Several mutants with aberrant trichome patterning and differentiation are known in Arabidopsis. The genes involved in these processes code for proteins with diverse functions such as transcription, protein degradation, microtubule arrangement, cell wall remodeling etc. Even though the role of these genes in trichome development have been studied by genetic and biochemical methods, the factors that in turn regulate them at the transcriptional, translational and post-translational levels have been less studied. Here, using mutational analysis, we have studied two such factors that control the trichome development genes and dissected the molecular mechanisms adopted by them.

In the first part of the work, we have addressed the role of class II *TCP* genes in general, and *TCP4* in specific, in trichome differentiation, and in the second part, we have addressed the role of *TNI/UBP14* in controlling the proteins involved in trichome development.

TCPs negatively regulate trichome development

The *TCP* genes encode DNA-binding transcription factors and regulate multiple aspects of plant development including leaf shape and size. The Arabidopsis genome encodes 24 *TCP* genes, classified into two major groups; class I and class II, based on their sequence similarity. Among the eleven class II members, five (*TCP2*, *3*, *4*, *10*, *24*) are post-transcriptionally regulated by miR319. Role of miR319-targeted *TCP* genes are well established in leaf morphogenesis at the cellular level as a negative regulators of cell proliferation and as a positive regulators of cellular differentiation. It has been shown that in *TCP* loss-of-function, there is prolonged cell division and in their gain-of-function, there is a precocious differentiation at both organ and cellular levels. However, these studies focused mainly on the role of the *TCP* genes in the pavement cell differentiation without much attention to other epidermal cells with specialized structure and function, namely trichomes and stomata.

Detailed phenotypic analysis of the class II *TCP* loss-of-function as well as and gain-of-function lines suggested that these *TCP* genes negatively regulate both trichome initiation and trichome differentiation. To learn more on the molecular mechanism of *TCP4*-mediated trichome development, we analyzed the results of the DNA microarray analysis where the transcriptome of the 9-day old seedlings of *jaw-D;pTCP4:mTCP4:GR* genotype was compared before and after TCP4 induction and identified four genes involved in trichome development, that was up-

regulated by TCP4. These genes are *GLABROUS INFLORESCENCE STEMS* (*GIS*), *TRICHOMELESS1 & 2* (*TCL1*, *2*) and *ZINC FINGER PROTEIN8* (*ZFP8*) that have been previously shown to regulate both trichome initiation and differentiation. Of the four genes, *GIS* is reported to regulate the trichome branching. Analysis of *GIS* transcript levels in the *TCP* loss-of-function mutants like *jaw-D* and *tcp2;4;10* showed a down-regulation while it was up-regulated in the gain-of-function lines *TCP4:VP16*, *pBLS:rTCP4:GFP* and upon TCP4 induction in the *pTCP4:mTCP4:GR* line. *GIS* transcript was also up-regulated when TCP4 was induced in the absence of additional protein synthesis, suggesting that the TCP4 protein is directly responsible for *GIS* activation. Further, TCP4 is capable of binding to its cognate sites present on the *GIS* locus. While *GIS* is massively activated in the *TCP4:VP16* line leading to reduced trichome branching, The TCP4:VP16 protein failed to suppress trichome branching in the absence of *GIS* as seen in the *gis*;*TCP4:VP16* line. Taken together, these results provide evidence that *GIS* is required for the *TCP4*-mediated inhibition of trichome differentiation.

Previous analysis of several trichome mutants showed that there is a strong correlation between the endoreduplication status of a trichome and its extent of branching. However, there are several other genes including *STI*, *BLT*, *NOK* and *GIS* that regulate trichome branching independent of endoreduplication. However TCPs, regulate *GIS* transcription, independent of endoreduplication pathway.

In addition to branching, analysis of trichome development in the *TCP* loss and gain-of-function mutants has shown that *TCPs* negatively regulate trichome initiation as well. From the analysis of the microarray results mentioned above, all the four identified genes - *TCL1*, *2*, *ZFP8*, *GIS* - are reported to be involved in regulating trichome density. Among these, *TCL1*, and *2* suppress trichome initiation whereas *ZFP8* & *GIS* promote it. It is possible that *TCPs* are involved in maintaining the homeostatic regulation of trichome density by activating both positive and negative regulators of trichome initiation. Gene expression analysis has shown that TCP4 directly up-regulate the expression of all these genes (Results in *Chapter IV*; Krishna Reddy Challa, PhD thesis, 2014). While *TCP4:VP16* suppressed trichome density in Col-0, *tcl1* mutation did not show increased trichome density, possibly because of a functional redundancy between *TCL1* and *TCL2*. However, the *tcl1;TCP4:VP16* plants failed to reduce trichome density, suggesting that TCP4 required *TCL1* for inhibition of trichome initiation and *TCL2* is

not capable of compensating for the loss of *TCL1*. Analysis of the *tcp2;4;10;zfp8* mutant showed that *ZFP8* acts downstream to the *TCP* genes. Taken together, we conclude that *TCPs* act upstream to *GIS*, *ZFP8*, *TCL1/2* to maintain a balanced distribution and differentiation of trichome cells.

TARANI (_TNI_) negatively regulates the trichome differentiation

Analysis of trichome development in the *tni* mutant showed that the TNI protein negatively regulates trichome differentiation alone, without affecting trichome density. *TARANI* encodes the deubiquitinase enzyme UBP14 in Arabidopsis (Premananda, K., PhD Thesis, 2014). Because of the G→A point mutation at the junction of third intron and fourth exon, two different transcripts are formed in the tni mutant; the wild type *TNI* transcript and an aberrant TNI^{intron} transcript where the 3rd intron is incorporated in frame. This result in a two-fold down-regulation of the *TNI* transcript in homozygous *tni* plants compared to Col-0. To determine whether the *tni* trichome phenotype is caused by this decrease in *TNI* level or by the presence of the aberrant transcript TNI^{intron}, we generated a transgenic line where *TNI*, TNI^{intron} and an artificial micro RNA that targets *TNI* specifically in the trichome cells under the control of *GL2* promoter. Analysis of the trichome-branching phenotype in these transgenic lines revealed that, interestingly, both under and over-expression of *TNI* leads to increased trichome branching, emphasizing the requirement for a balanced amount of TNI protein in the cell for proper trichome development.

Genetic analysis of *tni* with known trichome branching mutants has shown that *BLT* is epistatic to *TNI*. Based on the genetic and molecular data, we hypothesize that in *tni* mutant, there is an increased amount of BLT protein that leads to increased trichome branching. To test this, we have raised a *35S::HA-BLT* transgenic line, which shows hyper branched trichome phenotype. Analysis of the *tni;35S::HA-BLT* phenotype, and comparison of BLT protein level between Col-0 and *tni* using anti-HA antibody, would test our hypothesis that BLT is degraded by TNI to suppress trichome branching. We are currently in the process of generating the *tni;35S::HA-BLT* line and hope to obtain the data by the time of thesis defense examination.

Thus, we have added one more transcription factor, TCP4 to the existing trichome developmental pathway (Fig. 6). We have investigated the detailed molecular mechanism for

TCP-mediated regulation of trichome differentiation and have laid the foundation for further studies on role of *TCP* genes in trichome initiation. In the mutants of *TCP* genes, it is known that the pavement cell differentiation is reduced and the studies reported here have shown that the loss of *TCP* function has an opposite effect on trichome differentiation; the branching is increased in the *TCP* mutants. It is possible that the TCP proteins collaborate with different partners in these two cell types to bring about opposite effect on differentiation.

From the second part of this study, we have added a protein degradation factor, TNI/UBP14, to the existing trichome differentiation pathway (Fig. 6). We have shown that, the balanced amount of TNI protein is required for normal trichome development. Unpublished data from our laboratory has shown that TNI is involved in conversion of free polyubiquitin chains into monoubiquitin, which is an essential step in proteasome-mediated degradation of all target proteins (Parinita Majumdar and Utpal Nath). BLT could be one such target protein that is up-regulated in the *tni* mutant leading to hyper-branched trichomes. Increased trichome branching in the *35S::HA-BLT* supports this hypothesis. Comparative estimation of the BLT levels between Col-0 and *tni* plants would enable us to demonstrate this.

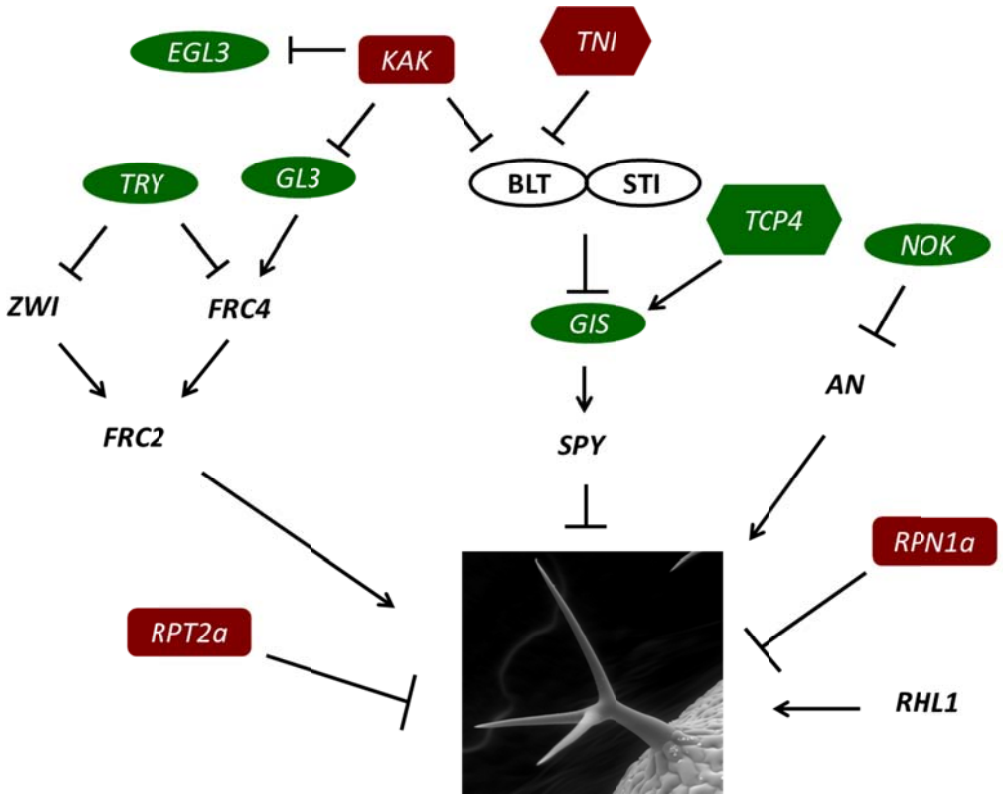

Figure 6 Genetic pathways regulating trichome branching.

We have placed *TCP4* (represented by the green hexagon) upstream to the *GIS* gene and *TNI* (represented by red hexagon) upstream to the BLT protein to the existing trichome pathway (discussed in detail in *Chapter V*). Lines with arrow heads and blunt ends indicate positive and negative regulation, respectively.