia forming on the dtm-cr5 apices were observed by stereomicroscopy and SEM (Supplementary Fig. 12c, d). Although the leaf formation rate was less affected in dtm-1, longitudinal sectioning revealed that two precocious leaves formed almost simultaneously on the flank of the SAMs at 6 DAG (Supplementary Fig. 13e–g).

LEAFLESS (LFS) is required for leaf formation and its expression predicts leaf initiation. Since dtm mutations affected leaf phyllotaxis, we used in situ hybridization to investigate whether LFS expression was altered in dtm mutants during leaf formation. Consistent with previous reports, we observed that LFS was expressed in the incipient regions of leaf primordia. Although LFS expression remained unchanged in both the dtm-1 and dtm-cr5 apices at 3 DAG, its expression was barely detectable after this time point (Supplementary Fig. 13h–p). This effect on LFS expression is consistent with the observation that leaf formation was accelerated in dtm-1 and dtm-cr5 after germination.

Discussion

It is well established that maintenance of SAM activity is primarily governed by the conserved CLV-WUS pathway, but it is not well understood how this pathway is modified to allow diversified growth patterns in different plant species. In this study, we characterized a tomato LITTLE ZIPPER protein, DTM, and elucidated its role in SAM maintenance by defining WUS and CLV3 expression domains.

Our data indicate that DTM is crucial for maintaining SAM activity in tomato, a model plant with a sympodial growth habit. Loss of, or weakened, DTM activity affects primary and secondary shoot meristem development, resulting in enlarged flattened SAMs, ectopic formation of axillary buds and leaves, and termination of SYMs on side shoots, as well as enlarged fasciated flowers and fruits. Such flower and fruit fasciation are often associated with failures in SAM maintenance, as observed in the tomato fasciated inflorescence, fasciated and branched and fas mutants. Furthermore, the defects in SAM development caused by dtm mutations are characteristic features of A. thaliana mutants containing mutations in CLV3 or genes involved in the transmission of the CLV3 peptide signal, such as clavata1 and the quadruple mutant of the CLAVATA3 INSENSITIVE RECEPTOR KINASE genes. Given that dtm mutants produce tricots, or fused cotyledons, DTM is also involved in embryogenic meristem formation. However, its primary role is to maintain post-embryogenic meristem development. The presence of multiple slightly domed SAM-like structures in the dtm seedlings indicates that DTM limits stem cell activity. Thus, impairing DTM activity causes overproduction of stem cells in the SAM. Under field growth conditions, the excessive number of stem cells ensures the development of extremely long inflorescences and large flowers.

Like tomato dtm mutants, the A. thaliana double mutant, zpr3–2 zpr4–2, shows a similar phenotypic abnormality resulting from SAM defects, including a high frequency of tricots, early leaf initiation and flower fasciation. This suggests that DTM and its A. thaliana homologs have conserved roles in SAM maintenance. However, DTM has an additional role in the regulation of cell differentiation in tomato because dtm mutations cause ectopic
trichomes to be formed on the SAM epidermis, as also observed in the petunia (Petunia hybridra) and pepper (Capsicum annuum) hairy meristem mutants that have mutations in GRAS family genes, which encode proteins that interact with WUS homologs9,12. The regulation of cell differentiation by DTM is likely CLV3-independent, since ectopic trichomes are still formed on the SAM epidermis of the double dtm-1 fas mutants.

Consistent with a function in the regulation of SAM activity, DTM is mainly expressed in the central zone of the SAM that marks the SCLV3 and SWUS expression domains28,29. Based on phenotypic observations and expression changes of these meristematic genes caused by the dtm mutations, we conclude that DTM regulates SAM maintenance through defining the expression domains of the meristem genes SCLV3 and SWUS. Disrupting DTM activity leads to SCLV3 expression spreading to the L1 layer in the SAM, where it is not expressed in wild type29. Furthermore, high SCLV3 activity likely causes the patchy SWUS expression observed in dtm SAMs, a phenomenon that has been observed in CLV3 overexpressing A. thaliana9.

Although previous studies have shown that CLV3 restricts WUS expression to a limited set of cells in the central zone of the SAM in A. thaliana9, high SCLV3 expression caused by dtm mutations does not repress overall SWUS transcription, consistent with a failure in CLV3 peptide signal transmission in the dtm mutants. Thus, the expanded SCLV3 expression caused by dtm mutations did not reduce SAM size, but instead led to enlarged SAMs. Such SAM enlargement, coupled with high CLV3 expression, is well recognized in A. thaliana mutants such as clavata1, receptor-like protein kinase2 and the quadruple mutant of the CLAVATA3 INSENSITIVE RECEPTOR KINASE genes, in which mutations disrupt the transmission of the CLV3 peptide signal33,34. Expanding CLV3 and WUS expression has also been observed in the enlarged shoot meristems of an A. thaliana HD-ZIP III triple mutant42.

Our data also demonstrated that DTM can interact with HD-ZIP III transcription factors in vitro and in vivo, in agreement with previous studies in A. thaliana25,27. Both dtm and zpr3–2 zpr4–2 have SAM defects similar to the A. thaliana phyv-1d mutant25,27. Thus, DTM regulates SAM activity in tomato through a similar mechanism to A. thaliana ZPR proteins, by acting as a competitor to suppress HD-ZIP III transcription factor activity. Ectopic AM formation in dtm mutants is also observed in tomato plants overexpressing miR166-resistant SIREY32. Furthermore, loss-of-function mutations in the tomato SIREV gene result in opposite meristem phenotypes, including SAM arrest and disordered leaf phyllotaxis, suggesting that DTM controls SAM development mainly through suppressing SIREV activity. This conclusion is further supported by attenuated and domain-shifted expression of the meristematic genes SCLV3 and SWUS in the SAMs of srev seedlings. Moreover, the characteristic SAM defect phenotypes of the dtm-cr5 srev-cr2 mutants suggest that DTM acts in a SIREV-dependent manner. We further observed that DTM competes for HD-ZIP III binding in a dosage-dependent manner, supporting the hypothesis that ZPR proteins form heterotramers, rather than heterodimers, with HD-ZIP III proteins43.

Given that HD-ZIP III transcription factors directly activate WUS and STM expression during de novo shoot regeneration and AM formation in A. thaliana20,21, we propose that the elevated expression of SWUS and SISTM in dtm apices is due to weak, or lost, inhibition of SIREV and other HD-ZIP III transcription factors that have yet to be identified. We conclude that a DTM-SIREV feedback loop plays an essential role in regulating the CLV-WUS signaling pathway by defining its expression domains.

Methods

Plant growth conditions and phenotypic analysis. dtm-1 was identified by screening for defective meristem mutants in an ethyl methanesulfonate mutagenesis population in the cultivated tomato L2A397 background. Since dtm-1 is female sterile, the mutant is maintained in a heterozygous state by backcrossing to wild type L2A397. Unless specified, all phenotypic observations and gene expression analysis were conducted with phytotron-grown seedlings or plants derived from self-pollination, or BC, heterozygotes. The phytotron growth conditions were maintained at 20–25 °C, a relative humidity of 70–80%, and 16 h daily illumination by 150 μmol m−2 s−1 light from metal halide and high-pressure sodium lamps. F2 mapping populations were grown under natural radiation in plastic greenhouses located in Songzhang, Shanghai.

Genetic mapping of the DTM gene and sequence analysis. dtm-1 was crossed with the wild relative S. pimpinellifolium accession LA1781 to produce a F1 hybrid population. Based on the lower phenotypes of the F1 progenies, dtm-1 plants identified from a total of 3,964 F2 progenies were used to map the DTM locus. Genomic DNA was extracted from 1–2 cm young leaves using a high-throughput miniprep method44. Leaves were ground in 350 μL DNA extraction buffer [100 mM Tris, 5 mM EDTA, 150 mM sorbitol, 30 mM sodium borate, 0.1% cetyltrimethylammonium ammonium bromide, 2% sarcosyl, pH 8.0] on a mixer mill MM 400 (Retsch). After extraction with 350 μL chloroform:isoamyl alcohol (24:1), DNA was precipitated with an equal volume of isopropanol from cleared supernatant by centrifugation and dissolved in 100 μL TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Molecular markers, comprising PCR and cleaved amplified polymorphism (CAPS) markers, were designed using a polymorphic sequence database from S. pimpinellifolium LA-1589, which were downloaded from SGN (https://solgenomics.net/). Marker information is provided in Supplementary Table 1.

After fine mapping, gene prediction was conducted in the 25.3 kb interval of the Heinz 1706 reference genome containing the DTM locus, using the FGENEsh program in Softberry (http://www.softberry.com/). To identify the causal dtm-1 mutation, genomic and complementary DNA (cDNA) sequences of the DTM genes from the mutant and the LA2397 wild type were determined by Sanger sequencing. Sequence assembly and comparisons were performed using the Sequencer® (Gene Codes Inc.) DNA analysis software. To identify the tomato LITTLE ZIPPER gene family members, protein sequences of DTM and A. thaliana ZPR proteins were used as queries in a BLAST X homology search of the ITAG2.5 genome database downloaded from SGN. Similarly, tomato HD-ZIP III protein homologs were identified by using protein sequences of the five A. thaliana HD-ZIP III proteins as queries. Maximum parsimony trees of tomato and A. thaliana DTM/ZPR and HD-ZIP III proteins were constructed using MEGA7 (ver. 7.0.26)45. Phylogenetic relationships were tested with 1000 bootstrap replications and the trees were inferred using the Subtree-Fusing-Regrafting (SPR) method embedded in the program.

Mutagenesis by CRISPR-Cas9. To create null dtm and dtl alleles using CRISPR-Cas9 genome editing technology, two single guide RNAs were designed using the CRISPR-P (http://cbi.hzau.edu.cn/crispr/) online tool46. After annealing, oligos were subsequently cloned into the psgr-Cas9-At vector37. Sequence-validated psgr-Cas9-At fragments containing guide sequences of the target genes were digested with the EcoRI and HindIII (New England Biolabs) restriction enzymes, and then cloned into the pCAMBIA1300 binary vector. The CRISPR-Cas9 system used to create the srev-cr mutants was constructed similarly, using a single guide RNA. These constructs were transformed into C. Moneymaker (LA2706) by Agrobacterium-mediated transformation36,46. Briefly, cotyledons harvested from 7–10 day old seedlings were co-cultured for two days on Murashige and Skoog medium containing 100 μM acetoxycongine with Agrobacterium strain GV3101 (OD600 = 0.5–0.6) harboring individual CRISPR-Cas9 constructs in the dark under standard tissue culture conditions. Then, the transformants were selected on Murashige and Skoog medium supplemented with 15 mg L−1 1-hygroycin B (H370, PhytoTechnology Laboratories®). Plants of the T0 generation were genotyped by using Cas9-specific primers and the mutants created by Cas9 were identified by sequencing. Cas9-free T1 plants harboring gene-edited mutations were backcrossed to Moneymaker, and their self F2 plants were used for phenotypic observations and gene expression analysis. Oligo and primer information used for vector construction and genotyping described here and hereafter is available in Supplementary Table 2.

Microscopy of SAM morphology. dtm-1, dtm-5r, and their corresponding wild type SAMs were dissected from seedlings at different developmental stages by removing any visible leaves and examined with a stereomicroscope (M125/DFC420, Leica). For SEM and histological analysis, the dissected meristems were fixed in formalin-acetic acid-alcohol (10% formaldehyde, 5% acetic acid, and 50% alcohol by volume). The meristems were gradually dehydrated by incubation in an ethanol series (30–100%). Half of these meristem samples were used for SEM analysis and so further dried using a critical-point dryer (Tousimis, USA), before being sputter coated in gold particles. SEM images were collected using a high-
resolution field emission SEM (Zeiss Merlin Compact, Zeiss or JSM-6360LV, JEOL). The other half were embedded in Paraplast (Sigma-Aldrich) for histological analysis and 8 μm sections made using a Leica microtome (Leica). The sections were briefly stained with 0.05% toluidine blue.

Gene expression analysis. Total 

Gene expression analysis was performed essentially as described in immunoprecipitation technical guide and protocols (Thermo Scientific) with some modifications. Before pulldown, 20 μl of anti-HA agarose resin (Sigma-Aldrich) (for a single reaction) was washed three times with 500 μl lysis buffer (10 m M HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl and 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100) containing 300 mM NaCl and once with 500 μl lysis buffer. The resin was suspended in 500 μl lysis buffer after the last wash and incubated with an aliquot of 50 μl freshly prepared lystate containing HA-HD-ZIP III proteins for 4 h at 4 °C. Anti-HA agarose resin conjugated with HD-ZIP III fusion proteins was collected by a short centrifugation at 5000 × g at 4 °C. The resin was then washed three times with 500 μl lysis buffer containing 300 mM NaCl at 4 °C, followed by three washes with 300 μl lysis buffer containing 0.5% Triton X-100. After washing, the resin was suspended in 500 μl lysis buffer and incubated with 50 μl cytosolic- or cymc-DTM or cymc-DTL lystate overnight at 4 °C. The resin containing the protein complex was then pelleted and washed as described above. The immunoprecipitation protein complex was released from the resin with 30 μl of 0.1 M glycine (pH 2.5) for 30 min at 4 °C.

For Western blot analysis, IP prepared proteins and aliquots of input controls (2% of the amount used for each IP reaction) were separated in 10% (cytc-DTL, -DTM and its mutants) or 12% (HA-HD-ZIP III proteins) SDS-PAGE gels and transferred to Amersham Hy bond P 0.45 PVDF membranes (GE Healthcare Life Science). The membranes were subsequently blotted with primary anti-HA (1:10,000, # 37255, CST) or anti-cmyc (1:2000, # A01728-40, Genscript) antibodies and then with secondary anti-rabbit HRP antibodies (1:5,000, # Ab6721, Abcam) as previously described. To detect the antibody signals, the membranes were incubated in the buffer provided with the Super Signal West Pico Chemiluminescent substrate kit (Pierce). All full-length blots and gels are shown in Supplementary Fig. 14–17.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay was performed essentially according to the procedures established previously with some modifications. Briefly, 2 μg purified SIreve (nucleotide 1–795) plasmid (pTNT™ vector, Promega) and PCR fragments of DTM, DTM, L8F, and DTM, L8F (L22A) coding sequences with the SP6 and T7 polymerase promoter sequences incorporated were used as transcription templates in the Wheat Germ Protein Expression System (Promega). Individual proteins were then synthesized in 10 μl reaction volumes using the TNT™ SP6 High-Yield Wheat Germ Protein Expression System (# S3261, Promega) following the supplier’s instructions. The DNA binding assay, 1 μl aliquots of the synthesized SIreve (1–265aa) protein was first incubated with 1, 2, and 4 μl of wild type DTM protein and its mutant forms DTM, L8F and DTM, L8F (L22A) in gel-shift binding buffer from the LightShift EMSA Optimization and Control Kit (Thermo). After 20 min incubation on ice, the Cy-5 labeled HB-9 DNA duplexes (5’-CAGATCTGTTAT- GATTACGAGAAT-3’) recognized by Rev2 were added and the reactions were incubated at room temperature for another 20 min. The mixtures were then subjected to electrophoresis in a 6% native PAGE gel.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Tomato sequence data for ZPR, HD-ZIP III, and other genes used in this study can be found at the Sol Genomics Network (SGN, https://solgenomics.net/) under the following accession numbers: DTM (Solyc09g009620), DTL (Solyc11g007100), SlZPR1 (Solyc01g91940), SlZPR2 (Solyc09g067570), SlZPR2B (Solyc09g078610), SlCIpla (Solyc09g069740), SlCIplt (Solyc02g046070), SlCIpl (Solyc02g069380), SlCIplt (Solyc09g066560), SlSIpla15A (Solyc03g120910), SlSIplt15B (Solyc02g044410), SwIUC (Solyc02g083950), SlCIpl3 (Solyc10g71380), StSIM (Solyc02g011420), LSFIpl5 (Solyc05g131540). A. thaliana ZPR and HD-ZIP III gene sequences can be found at the Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/index.jsp); ZPR1 (At2g34540), ZPR2 (At1g60809), ZPR3 (At3g52770), LFSIpl2 (At5g12097), ABAC15 (At1g25120), ABAC18 (At1g32880), REV (At1g60960), PHB (At2g42710) and PHV (At1g30490). Source data of floral organ number measurements used for making the boxplot in Fig. 1 is shown in Supplementary data table 1, and source data for expression analysis by qRT-PCR in Fig. 7 and Supplementary Fig. 10 is available in Supplementary data 2 and 3, respectively. All other data generated in this study are available from the authors upon request.

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**Author contributions**
Q.X., R.L., L.W., S.Y., M.L. performed the experiments and analyzed the data; H.X. conceived the study; Q.X., R.L. and H.X. wrote the paper.

**Additional information**
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