

Cell morphogenesis of trichomes in *Arabidopsis*: differential control of primary and secondary branching by branch initiation regulators and cell growth

Ulrike Folkers¹, Jürgen Berger² and Martin Hülskamp^{1,*}

¹Lehrstuhl für Entwicklungsgenetik, Universität Tübingen, Spemannstrasse 37-39, D-72076 Tübingen, Germany

²Max Planck Institut für Entwicklungsbiologie, Spemannstrasse 35, D-72076 Tübingen, Germany

*Author for correspondence (e-mail: mh@mail.mpi-tuebingen.mpg.de)

SUMMARY

Cell morphogenesis, i.e. the acquisition of a particular cell shape, can be examined genetically in the three-branched trichomes that differentiate from single epidermal cells on the leaves of *Arabidopsis thaliana*. In normal development, the growing trichome cell undergoes two successive branching events, resulting in a proximal side stem and a distal main stem which subsequently splits in two branches. Using new and previously described trichome mutants, we have analyzed the branching pattern in single and double mutants affecting branch number or cell size in order to

determine underlying mechanisms. Our results suggest that primary branching is genetically distinct from subsequent branching events and that the latter, secondary events are initiated in response to positive and negative regulators of branching as well as subject to control by cell growth. We propose a model of how trichome cell morphogenesis is regulated during normal development.

Key words: trichome, cell morphogenesis, *Arabidopsis*, *STACHEL*, *ANGUSTIFOLIA*

INTRODUCTION

During cell morphogenesis cells alter their intrinsic organization by establishing functionally distinct cytoplasmic or plasma membrane/cell wall regions which in turn facilitate morphological changes. While this process remains inconspicuous in many cell types simply due to the lack of morphological criteria, it becomes obvious in some cell types such as neurons (Mitchison and Kirschner, 1988; Bixby and Harris, 1991), polarized epithelial cells (Drubin and Nelson, 1996) or in unicellular organisms like *Dictyostelium* (Condeelis, 1993; Grebecki, 1994), *Acetabularia* (Berger et al., 1987; Menzel, 1996) or *Caulerpa* (Jacobs, 1994). These examples demonstrate that cells can develop complex and differentiated forms sometimes reminiscent of whole organisms – established on the cellular level. Although well studied, most of these experimental systems are not readily accessible to a functional or genetic approach. Most of our current understanding of cell morphogenesis is derived from studying the determination of cell division axes or the establishment of cell polarity (Chant, 1994; Drubin and Nelson, 1996). In both processes an apparently non-polarized cell chooses and determines an axis. Although these model systems provide the framework to understand the regulation of the spatial arrangement of cells along one axis, it is unknown whether even parts of those pathways are employed for setting up the three-dimensional architecture of cells.

We study cell morphogenesis using leaf trichome development in *Arabidopsis* as a genetic model system. The elaborate three-dimensional structure of trichomes provides clear criteria to judge changes of cell form in different mutants. Trichomes are specialized epidermal cells which are regularly distributed on leaves and stems (Uphof, 1962; Hülskamp et al., 1994; Marks, 1994). Trichome cells originate from specific positions in the epidermal layer, then undergo four rounds of endoreplication and a sequence of morphological changes resulting in the mature trichome. The mature leaf trichome is a large, polarized cell with two branch points and a large nucleus located at the lower branch point (Hülskamp et al., 1994). A number of genes have been identified that are required for trichome development (Redei, 1962; Lee-Chen and Steinitz-Sears, 1967; Feenstra, 1978; Koornneef, 1981; Koornneef et al., 1982; Haughn and Somerville, 1988; Marks et al., 1991; Hülskamp et al., 1994). Genetic and cytological analysis of the corresponding mutants resulted in the determination of a sequence of developmental steps for trichome morphogenesis: specification of the trichome cell, regulation of cell size, local outgrowth, extension growth and branching (Hülskamp et al., 1994).

In order to unravel how the trichome cell organizes its architecture we focused on mutants that specifically affect the branching pattern. These mutants provide clear criteria to distinguish changes in cell form. Based on the different relative positions of branch points in these mutants it has been

suggested that a leaf trichome cell may be considered to be composed of different pattern elements (Fig. 1; Hülkamp et al., 1994). The two stems resulting from the primary branching event acquire different qualities. The stem pointing towards the proximal end of the leaf becomes the side stem while the distal stem develops into the main stem which is characterized by the initiation of a secondary branch point. In this work we systematically studied double mutant combinations of mutants affecting branch number or cell size. We provide evidence that three regulation levels can be distinguished: specification of branching events, regulation of branch point number and regulation of branching by cell growth.

MATERIALS AND METHODS

Plants and plant culture

The wild-type strain used in this work was a Landsberg strain carrying the *erecta* mutation. The *gl3* mutant (Koorneef et al., 1982) was obtained from M. Koorneef, Agricultural University, Wageningen, The Netherlands. The following single and double mutants were isolated and have been described previously (Hülkamp et al., 1994): *sti-EMU*, *an-EM1*, *zwi-EM1*, *sta-23*, *try-EM1*, *gl3 an-EM1*, *gl3 try-EM1*, *sti-EMU an-EM1*, *sti-EMU try-EM1*. All plants were grown under constant illumination as described previously (Mayer et al., 1993).

Mutant screening and genetic characterization of mutants

The newly identified EMS induced mutants *sti-40* and *nok-122* were isolated as described previously (Mayer et al., 1991; Hülkamp et al., 1994). Double mutants were constructed by preselecting F₂ plants displaying one of the mutant phenotypes. The progeny of these plants was scored for the phenotype of the other mutant. Backcrosses with both parental lines were performed to confirm the genotype of the double mutants. Two double mutants, *angustifolia stachel* (*an sta*) and *stachel triptychon* (*sta try*), were found to be sporophytically male and female sterile and therefore could not be backcrossed. We used the narrow leaf phenotype of *an* mutants and the 'nest' phenotype of *try* mutants to unambiguously identify one mutant. The double mutants were identified by the new trichome phenotype and the new sterility phenotype. In addition, the segregating F₂ population revealed the respective single mutant and the new double mutant phenotypes with the approximate expected segregation ratio of 9:3:3:1. For *an sta* we found: 278 WT, 110 *an*, 79 *sta*, 22 *an sta* and for *sta try*: 276 WT, 61 *sta*, 62 *try* and 14 *sta try*. A complete list of double mutants constructed and analyzed in this study is shown in Table 1.

Measurements of trichome orientation and the position of branch points and the nucleus

For all measurements involving the position of the nucleus, young leaves were stained in a solution of 4',6-diamino-2-phenylindole (DAPI) at a concentration of 5 µg/ml in PBS for 30 minutes, washed in PBS, mounted and directly inspected with a Zeiss microscope (Axiophot). For measurements of the angle between branches or the relative proximodistal orientation of trichomes with respect to the leaf we used unfixed leaves. Images were photographed with a Kontron Elektronik ProgRes 3012 CCD camera and printed for subsequent measurements. The branch points were defined as the vertex between the two branches. The position of branch points and the nucleus were measured as their distance from the leaf surface. In order to facilitate a comparison between wild type and mutants, the relative proportions of the stalk and the branches and the relative position of the nucleus were determined for individual trichomes.

The orientation of trichomes with respect to the proximodistal leaf axis was defined as the angular distance between the mid rib of the leaf and the orientation of the trichome (in wild-type the orientation

of the side stem, in *an* and *sta* mutants the orientation of the two branches).

Scanning electron microscopy and graphics work

Scanning electron microscopy was done as previously described (Laux et al., 1996). Pictures were processed using Adobe Photoshop 3.0 and Aldus Freehand 7.0 software.

RESULTS

Specification of branching events

In wild-type plants leaf trichomes undergo two consecutive branching events (Fig. 2A-D). Upon emergence out of the epidermal surface the incipient trichome cell initiates primary branching (Fig. 2A,B). The two stems are oriented with an angle of 25 degrees with respect to the proximodistal orientation of the leaf (Fig. 3A). Slightly later, the stem pointing towards the distal end of the leaf, the main stem, undergoes secondary branching (Fig. 2C). Although mature trichomes vary considerably in their absolute size the relative spatial arrangement appears to be fairly regular. The two stems resulting from the primary branching event show an angle of about 119 degrees (Fig. 3). Secondary branching is initiated in a plane perpendicular to the primary branch plane (Fig. 2D). Secondary branches exhibit an angle of about 83 degrees. The nucleus is typically positioned at the lower branch point (Fig. 3).

The analysis of mutant phenotypes suggests that primary and secondary branching are genetically distinct. Our results suggest that two genes, *STACHEL* (*STA*) and *ANGUSTIFOLIA* (*AN*), are required for the specification of the primary and the secondary branching events respectively. This classification is based on four criteria: (1) the length of the stalk and the branches; (2) the angle between the two branches; (3) the orientation of the two branches with respect to the proximodistal axis of the leaf, and (4) the position of the nucleus.

an mutants seem to undergo primary branching but to skip secondary branching (Fig. 2I). This is suggested by the *an* mutant phenotype (Fig. 2F). In *an* mutants the branch point was found at approximately the same position as the primary branch point in wild-type trichomes (no significant difference was found in a Student's *t*-test, Fig. 3). The two branches form an angle of 116 degrees and are oriented with an angle of 26 degrees relative to the proximodistal axis of the leaf (Fig. 3). The nucleus is situated slightly lower than in wild-type at the base of the two branches (Fig. 3). In contrast primary branching appears to be lacking in *sta* mutants (Fig. 2E,H). In *sta* mutants trichomes show strikingly different proportions with a long stalk and two short branches. The branch point is slightly lower than the secondary branch point in wild-type but significantly higher than in *an* mutants (Student's *t*-test, $\alpha=0.001$). The two branches are arranged with an angle of 85 degrees (Fig. 3). Unlike in *an* mutants, no obvious orientation of the two branches relative to the leaf axis was observed (Fig. 3). The nucleus is located in the stalk below the branch point (Fig. 3, absolute and relative positions are significantly different between *sta* and *an* mutants, Student's *t*-test, $\alpha=0.001$).

These data suggest that the two branching events are specified separately by *AN* and *STA*. This was tested by analysis of *an sta* double mutants. *an sta* double mutants show unbranched trichomes (Fig. 2G) indicating that both genes are

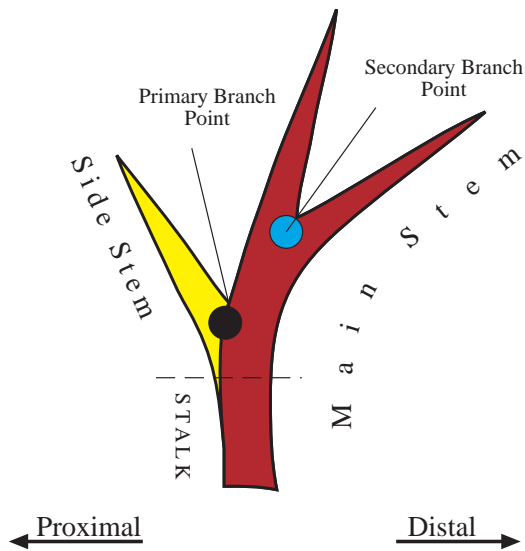


Fig. 1. Schematic representation of pattern elements of a trichome cell. Based on trichome mutants affecting the branching pattern, different pattern elements can be distinguished. The two branches resulting from the primary branch point (shown as a black circle) are of different quality. The branch pointing to the distal end of the leaf develops to the main stem (shown in red). The proximal branch is called the side stem (shown in yellow). The secondary branch point (shown as a blue circle) is initiated on the main stem.

required to get branching at all. In *an* mutants, the primary branching appears to be carried out through *STA*, while in *sta* mutants *AN* mediates the secondary branching. This suggests a genetically independent specification of primary and secondary branching events.

Regulation of primary and secondary branching

In order to analyze the regulation of primary and secondary branching we studied mutations in two genes, *STICHEL* (*STI*) and *NOECK* (*NOK*), that result in fewer or more branch points but do not affect cell size.

The *STI* gene has been previously described to play a role in the initiation of branching (Hülkamp et al., 1994). In the strong *stichel* (*sti*) mutant *sti-EMU* most trichomes do not develop any branch points (Fig. 4F,K; Table 1). Double mutant combinations with *an* and *sta* revealed completely unbranched trichomes suggesting that *sti* is epistatic (Table 1). A newly identified *sti* allele, *sti-40*, has one branch point resting on a long stalk (Table 1). Transheterozygous combinations of *sti-EMU* and *sti-40* display the branched *sti-40* phenotype. Since the unbranched phenotype of *sti-EMU* is recessive to the branched phenotype of *sti-40*, *sti-EMU* probably represents a strong *sti* allele. These findings suggest a quantitative requirement of *STI* activity for branch initiation.

Mutations in the *NOK* gene result in glassy trichomes with an increased branch point number. *nok* mutant trichomes are normal in size, as judged by nuclear size, but produce up to seven branch points (Fig. 4A-E; Table 1). These branch points are initiated sequentially: the incipient trichome initiates primary branching, then secondary branching starts, which is indistinguishable from wild type (Fig. 4A,B). Subsequently the developing trichome keeps initiating new branch points on

both the side and the main stem (Fig. 4C,D). A slight increase in branch point number can also be observed in heterozygous *nok* plants (Table 1).

The supernumerary branching events appear to be secondary branchings. This can be inferred from *an nok* and *sta nok* double mutants. In *an nok* double mutants trichomes display an *an* phenotype (Table 1; Fig. 4G), indicating that *an* is epistatic to *nok*. In contrast *sta nok* double mutants develop between two and four branch points (Table 1; Fig. 4H), indicating that supernumerary secondary branch points are initiated (Fig. 4J).

Mutations in *STI* and *NOK* result in opposite phenotypes: fewer branch points or supernumerary branch points. In order to understand the genetic interaction between *STI* and *NOK* we studied double mutant combinations of *nok* with the weak and strong *sti* alleles. Surprisingly we found that *nok* partially suppresses the strong *sti* phenotype resulting in *sta*-like trichomes (Table 1, Fig. 2I). The double mutant combination of *nok* with the weak *sti-40* allele gives rise to two branch points (Table 1). These findings suggest that mutations in the *NOK* gene rescue *sti* mutants in a quantitative manner.

Regulation of branching by cell growth

It has been observed previously that the number of branch

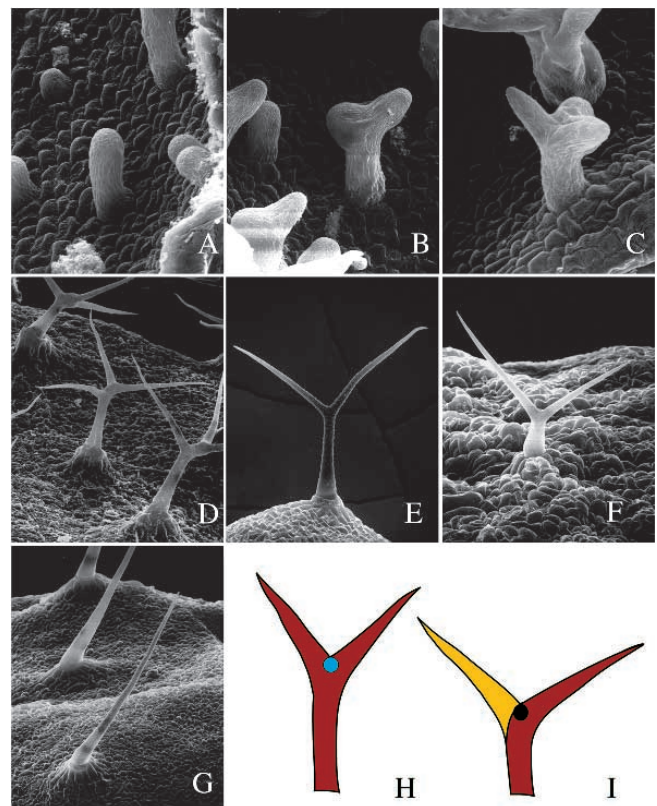


Fig. 2. Branch point specification in trichomes. (A-G) SEM micrographs of leaf trichomes. (A) Young unbranched wild-type trichome; (B) young wild-type trichome with the primary branch point; (C) young wild-type trichome with three incipient branches; (D) mature wild-type trichome; (E) mature *sta-23* trichome; (F) mature *an-EM1* trichome; (G) mature *sta-23 an-EM1* trichome; (H) schematic drawing of the *sta* phenotype; (I) schematic drawing of the *an* phenotype.

points is altered in trichome mutants in which the nuclear size is affected (Hülkamp et al., 1994). Trichome mutants with a reduced DNA content, such as *glabra3* (*gl3*) (Fig. 5D,G), have smaller cells with fewer branch points whereas trichome mutants with a larger DNA content as exemplified by *triptychon* (*try*) (Fig. 5A) show an increase in cell size and develop trichomes with more branch points. This correlation implies that the initiation of branch points is regulated or integrated by cell size or cell growth.

Double mutant analysis of *try* with branching pattern mutants revealed that in *try* mutants the initiation of secondary branching but not primary branching is promoted (Compare Fig. 5E,F, Table 1). While *sta try* mutant trichomes have two or even three branch points (Fig. 5F) *an try* double mutants show only one branch point (Fig. 5E).

The function of *sti* and *nok* was found to be independent of *TRY*. The double mutant combination of *sti* and *try* results in larger but unbranched trichome cells (Fig. 5B) indicating that *sti* is epistatic to *try*. In contrast *nok try* double mutants show an additive phenotype resulting in trichomes with up to 11 branch points (Fig. 5C).

To determine whether the same regulatory rules apply when trichome growth and final size is reduced we analyzed double mutant combinations of *gl3* with all branching mutants. The *gl3* allele used in this study results in about 85% of all trichomes

having one branch point. Both double mutant combinations of *gl3* with *an* and *sta* resulted in a reduced, but still prominent class of branched trichomes (Table 1). Therefore, it is not possible to assign a specific quality to the branch point found in *gl3* mutants. This result suggests that either of the two branch points is initiated with a certain probability when a critical minimum cell size is reached. This view is supported by the finding that double mutants of *gl3* with mutations promoting the initiation of additional secondary branching, *nok* and *try*, do not result in more branch points than *gl3* mutant trichomes (Table 1).

Regulation of branching competence

Mutations in *ZWICHEL* (*ZWI*), result in a phenotype reminiscent of *an* (compare Figs 1F and 6A). However, *zwi* trichomes frequently are more stunted and have a short stalk (Oppenheimer et al., 1997). Moreover, the branching pattern ranges from unbranched trichomes, trichomes with an extremely reduced distal stem (Fig. 6B) to trichomes with two stems of equal length (Fig. 6A). This phenotypic spectrum suggests that in *zwi* trichomes the development of the main stem as opposed to the initiation of a specific branch point is affected (Fig. 6F). If this assumption is correct one would expect that the general ability to initiate primary or secondary branch points is not generally affected in *zwi* trichomes. We tested this in double mutant combinations of *zwi* with *an* and *sta*. In *sta zwi* double

Table 1. Number of branch points in wild type and trichome mutants

	Number of branch points†											Total*	
	0	1	2	3	4	5	6	7	8	9	10		11
Ler			94	6									379
sti-EMU	86	14											591
sti-40	5	95											485
an-EM1	2	97	1										294
zwi-EM1	7	93	<1										563
sta-23	2.5	95	2.5										238
nok-122			<1	18	51	20	10	1					552
nok-122 het‡			51	48	1								822
try-EM1			4	25	44	24	4						552
try-EM1 het‡			59	41									594
gl3	13	85	2										685
try-EM1 sti-EMU	92	8											291
try-EM1 an-EM1	2	89	9										403
try-EM1 zwi-EM1	4	93	3										347
try-EM1 sta-23		<1	88	9	2								164
try-EM1 nok-122					<1	3	12	30	22	18	10	4	233
try-EM1 gl3	7	70	23										387
nol-122 sti-EMU	12	83	5										367
nok-122 sti-40		7	86	7									437
nok-122 an-EM1	<1	99	1										396
nok-122 zwi-EM1	5	92	3										341
nok-122 sta-23		2	63	31	3		<1						204
nok-122 gl3	5	83	12										105
gl3 sti-EMU	100												415
gl3 an-EM1	79	21											266
gl3 zwi-EM1	91	9											576
gl3 sta-23	63	37											191
sti-EMU an-EM1	100												293
sti-EMU zwi-EM1	95	5											499
sti-EMU sta-23	100												212
zwi-EM1 an-EM1	96	4											432
zwi-EM1 sta-23	69	31											286
an-EM1 sta-23	96	4											252

*Total number of trichomes counted on leaves 3 and 4 of more than 10 plants.

†in %.

‡het=heterozygous, F₁ plants derived from the cross between the homozygous mutant parent and Ler.

mutants we found trichomes displaying a branching pattern similar to that in *sta*. Although the frequency of branched trichomes was reduced compared to the single mutants (Table 1), this shows that *zwi* trichomes are able to initiate a secondary branching. In double mutant combinations of *an* and *zwi* most trichomes display an unbranched phenotype. This suggests either that *zwi* trichomes can not initiate primary branching in the absence of AN or that AN and ZWI act in a redundant fashion to facilitate main stem growth. The latter is supported by the finding that the few branched trichomes found in the double mutant have a very low branch point. In these trichomes the stalk is extremely short or not visible at all. This suggests that stalk growth in *an zwi* trichomes is even more reduced than in *zwi* mutants.

The phenotype of the *zwi nok* double mutant indicates that in *zwi* mutants not only the main stem is affected but also the side stem. While in *nok* mutants extra secondary branch points are initiated on both the main and the side stem, no additional branch points are initiated on either stem in the *zwi nok* double mutant. Instead the main stem was often found to be twisted (Fig. 6C). Taking into account that *zwi* trichomes are able to initiate secondary branch points (as shown in the *sta zwi* double mutant) the lack of additional branch points on both stems in the *zwi nok* double mutant indicates that reduced growth and branching competence is not specific to the main stem but a more general affect in *zwi* mutants.

Similarly *zwi try* double mutants are larger, but have only one branch point indicating that *zwi* is epistatic to *try*. Occasionally we found a branched side stem (Fig. 6D) and sometimes the main stem was twisted (Fig. 6E). These data suggest that branching competence in *zwi* mutants is not rescued by increased cell size or cell growth.

DISCUSSION

Leaf trichome development in *Arabidopsis* involves a temporally defined sequence of events: initiation, three rounds of endoreplication, local outgrowth, initiation of primary branching, another round of endoreplication and secondary branching. Branching occurs when a critical cell size is reached. Primary branching is initiated after three rounds of endoreplication resulting in a proximal and a distal branch, pointing towards the base and the tip of the leaf respectively. Subsequently the distal stem becomes the main stem and initiates secondary branching (Hülskamp et al., 1994). The different development of the proximal and the distal stem reflects an intrinsic polarity of the trichome.

The identification of mutants affected in distinct aspects of trichome cell morphogenesis suggests independent genetic requirements for different steps. Based on the phenotype of

these mutants the architecture of a trichome cell can be considered to be composed of different pattern elements (Fig. 1). In order to define pattern elements and to analyze their regulation we studied single and double mutants affecting trichome branching or cell size.

Primary and secondary branching are genetically distinct

Although the primary and secondary branch points are initiated successively they do not depend on each other. The finding that *an sta* double mutants are unbranched indicates that the primary and secondary branch points result from genetically independent

A	Wild type	<i>sta</i>	<i>an</i>
Position of the primary branch point (μm)	166 \pm 28	–	159 \pm 30
Position of the secondary branch point (μm)	200 \pm 30	188 \pm 32	–
Position of the nucleus (μm)	155 \pm 35	122 \pm 33	129 \pm 30
Length of the side stem (μm)	185 \pm 37	–	320 \pm 56
Length of the secondary branches (μm)	112 \pm 49	237 \pm 44	–
Angle between side and main stem	109 \pm 6	–	116 \pm 10
Angle between secondary branches	83 \pm 9	85 \pm 10	–
Angle between trichome orientation and proximodistal leaf axis	25 \pm 12	43 \pm 26	26 \pm 15

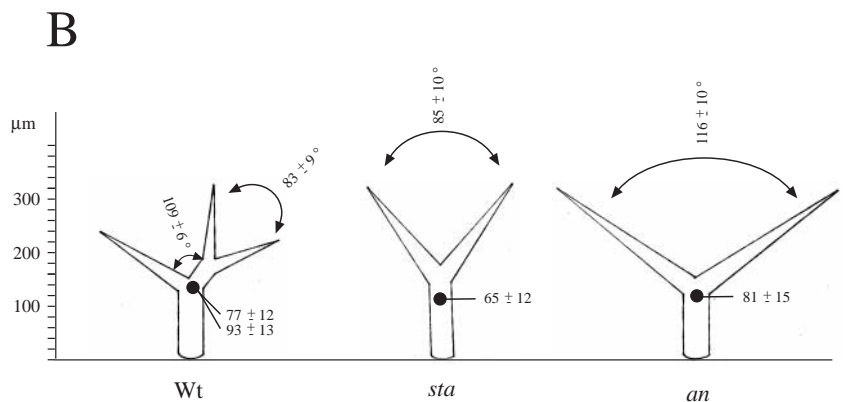


Fig. 3. Measurements of the orientation of trichomes with respect to the leaf axis, the stem length, the branch length, the position of the nucleus and the angle between two branches. (A) Absolute measurements. The means and standard deviations are shown for different parameters and represent measurements of 100 individual trichomes. All length measurements are shown in μm . (B) Relative proportions. The large variation in size makes it difficult to compare the different parameters between wild type and mutants. To facilitate a comparison, the trichomes shown in B are drawn to scale. The values indicate the relative position of the nucleus. The distance between the leaf surface and the branch points is defined as 100%. The means of the relative positions and the standard deviations are shown. For wild-type the relative positions of the nucleus are calculated with respect to the primary (77 ± 12) and the secondary (93 ± 13) branch point.

branching events regulated by STA and AN. Hence these two genes are required for initiating and/or assembling specific branch points (Figs 1 and 7).

Primary and secondary branching is differentially regulated by cell growth

A critical minimal cell size appears to be required to initiate primary and secondary branching. In *gl3* mutants trichomes fail to undergo the fourth round of endoreplication resulting in a smaller cell that usually shows only one branching event. In wild-type trichomes initiation of the primary branch point coincides with the completion of the third round of endoreplication. This suggests that in *gl3* mutants primary branching but not secondary branching takes place. Double mutants of *gl3* with *an*, however, show a greatly reduced frequency of branched trichomes. This implies that most *gl3* mutant trichomes do not reach the critical cell size required to initiate primary branching. The finding that *gl3 sta* double mutants also show a prominent class of branched trichomes suggests

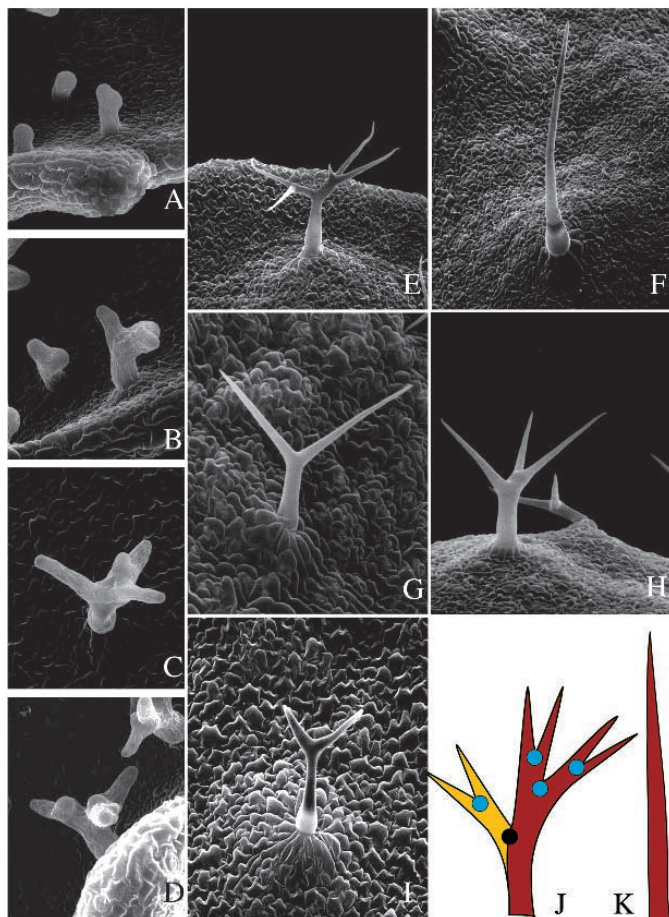


Fig. 4. Regulation of branch point initiation in trichomes. (A-I) SEM micrographs of leaf trichomes. (A) Young *nok-122* trichome with one branch point. (B) Young *nok-122* trichome with two branch points. (C) Young *nok-122* trichome with three branch points. (D) Young *nok-122* trichome with four branch points. (E) Mature *nok-122* trichome. (F) Mature *sti-EMU* trichome. (G) Mature *an-EM1 nok-122* trichome with no extra branch points. (H) Mature *sta-23 nok-122* trichome with two branch points. (I) Mature *sti-EMU nok-122* trichome with one branch point. (J) Schematic drawing of the *nok* phenotype. (K) Schematic drawing of the *sti* phenotype.

that secondary branching can also occur if primary branching fails. It is important to note that secondary branching can be initiated at a much smaller cell size than during wild-type development.

In contrast, increased cell size and cell growth, as caused by mutations in the *TRY* gene, results in supernumerary branch points. Additional branching, however, depends on the function of AN but not STA. Therefore the supernumerary branch points found in *try* trichomes have the quality of secondary branch points (Fig. 7).

The number of secondary branching events clearly depends on cell size or cell growth, though it is not obvious by what mechanisms this might work. The simplest assumption is that secondary branching is initiated in response to the amount of a branching promoting factor. The amount of such a factor would be reduced or increased in smaller or larger cells, respectively and, as a consequence, would affect the number of secondary branching events (Fig. 7).

Secondary branching events are initiated in response to positive and negative regulators

Mutations in *STI* and *NOK* result in fewer or more branch points respectively. Cell size, however, is not affected in either

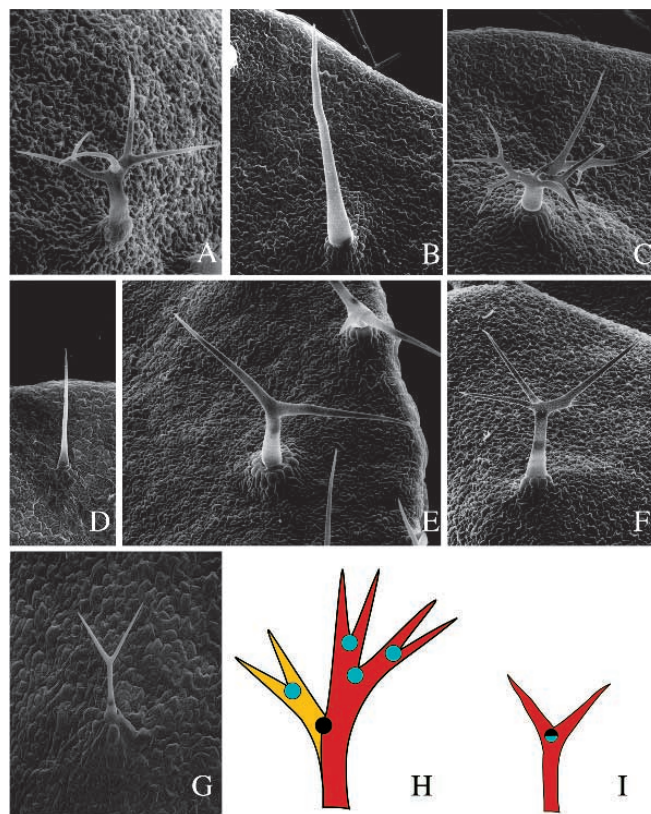


Fig. 5. Regulation of the branch pattern by cell growth. (A-G) SEM micrographs of leaf trichomes. (A) Mature *try-EM1* trichome with four branch points. (B) Mature *try-EM1 sti-EMU* trichome. (C) Mature *try-EM1 nok-122* trichome with eight branch points. (D) Mature *gl3* trichome. (E) Mature *try-EM1 an-EM1* trichome. (F) Mature *try-EM1 sta-23* trichome with three branch points. (G) Mature *gl3* trichome with one branch point. (H) Schematic representation of the *try* phenotype. (I) Schematic drawing of the *gl3* phenotype; note that the branch point is indicated as a primary and secondary branch point without a clear identity.

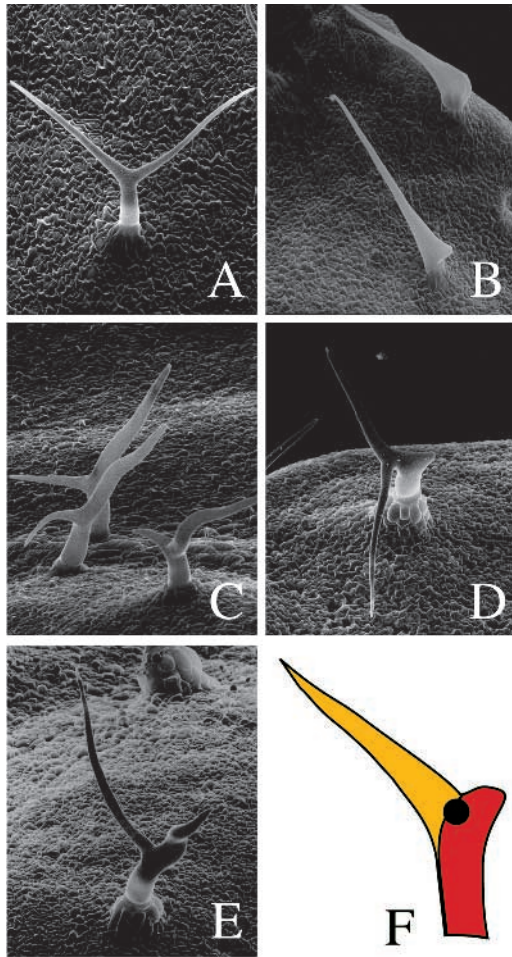


Fig. 6. Regulation of main stem growth. (A-E) SEM micrographs of leaf trichomes. (A) Mature *zwi-EM1* trichome, both branches are equal in size. (B) Mature *zwi-EM1* trichome with an extremely reduced main stem. The main stem was recognized due to orientation relative to the proximal-distal axis of the leaf. (C) Mature *zwi-EM1 nok-122* trichome with a slightly twisted main stem. (D) Mature *zwi-EM1 try-EM1* trichome with a branched side stem. (E) Mature *zwi-EM1 try-EM1* trichome with a twisted main stem. (F) Schematic drawing of the *zwi* phenotype.

mutant. One could describe these mutants by considering them to affect the availability or stability of a branching promoting factor.

In this scenario *sti* mutants have no or little branching promoting activity. Differences in the allelic strength of *sti* alleles could easily be explained by different levels of the branching promoting factor. This view is also supported by the finding that increased cell size (i.e. in *try sti* double mutants) does not affect the *sti* phenotype. In contrast *nok* mutants would be expected to have higher levels of branching promoting activity and hence more secondary branch points than wild type. Consistent with this idea the *nok* phenotype is enhanced in double mutants which results in an increased cell size (i.e. in *nok try* double mutants). However, a certain minimal cell size appears to be necessary since *nok gl3* double mutants show a *gl3* phenotype.

Secondary branching is regulated by two independent pathways, a branch initiation pathway and a cell growth

pathway (Fig. 7). Both pathways involve positive and negative regulators of branching that show strikingly different regulatory interactions. Cell growth is positively regulated by GL3 and negatively controlled by TRY with *gl3* being epistatic to *try*. Hence it is likely that TRY exerts its effect on branching indirectly through its function as an inhibitor of GL3 (Fig. 7). By contrast, in the branch initiation pathway, the negative regulator NOK and the positive regulator STI counteract each other (Fig. 7). We found that the lack or reduction of the positive regulator STI can be partially rescued by removing the negative regulator NOK. Moreover, rescue of the *sti* phenotype in *nok sti* double mutants was found to be dependent on the phenotypic strength of the *sti* allele, suggesting a quantitative mode of regulation for one or both activities.

Regulation of primary branching

While the regulation of secondary branching depends on cell growth as well as on positive and negative regulation factors, primary branching only requires a minimal critical cell size and the activity of STI (Fig. 7). However, the requirement of STI for both branching events indicates a partial regulatory overlap between primary and secondary branching. An attractive explanation for this overlap is that primary and secondary branching occur at different levels of STI activity. This would imply that STI provides spatial information for branch initiation. Alter-

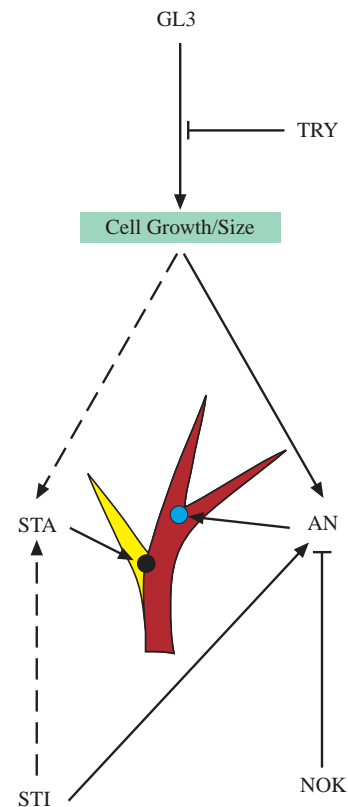


Fig. 7. Model for the genetic regulation of branching in *Arabidopsis* trichomes. Branching is regulated by positive and negative regulatory factors and by cell growth. Positive regulatory interactions are indicated by lines ending in arrows. Negative regulatory interactions are indicated by lines terminating in a vertical bar. Dashed lines indicate a qualitative requirement, solid lines indicate quantitative regulation.

natively it is possible that STI is qualitatively required such that STI and STA act in concert to initiate primary branching.

Regulation of branching competence and growth maintenance by ZWI

A qualitative distinction between a proximal and a distal stem is evident from the competence of the distal stem to initiate secondary branching. This view is supported by the finding that in *zwi* mutants specifically main stem growth but not side stem growth is affected. In principle this difference could either reflect a qualitative difference in branching competence or a quantitative difference such as preferential growth of the main stem and, as a consequence, additional branching. The latter view is supported by the finding that both mutants producing supernumerary branch points, *nok* and *try*, initiate extra branch points on both the proximal and the distal stem.

The role of ZWI for main stem growth and branch initiation, however, remains unclear. On the one hand *zwi* mutant trichomes are capable of forming secondary branches (as shown by the *sta zwi* double mutant). On the other hand the *zwi* phenotype cannot be suppressed in double mutant combinations with mutations that result in supernumerary secondary branch points. Unexpectedly, also the proximal stem did not initiate extra secondary branches in *nok zwi* and *try zwi* double mutants. This suggests that growth and branching competence in *zwi* trichomes is not only affected in the main stem but generally in the whole trichome cell. The recent cloning of the ZWI gene sheds some light on the dual role in growth and branching (Oppenheimer et al., 1997). The ZWI gene encodes a member of the kinesin-like microtubule motor proteins containing a calmodulin-binding site. This suggests that ZWI is probably involved in Ca²⁺ dependent intracellular transport processes required for branching and growth. The genetic finding that ZWI acts in parallel with all other branching mutants may suggest that the directionality and spatial orientation of ZWI dependent transport processes is controlled by genes like *AN*, *STA*, *STI* and/or *NOK*.

Perspective

Cell morphogenesis has been studied in a wide range of organisms and for different cell types. In plants, most of our knowledge is derived from studying the growth of pollen tubes (Heslop-Harrison, 1987; Mascarenhas, 1993), and root hairs (Sievers and Schnepf, 1981). Generally speaking, it is thought that growth direction is regulated by localizing and directing the cytoskeleton, which subsequently will direct the orientation of growth. Different mechanisms have been suggested to be involved in the spatial regulation, including asymmetric distribution of Ca²⁺ (Pierson and Cresti, 1992; Feijo et al., 1995) or localized activity of small GTPases (Lin et al., 1996). The initial growth of trichomes out of the epidermis is reminiscent of tip growing cells such as pollen tubes and root hairs. To date the mechanisms underlying the growth and development of trichome cells are largely unknown. The regulatory network described in this study provides the framework for a cellular and molecular analysis of cell morphogenesis in trichomes.

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REFERENCES

- Berger, S., Groot, E. J. d., Neuhaus, G. and Schweiger, M. (1987). Acetabularia: a giant single cell organism with valuable advantages for cell biology. *Europ. J. Cell Biol.* **44**, 349-370.
- Bixby, J. L. and Harris, W. A. (1991). Molecular mechanisms of axon growth and guidance. *Annu. Rev. Cell Biol.* **7**, 117-159.
- Chant, J. (1994). Cell polarity in yeast. *Trends Genet.* **10**, 328-333.
- Condeelis, J. (1993). Understanding the cortex of crawling cells: insights from *Dictyostelium*. *Trends Cell Biol.* **3**, 371-376.
- Drubin, D. G. and Nelson, W. J. (1996). Origins of cell polarity. *Cell* **84**, 335-344.
- Feenstra, W. J. (1978). Contiguity of linkage groups 1 and 4, as revealed by linkage relationships of two newly isolated markers *dis-1* and *dis-2*. *Arabid. Inf. Serv.* **15**, 35-38.
- Feijo, J. A., Malho, R. and Obermeyer, G. (1995). Ion dynamics and its possible role during in vitro pollen germination and tube growth. *Protoplasma* **187**, 155-167.
- Grebecki, A. (1994). Membrane and cytoskeletal flow in motile cells with emphasis on the contribution of free-living amoeba. *Int. Rev. Cytol.* **148**, 37-79.
- Haughn, G. W. and Somerville, C. R. (1988). Genetic control of morphogenesis in *Arabidopsis*. *Dev. Genet.* **9**, 73-89.
- Heslop-Harrison, J. (1987). Pollen germination and pollen-tube growth. *Int. Rev. Cytol.* **107**, 1-78.
- Hülskamp, M., Misera, S. and Jürgens, G. (1994). Genetic dissection of trichome cell development in *Arabidopsis*. *Cell* **76**, 555-566.
- Jacobs, W. P. (1994). Caulerpa. *Scientific American* **271**, 100-104.
- Koornneef, M. (1981). The complex syndrome of *ttg* mutants. *Arabid. Inf. Serv.* **18**, 45-51.
- Koornneef, M., Dellaert, L. W. M. and Veen, J. H. V. d. (1982). EMS- and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana*. *Mutat. Res.* **93**, 109-123.
- Laux, T., Mayer, K. F. X., Berger, J. and Jürgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* **122**, 87-96.
- Lee-Chen, S. and Steinitz-Sears, L. M. (1967). The location of linkage groups in *Arabidopsis thaliana*. *Can. J. Genet. Cytol.* **9**, 381-384.
- Lin, Y., Wang, Y., Zhu, J. and Yang, Z. (1996). Localization of a rho GTPase implies a role in tip growth and movement of the generative cell in pollen tubes. *Plant Cell* **8**, 293-303.
- Marks, M. D., Esch, J., Herman, P., Sivakumaran, S. and Oppenheimer, D. (1991). A model for cell-type determination and differentiation in plants. In *Molecular Biology of Plant Development* Symposia of the Soc. Exp. Biol. No. XLV. Cambridge, UK: Company of Biologists Ltd.
- Marks, M. D. (1994). The making of a plant hair. *Current Biology* **4**, 621-623.
- Mascarenhas, J. P. (1993). Molecular mechanisms of pollen tube growth and differentiation. *Plant Cell* **5**, 1303-1314.
- Mayer, U., Ruiz, R. A. T., Berleth, T., Misera, S. and Jürgens, G. (1991). Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* **353**, 402-407.
- Mayer, U., Berleth, T., Ruiz, R. A. T., Misera, S. and Jürgens, G. (1993). *Pattern Formation During Arabidopsis Embryo Development*. New York: Plenum Publishing.
- Menzel, D. (1996). The role of cytoskeleton in polarity and morphogenesis of algal cells. *Curr. Op. Cell Biol.* **8**, 38-42.
- Mitchison, T. and Kirschner, M. (1988). Cytoskeletal dynamics and nerve growth. *Neuron* **1**, 761-772.
- Oppenheimer, D. G., Pollock, M. A., Vacik, J., Szymanski, D. B., Ericson, B., Feldmann, K. and Marks, M. D. (1997). Essential role of a kinesin-like protein in *Arabidopsis* trichome morphogenesis. *Proc. Natl. Acad. Sci. USA* **94**, 6261-6266.
- Pierson, E. S. and Cresti, M. (1992). Cytoskeleton and cytoplasmic organization of pollen and pollen tubes. *Int. Rev. Cytol.* **140**, 73-125.
- Redei, G. P. (1962). Single locus heterosis. *Zeitschrift für Vererbungslehre* **93**, 164-170.
- Sievers, A. and Schnepf, E. (1981). Morphogenesis and polarity of tubular cells with tip-growth. *Cell Biol. Monogr.* **8**, 265-299.
- Uphof, J. C. T. (1962). *Plant Hairs*. Berlin, Gebr. Bornträger.