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Epidermal differentiation: trichomes in *Arabidopsis* as a model system

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ABSTRACT ***Arabidopsis*** **trichomes are an excellent model system to study all aspects of cell differentiation including cell fate determination, cell cycle regulation, cell polarity and cell expansion. Genetic analysis had initially identified mutants affecting trichome development at different developmental stages. During recent years, molecular analysis of the corresponding genes has revealed a first glimpse of the underlying molecular mechanisms. This paper summarizes some of the recent insights regarding the mechanisms of trichome development.**

KEY WORDS: *trichome, Arabidopsis, cytoskeleton, patterning, differentiation*

The analysis of the development of single cells has been very successful to unravel the interplay of specific developmental regulation events and of more general cellular functions. In the model plant *Arabidopsis* in particular root hairs, pollen tubes, stomata cells and leaf trichomes have been studied in great detail. Although this review focuses on trichome development references to other cell types are included due to the fact that some mutations also affect the development of other cell types in a characteristic manner.

Development of *Arabidopsis* trichomes

Trichomes are thought to be important to protect the plant against herbivores, loss of water through transpiration and UV irradiation (Johnson, 1975; Mauricio and Rausher, 1997). In *Arabidopsis* trichomes are found on most aerial parts of the plant including rosette leaves, stem and cauline leaves but not on the hypocotyl and the cotyledons. Morphology and density differs dramatically on different organs. While trichomes on rosette leaves have 3-4 branches, cauline trichomes show reduced branching and stem trichomes are unbranched. Trichome density on the abaxial side of leaves increases significantly with the plant changing from vegetative to reproductive growth and serves as a convenient marker to search for mutants affecting the transition (Telfer *et al.,* 1997).

The analysis of trichome development has mainly focused on trichomes growing on rosette leaves (Marks, 1997;Hulskamp *et al.,* 1999; Larkin *et al.,* 2003). Here they develop at the base of young leaves in a field of rapidly dividing protodermal cells, some of which are chosen to differentiate into trichomes. Trichome initials are well spaced and almost never occur next to each other. As clustered trichomes would be expected in case of a random distribution it is evident that a mechanism regulating the spacing must exist (Larkin *et al.,* 1996). Incipient trichome cells switch from mitotic divisions to replication cycles without cell divisions (endoreduplication) (Hulskamp *et al.,* 1994). The trichome cell undergoes four endoreduplication cycles resulting in a DNA content of 32C. The trichome enlarges, expands away from the surface and initiates two or more branching events. The orientation of the branches is co-aligned with respect to the leaf basal-apical axis and also the angles between the branches are very regular (Folkers *et al.,* 1997).

Mutational analysis of trichome development

A large number of mutants were isolated in different labs that show defects in trichome development (Marks, 1997; Hulskamp *et al.,* 1999; Larkin *et al.,* 2003). Although the initial screens have identified the major classes of genes required for the key steps of trichome development the number of genes identified to be important for trichome development is steadily growing. This is not only due to higher saturation reached by more intensive screens but also to the application of more refined criteria for mutant evaluations. Initially the mutants were categorized according to the developmental steps that were affected (Hulskamp *et al.,* 1994). A first class of mutants affects trichome initiation such that fewer or more trichomes are formed. A second class of mutants caused aberrations in the regulation of endoreduplication including alterations in the number of endoreduplication cycles or the initial switch

Abbreviations used in this paper: F-Actin, filamentous actin; WAVE, Wiscott-Aldrich syndrome protein family Verprolin-homologous protein.

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from mitosis to endoreduplication. A third class of mutants resulted in an increased or decreased number of branches. A fourth class of mutants showed randomized growth suggesting that they are involved in the directionality of expansion growth. A fifth class of mutants appears to generally affect the maturation of trichomes. This class of mutants will not be considered here as no deeper analysis on these has been reported to date. The molecular and genetic analysis has revealed that each class of genes is very heterogeneous with respect to the underlying cell biological and biochemical mechanisms.

Selecting trichome fate in the epidermis

The regular distribution of trichomes on the leaf epidermis is not obviously linked to any underlying pattern of other cell types or structures in the leaf and hence its establishment requires a mechanism that creates a pattern de novo. Clonal analysis experiments rule out the possibility that a stereotype cell division pattern creates the distribution pattern (Larkin *et al.,* 1996; Schnittger *et al.,* 1999). Therefore the pattern has to be created by intercellular communication. The current models that are based on a theoretical model formulated by Hans Meinhardt (Meinhardt, 1982; Meinhardt, 1994) assume that initially all cells express trichome promoting factors and that these activate their own repressors that can move between cells thereby initiating a competition between the cells (Larkin *et al.,* 2003; Pesch and Hulskamp, 2004). Theoretical considerations require that the trichome promoting factors can self-enhance to enable the rapid amplification of small differences arising from small and random fluctuations.

Genetically, several genes were identified that classify as trichome promoting or trichome repressing factors and generally,

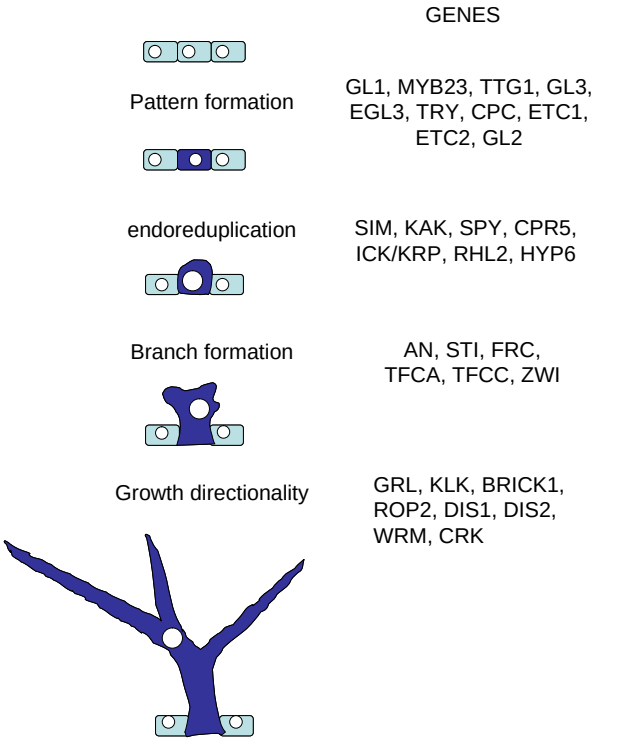

Fig. 1. Trichome development and mutants affecting each developmental stage.

their genetic interactions are consistent with the above mentioned model (Schnittger *et al.,* 1998; Szymanski and Marks, 1998; Schnittger *et al.,* 1999).

Mutations in three genes result in a reduced number of trichomes, *GLABRA3* (*GL3*), or the complete absence of trichomes, *GLABRA1* (*GL1*), *TRANSPARENT TESTA GLABRA1* (*TTG1*) and are therefore considered positive regulators of trichome development (Koornneef, 1981; Koornneef *et al.,* 1982; Hulskamp *et al.,* 1994). The function of *GL3* is redundant with its close homolog *ENHANCER OF GLABRA3* (*EGL3*). If both genes are mutated, the plant is glabrous (Zhang *et al.,* 2003). *GL1*, *GL3* and *EGL3* are likely to act as transcriptional regulators as *GL1* encodes a MYB-related transcription factor and *GL3* and *EGL3* encode helix-loop-helix factors (Oppenheimer *et al.,* 1991; Payne *et al.,* 2000; Zhang *et al.,* 2003). *TTG1* encodes a WD40 domain protein (Walker *et al.,* 1999) and was shown to interact with *GL3* suggesting that it acts as a transcriptional coregulator (Payne *et al.,* 2000).

TRIPTYCHON (*TRY*) was the first identified negative regulator of trichome development. The *try* mutants show clusters of trichomes suggesting that the lateral suppression of trichome fate is affected. *TRY* encodes a small single R3-MYB-repeat protein that has no obvious activation domain (Schellmann *et al.,* 2002). The function of *TRY* is controlled redundantly by at least four genes that are close homologs: The root hair patterning gene *CAPRICE* (*CPC*) turned out to function also during trichome patterning such that *cpc* mutants have more trichomes than wild type. The *try cpc* double mutant showed extremely enlarged clusters containing up to 40 trichomes. Recently two more TRY/CPC-like genes, ENHANCER OF TRY AND CPC 1 (ETC1) and ETC2 have been shown to act redundantly with TRY and CPC. The *try cpc etc2* triple mutant has additional trichomes at the leaf borders (Kirik *et al.,* 2004a) and the *try cpc etc1* triple mutant has leaves that are almost completely covered with trichomes (Kirik *et al.,* 2004b).

Yeast two hybrid interaction assays have revealed that the patterning proteins interact with each other in a characteristic manner. GL3 and EGL3 interact with TTG1 and GL1 but GL1 and TTG1 do not directly interact (Payne *et al.,* 2000; Zhang *et al.,* 2003). In addition TRY was shown to interact with GL3 and the recent finding by Esch and coworkers that TRY and GL1 compete for binding to GL3 suggests a model according to which in trichomes an active complex consisting of GL1, GL3 and TTG1 is operating and in non-trichome cells the complex consisting of TRY, GL3 and TTG1 is inactive (Esch *et al.,* 2003). The cellular interactions required for the cells to interact are thought to be mediated by the negative regulators moving between the cells. The ability to move between cells is so far only documented for CPC. When CPC:GFP fusion protein is expressed under the *CPC* promoter that confers expression only in non-hair cell-files (atrichoblasts), the fluorescence of the fusion protein was not only found in the atrichoblasts but also in the trichoblasts demonstrating the movement ability of CPC (Wada *et al.,* 2002).

The combined action of the patterning genes results in the trichome specific expression of *GLABRA2* (*GL2*). *GL2* encodes a homeobox transcription factor and is thought to be the first target gene of the patterning machinery and responsible for mediating their input into trichome differentiation (Rerie *et al.,* 1994; Cristina *et al.,* 1996; Szymanski *et al.,* 1998). A role of *GL2* in cell differentiation is indicated by the finding that *GL2* regulates the

signal transduction factor phospholipase Dζ1 during root hair development and that phospholipase Dζ1 in turn controls cell differentiation in the root epidermis (Ohashi *et al.,* 2003). In addition *GL2* seems also to be involved in earlier patterning events as the introduction of additional copies of *GL2* in wild type plants results in an altered trichome pattern (Ohashi *et al.,* 2002).

The first step into cell differentiation: the switch from mitosis to endoreduplication

The first visible morphological change of a trichome initial is the increased nucleus resulting from the first endoreduplication cycle. Two types of experiments allow a first glimpse of how this switch from mitosis to endoreduplication is controlled. Mutations in the *SIAMESE* (*SIM*) gene lead to multicellular trichomes (Walker *et al.,* 2000); the molecular nature of the *SIM* gene has not been reported yet. A second approach towards an understanding of how the switch from mitosis to endoreduplication is regulated is mis-expression studies. Trichome-specific expression of known cell cycle genes allowed to circumvent gametophytic or embryonic lethality and to analyze the affect of their overexpression on trichome development. Overexpression of a specific B-type cyclin, which normally triggers the transition from G2 to mitosis caused a similar phenotype as *sim* mutants (Schnittger *et al.,* 2002b). GL2:CYCB1,2 plants have multicellular trichomes with a total amount of DNA similar to wild type. Also D-type cyclin overexpression led to multicellular trichomes. This was surprising as D-type cyclins normally regulate the transition from G1 to S phase, though consistent with this latter function the total amount of DNA per trichome was also increased (Schnittger *et al.,* 2002a).

Stopping growth: regulation of the number of endoreduplication cycles

Several trichome mutants show either reduced or increased ploidy levels (Hulskamp *et al.,* 1994; Jacobsen *et al.,* 1996; Perazza *et al.,* 1999; Kirik *et al.,* 2001; Sugimoto-Shirasu *et al.,* 2002). The genetic and molecular analysis revealed no coherent regulation scheme. The ploidy level is rather controlled independently by various pathways. The first pathway controlling the ploidy level is utilizing the above described patterning genes. Mutations in *GL3* and *TRY* result in reduced and increased ploidy levels respectively suggesting that the patterning genes have a second role in the regulation of the progression of endoreduplication cycles (Hulskamp *et al.,* 1994; Esch *et al.,* 2003). A second pathway is likely to be controlled by the regulation of protein degradation. The *KAKTUS* (*KAK*) gene is important to repress endoreduplication after the fourth endoreduplication cycle; the *kak* mutants have a DNA content of 64 C. The recent cloning of the *KAK* gene revealed that the gene encodes a putative E3 ligase suggesting that *KAK* regulates the ploidy level by ubiquitinating proteins normally promoting the progression of endoreduplication cycles (Downes *et al.,* 2003; El Refy *et al.,* 2004). Third, the plant hormone gibberellin seems to act as a positive regulator of the ploidy level in trichomes. The *spindly* (*spy*) mutant, which behaves as a constitutive gibberellin response mutant, has trichomes with twice the ploidy level as wild type (Jacobsen and Olszewski, 1993). Fourth, one pathway seems to be linked to the cell-death regulation. One line of evidence for this is the finding that the *constitutive pathogen response5* (*cpr5*) mutant exhibits trichomes with reduced ploidy (Kirik *et al.,* 2001). The second line of evidence is the observation that overexpression of *INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASES/KIP-RELATED PROTEINS* (*ICK/KRP*) causes reduced ploidy in trichomes and eventually cell death (Schnittger *et al.,* 2003). Finally, also DNA-topoisomerases involved in the ATP-dependent separation of entangled DNA are required for the progression of endoreduplication cycles. *ROOT HAIRLESS2* (*RHL2*) and *HYPOCOTYL6* (*HYP6*) encode components of the DNA TOPOSOMERASE VI complex and both promote the progression of endoreduplication cycles (Hartung *et al.,* 2002; Sugimoto-Shirasu *et al.,* 2002). One explanation is that in the absence of the isomerase activities a physical block inhibits further replication. Alternatively, the isomerases may be regulators that activate specific cell cycle checkpoints.

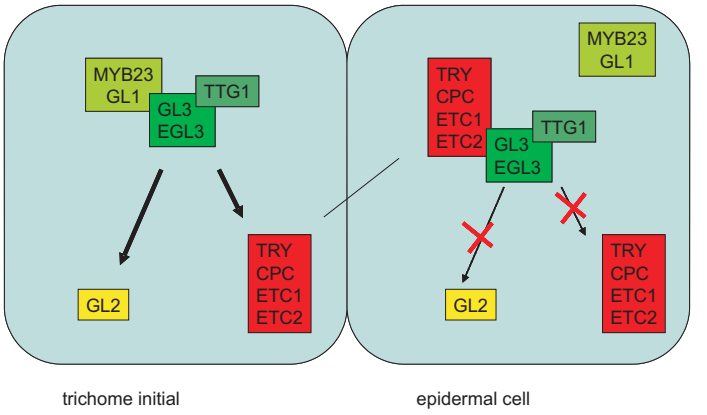

Fig. 2. Patterning Model. *Activators of trichome fate are depicted in green shades, inhibitors are in red. Arrows indicate transcriptional activation. In trichome cells the inhibitors are activated by the activating complex and move into neighboring cells, where they block the activity of the activating complex thereby inhibiting trichome fate.*

Regulation of branch number

A large number of branching mutants have been identified in which the number of branches is either increased or reduced (Folkers *et al.,* 1997; Luo and Oppenheimer, 1999). Generally, mutants with altered ploidy levels are also affected in branch number such that mutants with a reduced DNA content have fewer branches and mutants with increased ploidy levels have more branches (Hulskamp *et al.,* 1994; Folkers *et al.,* 1997). This correlation between ploidy level and branch number indicates that either the size or the growth time is relevant for the initiation of branches. The analysis of drug-treated trichomes and mutant analysis both point to the microtubules as an important regulator of branch initiation. Microtubule drugs not only result in isotropic growth but also inhibit the formation of branches. Conversely the stabilization of microtubules can rescue the unbranched mutant *stichel* (*sti*) (Mathur and Chua, 2000). Several mutations in factors regulating the microtubules were characterized that have little effect on general growth and more specific branching phenotypes enabling to analyze the role of microtubules more specifically. Mutations in the *TUBULIN FOLDING COFACTOR* (*TFC*) *C* and *TFCA* cause an underbranched trichome phenotype (Kirik *et al.,* 2002a; Kirik *et al.,* 2002b). These mutants are expected to interfere

with the making of assembly competent α/β tubulin dimers and are likely to be compromised in the assembly of new microtubules. Thus, the formation of new microtubules is likely to be important for the formation of new branches. Also mutations in factors required for cutting of already existing microtubules, the katanins result in reduced trichome branching. Taken together it is therefore likely that local destruction and new synthesis of microtubules is important to initiate branching. The spatial organization of microtubules is another level of regulation. Mutations in *FASS/TONNEAU2* and *SPIKE* lead to defects in microtubule organization (Traas *et al.,* 1995; Qiu *et al.,* 2002). *FASS/TONNEAU2* encodes a phosphatase-2A subunit and regulates the microtubules by phosphorylation of other proteins (Camilleri *et al.,* 2002). The *SPIKE* gene encodes a protein related to CDM-family (Caenorhabditis elegans CED-5; Homosapiens DOCK180) adaptor proteins, which trigger further downstream events through small RHO-like GTPases (Qiu *et al.,* 2002). The mechanisms by which *FASS/TONNEAU2* and *SPIKE* regulate the microtubule cytoskeleton and branching of trichomes in *Arabidopsis* are still elusive. Finally, also transport along the microtubules may be a specific aspect of branch initiation as mutations in the kinesin motor molecule *ZWICHEL* (*ZWI*) lead to reduced branching (Oppenheimer *et al.,* 1997). *ZWICHEL* was shown to bind to microtubules in a calmodulin-dependent manner and its activity is controlled by the KIC protein, which binds to *ZWI* in a calmodulin-dependent manner (Deavours *et al.,* 1998; Reddy *et al.,* 2004). This implies that Ca2+ may also be involved in the regulation of branch formation. The *ANGUSTIFOLIA* (*AN*) gene appears to be involved in creating a high concentration of microtubules at the tip of the trichome cell. How this is achieved is still mysterious. On the one hand *AN* interacts with *ZWI* in yeast two hybrid essays suggesting a direct involvement. On the other hand *AN* encodes a protein with sequence similarity to CtBP/BARS which would suggest a rather indirect regulation of microtubules by *AN* (Folkers *et al.,* 2002; Kim *et al.,* 2002). CtBP (C-terminal binding proteins) are transcriptional co-factors with a functional overlap to factors called BARS (Brefeldin A Ribosylated Substrates) (Matteis *et al.,* 1994; Nibu *et al.,* 1998).

The only trichome branching gene with undisturbed microtubule organization is the *STICHEL* (*STI*) gene. *STICHEL* appears to regulate branching in a dosage dependent manner as the strength of the phenotype is correlated with the activity of the STI-activity. The molecular function of *STICHEL* is unknown. The *STI* gene encodes a protein with sequence similarity to the DNA-polymerase-III subunits, but no replication phenotype is observed in the *sti* mutants (Ilgenfritz *et al.,* 2003).

Growing straight: trichome expansion is regulated by actin

A group of eight genes, that are collectively referred to as "distorted" mutants share a common trichome phenotype: trichomes develop fairly normal until the branches have been initiated and with the onset of cell expansion branches grow in a randomized manner such that they may twist and bulge or do not grow at all (Hulskamp *et al.,* 1994; Schwab *et al.,* 2003). In addition to the trichome phenotype for some of the mutants also other cell types show morphological defects. These include aberrant root hair growth, the formation of lobes in the pavement cells and the bending of hypocotyl cells out of the epidermal cell layer (Le *et al.,* 2003; Mathur *et al.,* 2003a; Mathur *et al.,* 2003b; El-Assal *et al.,* 2004b; Saedler *et al.,* 2004). The two observations that trichomes treated with drugs interfering with actin function and that most of the "distorted" mutants show an aberrant actin organization strongly suggested that the "distorted" genes regulate cell expansion through the actin cytoskeleton (Mathur *et al.,* 1999; Szymanski *et al.,* 1999). Consistent with this four of the "distorted" genes encode components of the ARP2/3 complex (Li *et al.,* 2003; Mathur *et al.,* 2003a; Mathur *et al.,* 2003b; El-Assal *et al.,* 2004b Saedler *et al.,* 2004). The ARP2/3 complex is known to promote actin formation by initiating F-actin polymerization of new actin filaments on already existing actin (Mullins *et al.,* 1998; Svitkina and Borisy, 1999). The cell biological analysis revealed that actin function is not generally impaired in arp2/3 mutants as peroxisome and Golgi movement along the actin cytoskeleton is still observed. Locally, striking differences were observed in that the velocity was higher in growth regions then in non-growth regions (Mathur *et al.,* 2003a; Mathur *et al.,* 2003b). Another cellular phenotype seen in arp2/3 mutant trichomes is that the fusion of the central vacuole did not occur and as a consequence multiple smaller vacuoles are formed (Mathur *et al.,* 2003a).

The central question of how ARP2/3 activity is spatially controlled is largely unresolved, though evidence is accumulating that suggests that a pathway related to the "WAVE" pathway in animals may exist. The WAVE pathway begins with the perception of signals that regulate the activity of small RHO and RAC-like GTPases. The activated forms bind to a large complex consisting of the two ARP2/3 activating proteins HSPC300 and WAVE and the three repressors PIR121, NAP125 and Abi2. Binding of the small GTPase to the complex releases the three inhibitors and results in the activation of HSPC300

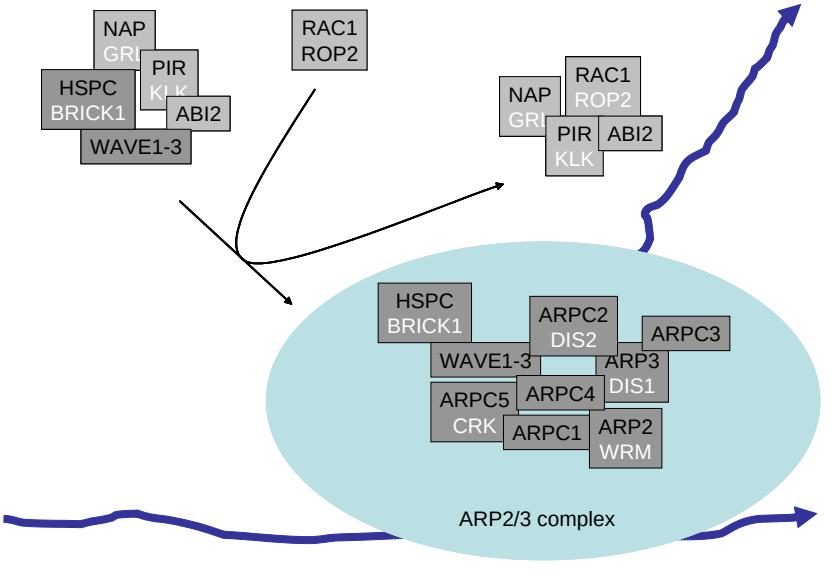

Fig. 3. Regulation of actin by the Arp2/3 complex. *(Explanation in the text). The growing actin strands are depicted in blue, arrowheads indicate the growth direction. Names of plant proteins homologous to the animal proteins are mentioned in white.*

and WAVE. In plants small GTPases related to RHO and RAC/CDC42 called ROPs are known and were shown to be involved in the control of actin organization (Yang, 2002; Mathur and Hulskamp, 2002; Smith, 2003). Also two of the immediate targets of ROPs, NAP125, PIR121, were identified (Basu *et al.,* 2004; Brembu *et al.,* 2004; Deeks *et al.,* 2004; El-Assal *et al.,* 2004a; Li *et al.,* 2004; Zimmermann *et al.,* 2004). The corresponding mutants show a similar phenotypic range as the arp2/3 mutants indicating that they are acting in the same pathway. In addition the HSPC300 homolog (BRICK1) was found in maize (Frank and Smith, 2002). The existence of a functional WAVE homolog is still not demonstrated though putative homologs were described based on sequence similarity comparisons (Brembu *et al.,* 2004).

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