Development 126, 5547-5557 (1999)
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DEV0265

Genetic control of trichome branch number in *Arabidopsis*: the roles of the *FURCA* loci

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Accepted 5 October; published on WWW 24 November 1999

SUMMARY

We are using trichome (hair) morphogenesis as a model to study how plant cell shape is controlled. During a screen for new mutations that affect trichome branch initiation in *Arabidopsis*, we identified seven new mutants that show a reduction in trichome branch number from three branches to two. These mutations were named *furca*, after the Latin word for two-pronged fork. These seven recessive mutations were placed into four complementation groups that define four new genes: *FURCA1*, *FURCA2*, *FURCA3* and *FURCA4*. The trichome branch number phenotype indicates that the *FURCA* genes encode positive regulators of trichome branch initiation. Analysis of double mutants suggests that primary and secondary branch initiation events are not genetically distinct, but rely on the levels of partially redundant groups of regulators of trichome branch initiation. Based on the analysis of both epistatic and additive genetic interactions between the *FURCA* genes and other genes that control trichome branch number, we propose a model that explains how these genes interact to control trichome branch initiation. This model successfully predicts the phenotypes of all the single and double mutants examined and suggests points of control of the trichome branch pathway.

Key words: *Arabidopsis thaliana*, Trichome, Cell shape, Cell differentiation, Cell expansion

INTRODUCTION

A fundamental question in developmental biology is how is cell shape controlled. In plants, cell shape is important not only for the function of the individual cell, but also for its contribution to the generation of plant form. Because plant cells are surrounded by a cell wall any changes in cell shape must be coupled with appropriate expansion of the cell wall. Although much has been learned about the mechanisms of cell expansion (Cosgrove, 1997a,b; Giddings and Staehelin, 1991; McQueen-Mason, 1997; Nicol and Höfte, 1998; Pennell, 1998), the molecular events underlying the initiation of localized cell expansion events remain obscure. However, comparisons with fungal and algal model systems provide insights into the initiation of localized expansion events in higher plants (Fowler and Quatrano, 1997). First, a cortical site is chosen at the position of future cell expansion. Then, targeted secretion modifies the site of expansion. Finally, interactions with the cytoskeleton place the cell wall synthesis machinery at the selected site and localized expansion ensues.

To uncover the mechanisms by which cell expansion events are initiated, we have studied the initiation of branches on trichomes (epidermal hairs) of *Arabidopsis* as a model. *Arabidopsis* trichomes are large, single cells that project from the epidermis of leaves, stems and sepals. Trichome development begins when a protodermal cell, in response to a complex interplay of tissue- and organ-specific regulators, adopts the trichome cell fate (Chien and Sussex, 1996; Larkin et al., 1999, 1996; Perazza et al., 1998; Schnittger et al., 1998; Szymanski et al., 1988; Telfer et al., 1997). Once the trichome fate is determined, the nascent trichome undergoes several rounds of endoreplication concomitant with cell enlargement and expansion out of the plane of the leaf blade (Hülskamp et al., 1994). When the developing trichome has expanded to the shape of a small cylinder, branching is initiated as a bulge on the distal face of the developing trichome – not at the tip of the cylinder but approximately halfway between the base and the tip of the cylinder (Folkers et al., 1997; Hülskamp et al., 1994). The bulge expands to become the first trichome branch while the tip of the cylinder continues to expand to form a second branch. As the first branch continues to expand, a third branch is initiated at its base (Folkers et al., 1997; Hülskamp et al., 1994). Expansion continues until the trichome is 300 to 500 μm tall. The final phase of trichome differentiation is maturation. During maturation, the trichome cell wall thickens, accessory cells differentiate around the base of the trichome, and the surface of the trichome develops numerous papillae (Hülskamp et al., 1994; Marks et al., 1991).

Previous studies of trichome development have identified a number of mutations that affect trichome branch number (Hülskamp et al., 1994; Folkers, et al., 1997; Perazza et al., 1999). Mutations in *NOEK* (*NOK*), *TRIPTYCHON* (*TRY*),

KAKTUS (*KAK*), *POLYCHOME* (*PYM*), *RASTAFARI* (*RFI*) and *SPINDLY* (*SPY*) lead to an increase in trichome branch number, whereas mutations in *STICHEL* (*STI*), *ANGUSTAFOLIA* (*AN*), *ZWICHEL* (*ZWI*), *GLABRA3* (*GL3*) and *STACHEL* (*STA*) lead to trichomes with fewer than normal branches. The first genetic analysis of trichome branching was conducted by Folkers et al. (1997) who studied branching in single and double mutant combinations of *an*, *zwi*, *sti*, *gl3*, *sta*, *nok* and *try*. Based on their results, Folkers et al. (1997) proposed that trichome branching was controlled by trichome cell growth (or level of endoreplication). In addition, Folkers et al. (1997) proposed that primary and secondary branching were genetically distinct events.

To further examine the genetic control of trichome branching, we screened for additional mutants that showed an altered trichome branch number. Seven new mutations that define four new genes were isolated; these mutations cause a decrease in trichome branch initiation resulting in trichomes with fewer than normal branches. Thus, these four new genes act as positive regulators of trichome branch initiation. The new mutations cannot be clearly assigned to roles in primary and secondary branching which suggests that this distinction is an oversimplification. Our data suggest instead that trichome branching is regulated by parallel, partially redundant pathways. Furthermore, our results from the analysis of double mutants suggest that cell growth and the control of trichome branch number are most likely independent processes.

MATERIALS AND METHODS

Plant strains and growth conditions

The plant strains used in this study are summarized in Table 1. Mutants were backcrossed at least once to wild-type plants of their respective ecotypes. Plants were grown under constant illumination as previously described (Krishnakumar and Oppenheimer, 1999) and fertilized twice with a complete nutrient solution (Pollock and Oppenheimer, 1999).

Genetic mapping

A representative allele from each of the four *furca* (*frc*) complementation groups was mapped using classical and molecular markers. To determine the genetic map position of the *FRC* genes relative to classical phenotypic markers, the *frc* mutants were crossed to the following strains (obtained from the *Arabidopsis* Biological Resource Center, The Ohio State University; strain [marker scored]): cs124 (*tt1-1*), cs35 (*cer5-1*), cs30 (*bp-1*), cs34 (*cer4-1*), cs38 (*cer7-1*), cs85 (*tt4-1*), cs75 (*ms1-1*), cs137 (*cer3-1*) and Ler (*er*). Plants showing the *frc* phenotype were selected from the subsequent F_2 population and scored for the phenotype of the classical phenotypic marker. The frequency of recombination between the *frc* mutation and the classical marker was used to determine the approximate map position of each *FRC* locus. Map positions were confirmed by using molecular markers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993).

Construction of double mutants

Double mutants were constructed by intercrossing the strains of interest and letting the F_1 plants self. Plants displaying only one of the parental phenotypes were collected from the F_2 population and allowed to self. Putative double mutants (plants with a novel phenotype) were selected from the F_3 population. Putative double mutants were confirmed by complementation tests with each of the original parents. In addition, double mutants were backcrossed to wild-type plants, and the subsequent F_2 population was examined for segregation of both of the original parental phenotypes.

Physical characterization of the *frc* mutants

Trichomes on wild-type and mutant plants were examined by scanning electron microscopy (SEM) as previously described (Oppenheimer et al., 1997). To quantify the trichome branch numbers for each mutant, all the trichome branches were counted on the adaxial side of either the third or fourth leaf from at least eight plants.

RESULTS

Isolation of new trichome branch number mutants

To identify additional genes that control trichome branch initiation, we screened for *Arabidopsis* mutants that showed an altered trichome branch number. We isolated seven mutants that showed a reduction in the number of trichome branches (Table 1; Fig. 1). These mutants were named *furca* (*frc*). To determine if the mutations were dominant or recessive, we backcrossed the *frc* mutants to wild-type plants. All the crosses produced wild-type F_1 progeny which demonstrated that all the *frc* mutations were recessive. Furthermore, analysis of the segregation ratios of the *frc* plants to wild-type plants in the F_2 population from each backcross indicated that each of the *frc* mutations was monogenic.

To determine if the *frc* mutations represented new trichome loci or previously identified loci, we performed complementation tests with *sti*, *an*, *zwi*, *gl3*, *sta*, *nok* and *try* mutants. We also crossed the *frc* mutants to each other to determine if any were allelic. The results of the pairwise complementation tests showed that the seven *frc* mutations represent four new genes that control trichome branch number. The new trichome branch number genes defined by the four complementation groups were named *FRC1* (two alleles), *FRC2* (three alleles), *FRC3* (one allele) and *FRC4* (one allele).

In addition to the complementation tests, we determined the genetic map positions of each of the *FRC* genes to confirm that each of the *FRC* complementation groups represented independent loci. The *FRC1* locus is located near the bottom of chromosome III, tightly linked to the classical genetic marker *CER7* (we found no *frc1-1 cer7* recombinants out of 680 *frc1-1* plants scored). The *FRC2* locus is approximately 12

Table 1. Name and origin of the trichome branch number mutants used in this study

Strain	Mutagen	Ecotype	Source (reference)
frc1-1	EMS	Col	This study
frc1-2	EMS	Col	This study
frc2-1	EMS	Col	Joy Chien
frc2-2	EMS	Col	This study
frc2-3	Fast neutrons	RLD	This study
frc3-1	Fast neutrons	RLD	This study
frc4-1	Fast neutrons	RLD	This study
sta-23	EMS	Ler	M. Hülskamp (Hülskamp et al., 1994)
try-240	EMS	Ler	M. Hülskamp (Hülskamp et al., 1994)
an-496	EMS	RLD	This study
zwi-3	EMS	Col	Krisnakumar and Oppenheimer (1999)
zwi-9311-11	Fast neutrons	RLD	Krisnakumar and Oppenheimer (1999)
zwi-W2	EMS	RLD	Krisnakumar and Oppenheimer (1999)
sti-9507-1	Fast neutrons	RLD	This study
nok-9310-11	Fast neutrons	RLD	This study
gl3	EMS	Ler	Koornneef et al. (1982)

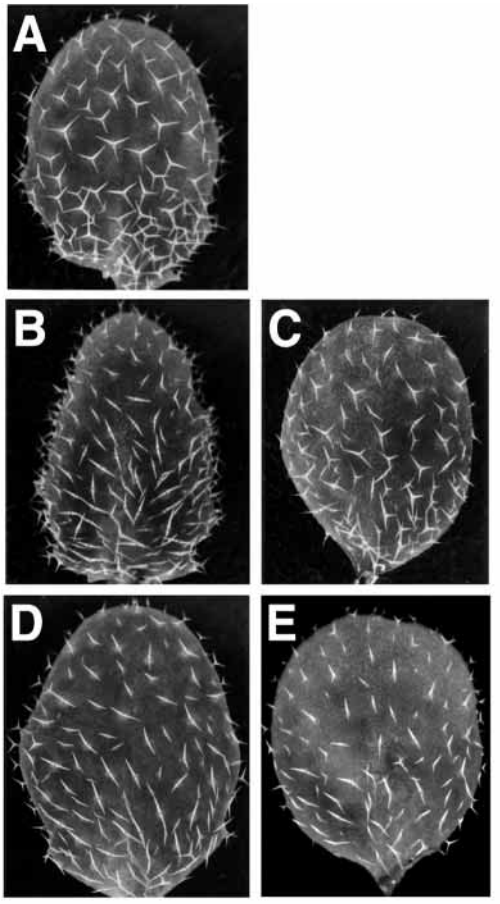


Fig. 1. Light micrographs of developing leaves of wild-type and *frc* mutants showing mature trichomes. (A) Fifth leaf of a Col wild-type plant showing mostly three-branched trichomes. (B) Fifth leaf of a *frc1-1* plant. (C) Fifth leaf of a *frc2-3* plant. (D) Fifth leaf of a *frc3-1* plant and (E) Fifth leaf of a *frc4-1* plant.

cM south of classical marker *cer5*, and approximately 23 cM south of SSLP marker nga280 on chromosome I. The map position of the *FRC3* locus is approximately 3 cM south of the molecular marker g4539 on chromosome IV. *FRC4* is located on chromosome II, linked to the classical marker *er* (we found no *frc4-1 er* recombinants out of 232 *frc4-1* plants scored). Because no known trichome branch mutants have been mapped to these positions, these results demonstrate that each of the *FRC* complementation groups represents a new gene.

Phenotypic analysis of the *frc* mutants

To determine if any of the *frc* mutations affect trichome number, we counted the number of trichomes on either the third or fourth leaf of at least 10 plants of each of the *frc* mutants. No significant difference was observed between the *frc* mutants and wild-type plants of the same ecotype (data not shown). This result suggests that the *FRC* genes play no role in cell fate decisions.

Wild-type plants generally have three or four trichome branches whereas most of the trichomes on *frc* mutant plants have only two branches. All of the branched trichomes on the plants were affected by the *frc* mutations including trichomes on the abaxial sides of the leaves and the few branched trichomes found on the floral stem. The unbranched stem and sepal trichomes were unaffected by the *frc* mutations. To quantify the extent of the trichome branch number reduction, trichome branches were counted on leaves of *frc* mutants and wild-type plants (Table 2). The *frc2* mutants have the weakest phenotype of the *frc* mutations. Generally, only about 60% of the trichomes on *frc2* mutants have two branches whereas more than 90% of the trichomes on the other *frc* mutants have two branches (Table 2). However, all the *frc* mutants display a significant decrease in trichome branch number compared to wild-type plants, and their segregation can be easily followed in crosses. Because the *frc* mutations are likely to be loss-of-function mutations, this result suggests that the *FRC* genes act as positive regulators of trichome branch initiation.

Table 2. Number of branches on wild-type and *frc* mutant trichomes

	Trichome branch points*					
Genotype (ecotype)	0	1	2	3	4	Total‡
Wild type (RLD)	0.25	0.84	80.9	18	<0.1	1192
Wild type (Col)	0	0	75.3	24.7	0	1060
frc1-1 (Col)	3	96.1	0.9	0	0	2111
frc2-1 (Col)	0	59.5	38.7	1.8	0	2217
frc2-3 (RLD)	0	38.3	58.2	3.5	0	711
frc3-1 (RLD)	0	93.2	6.8	0	0	3101
frc4-1 (RLD)	0.3	98.1	1.6	0	0	1975

*% of trichomes having the indicated number of branch points (1 branch point indicates a trichome with two branches).
‡Total number of trichomes counted.

We also used SEM to examine the trichomes of the *frc* mutants (Fig. 2). Only trichome branch initiation appears to be affected in the *frc* mutants; trichome maturation is not affected. Variation in the size and/or morphology of the accessory cells in *frc* mutants is similar to that observed in wild-type plants. The stalk height of *frc* mutant trichomes was within the normal variation seen in wild-type plants.

Plant size, growth rate, flowering time, fertility and general appearance of the *frc* mutants were also examined. Plants homozygous for either *frc1* or *frc3* are wild type in overall appearance, growth rate, flowering time and fertility. Plants homozygous for either the *frc2-1* or the *frc2-3* alleles, however, show decreased fertility compared to wild-type plants (data not shown). This decrease in fertility appears to be caused by a premature extension of the pistil from the unopened flower before the anthers mature. In outcrosses, *frc2* plants showed no obvious decrease in fertility either when used as the female parent or as the male parent (data not shown). These results suggest that the fertility defect is a pleiotropic effect of the *frc2* mutation and not a secondary mutation linked to the *frc2* mutation.

Plants homozygous for the *frc4-1* mutation also show apparent pleiotropic effects. The *frc4* mutant plants grow slower than wild-type plants, and produce abnormally short and bushy bolts at the time of flowering (data not shown). In addition, *frc4* mutants also show a decrease in fertility (data not shown). However, because we isolated only one mutant allele of *frc4*, we cannot rule out tightly linked, secondary mutations as the cause of the pleiotropic effects of *frc4-1*.

Double mutants

Genetic interactions among the *frc* mutations

To understand the genetic relationships among the *frc* mutations, pairwise combinations of *frc* double mutants were constructed.

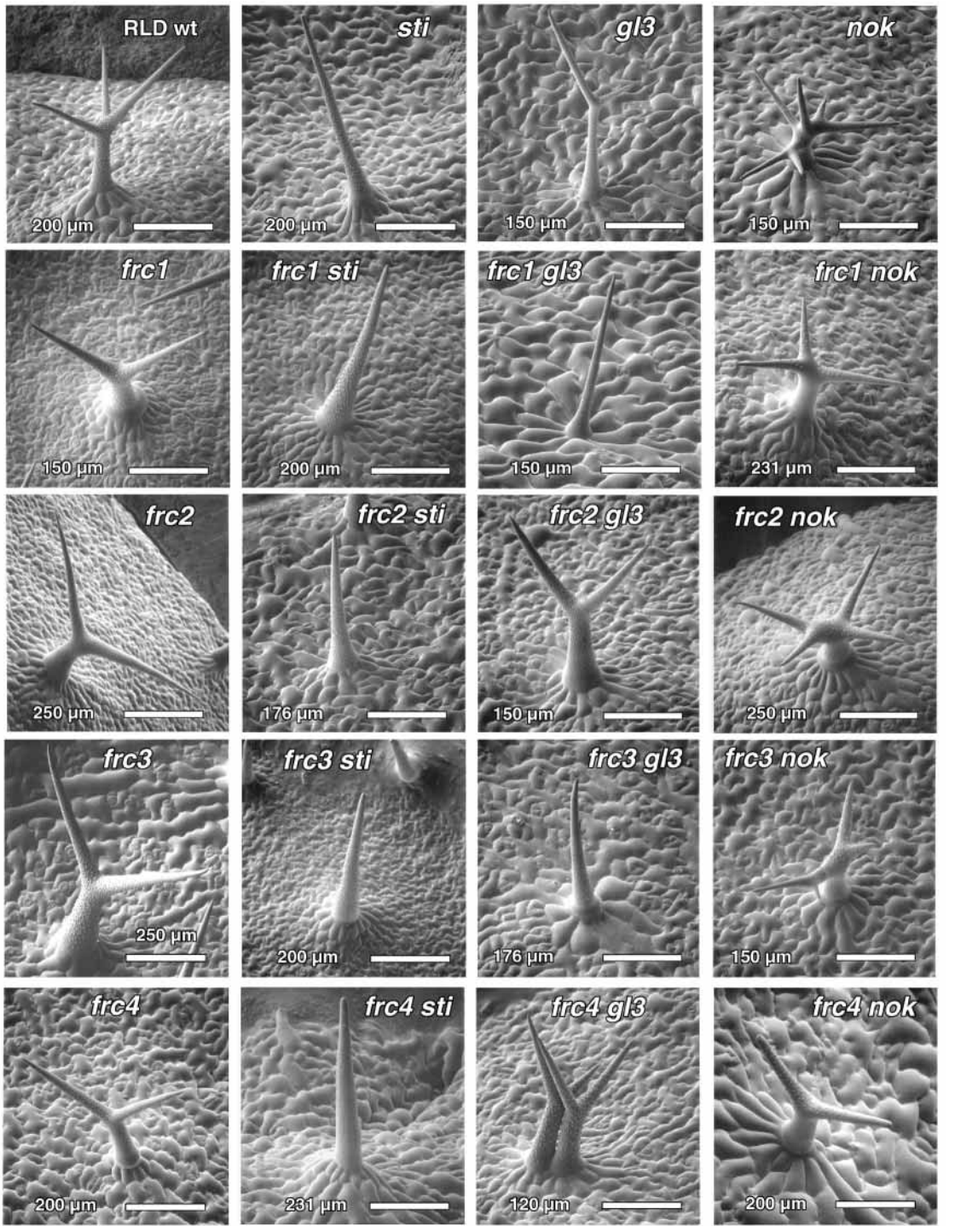
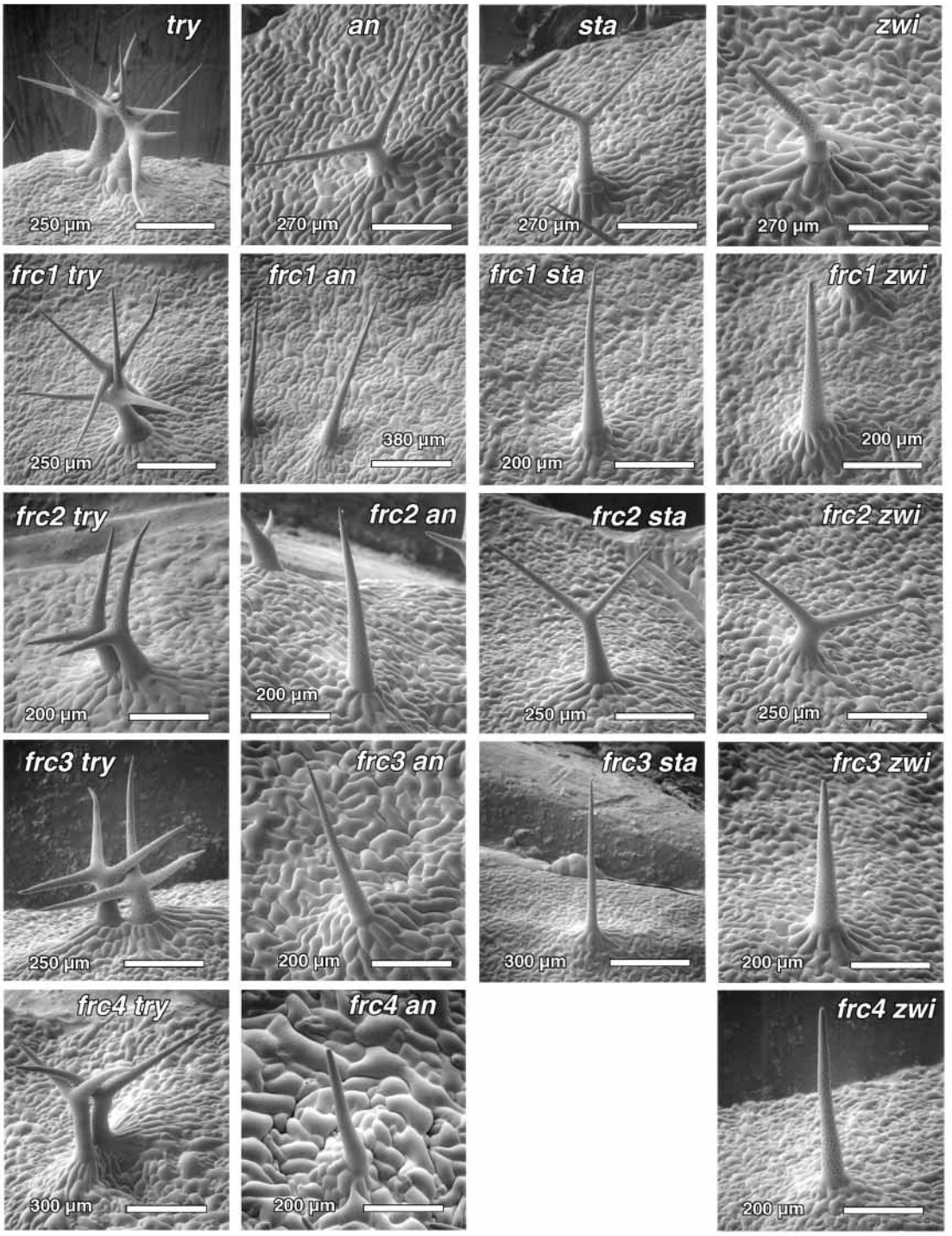


Fig. 2. Scanning electron micrographs of mature leaf trichomes of wild-type, *frc* and other trichome branch number mutants.

If the double mutant phenotype was similar to either one of the single mutants, then the interaction was interpreted as being epistatic. If the double mutant phenotype was more severe than either single mutant, then the interaction was interpreted as being additive. Mild effects were disregarded because they were indistinguishable from background effects. The *frc1-1 frc3-1* and *frc1-1 frc4-1* double mutants produce predominantly unbranched trichomes, and *frc3-1 frc4-1* double mutants have exclusively unbranched trichomes (Table 3; Fig. 3). These results suggest that *FRC1*, *FRC3* and *FRC4* act independently in the control of trichome branch number because the effects of mutations in these three genes are additive. The phenotype of *frc2-1 frc4-1* double mutants is the same as that of the *frc4-1* single mutants (Table 3; Fig. 3); therefore, *frc4-1* is epistatic to *frc2-1*. Although more than 60% of the trichomes on *frc2-1 frc1-1* double mutants and the *frc2-3 frc3-1* double mutants have two branches, at least 30% of the trichomes on these double mutants are unbranched (Table 3). Therefore, the effect of *frc2-1* in combination with



either *frc1-1* or *frc3-1* is additive. These results suggest that the *FRC2* gene is likely to act independently of both the *FRC1* and the *FRC3* genes.

Genetic interactions between the *frc* mutations and the other mutations that control trichome branch number

To determine the genetic relationships between the *frc* mutations and the other mutations that control trichome branch number, we constructed pairwise combinations of double mutant strains. We uncovered epistatic relationships among several pairs of branch mutant alleles. These are described below.

frc sti

Plants homozygous for both the *sti* mutation and any of the *frc* mutations produced only unbranched trichomes (Table 3; Fig. 2). However, *sti* single mutants also produced exclusively unbranched trichomes. Therefore, additive effects could not be distinguished from epistatic effects (see Discussion).

frc gl3

The *gl3* mutation has pleiotropic effects on trichome development; *gl3* mutants have aborted trichomes on the first leaf pair (D. G. O., unpublished results), a reduction in the

Table 3. Number of trichome branches on *frc* double mutants

Genotype	Trichome branch points*						Total‡
	0	1	2	3	4	>4	
frc1-1 frc2-1	31.3	68.7	0	0	0	0	1407
frc1-1 frc3-1	95.8	4.2	0	0	0	0	1722
frc1-1 frc4-1	99.8	0.2	0	0	0	0	1801
frc2-3 frc3-1	39	61	0	0	0	0	1018
frc2-1 frc4-1	0.9	99.1	0	0	0	0	867
frc3-1 frc4-1	100	0	0	0	0	0	719
sti-9507-1	100	0	0	0	0	0	929
frc1-1 sti-9507-1	100	0	0	0	0	0	649
frc2-1 sti-9507-1	100	0	0	0	0	0	640
frc3-1 sti-9507-1	99.9	0.1	0	0	0	0	1253
frc4-1 sti-9507-1	100	0	0	0	0	0	558
gl3	29	70.3	0.7	0	0	0	683
frc1-1 gl3	98	2	0	0	0	0	1477
frc2-1 gl3	3.6	95.7	0.7	0	0	0	921
frc3-1 gl3	98.1	1.9	0	0	0	0	529
frc4-1 gl3	22.3	77.6	0.1	0	0	0	916
nok-9310-11	0	0.5	16.5	27.5	28.1	27.4	1449
frc1-1 nok-9310-11	1.7	31.1	44.8	22.2	<0.1	0	1026
frc2-1 nok-9310-11	0	4.8	37.6	45.1	10.3	2.2	505
frc3-1 nok-9310-11	0	19.9	30.5	41.9	7.4	0.4	793
frc4-1 nok-9310-11	0	91.5	8.1	0.4	0	0	1148
try-240	0	0	1.5	20	41.6	36.4	534
frc1-1 try-240	0	20.1	79.3	0.6	0	0	1270
frc2-1 try-240	0	37.7	55.8	6.3	0.2	0	1120
frc3-1 try-240	0	5.9	44.9	41.9	7.2	0	781
frc4-1 try-240	0.3	92.3	7.4	0	0	0	782
an-496	11.5	86.6	1.9	0	0	0	1269
frc1-1 an-496	82.4	17.6	0	0	0	0	883
frc2-1 an-496	82.4	17.6	0	0	0	0	795
frc3-1 an-496	95.8	4.2	0	0	0	0	922
frc4-1 an-496	100	0	0	0	0	0	919
sta-23	0	79.2	20.8	0	0	0	715
frc1-1 sta-23	43.1	56.9	0	0	0	0	1023
frc2-1 sta-23	0.7	87.1	12.2	0	0	0	549
frc3-1 sta-23	58.4	41.6	0	0	0	0	562
zwi-9311-11	40.4	59.6	0	0	0	0	1117
frc1-1 zwi-9311-11	98	2	0	0	0	0	961
frc2-1 zwi-9311-11	56	43.8	0.1	0	0	0	728
frc3-1 zwi-9311-11	93.7	6.3	0	0	0	0	2407
frc4-1 zwi-9311-11	100	0	0	0	0	0	877

*% of trichomes having the indicated number of branch points (1 branch point indicates a trichome with two branches).
‡Total number of trichomes counted.

number of trichome branches (Table 3 and Folkers et al., 1996), a reduction in the amount of nuclear DNA (Hülskamp et al., 1994), a decrease in apparent trichome cell size (Hülskamp et al., 1994), and an increase in trichome clusters (D. G. O., unpublished results). Double mutant strains containing *gl3* and *frc1* or *frc3* produce predominantly (98%) unbranched trichomes (Table 3; Fig. 2). However, in these mutants, aborted trichomes are produced on the first leaf pair, and trichome clusters are present at the same frequency as seen in *gl3* mutants (data not shown). All aspects of the *gl3* phenotype appear to be epistatic to the *frc1* or *frc3* mutations except the trichome branch number reduction – the effect of which was additive in combination with either *frc1* or *frc3*. This result suggests that *GL3* may independently control different aspects of trichome development, and that *FRC1* and *FRC3* contribute independently to the control of trichome branch number.

The *gl3* mutation is completely epistatic to the *frc2* and *frc4* mutations: the *gl3 frc2-1* and *gl3 frc4-1* double mutants have

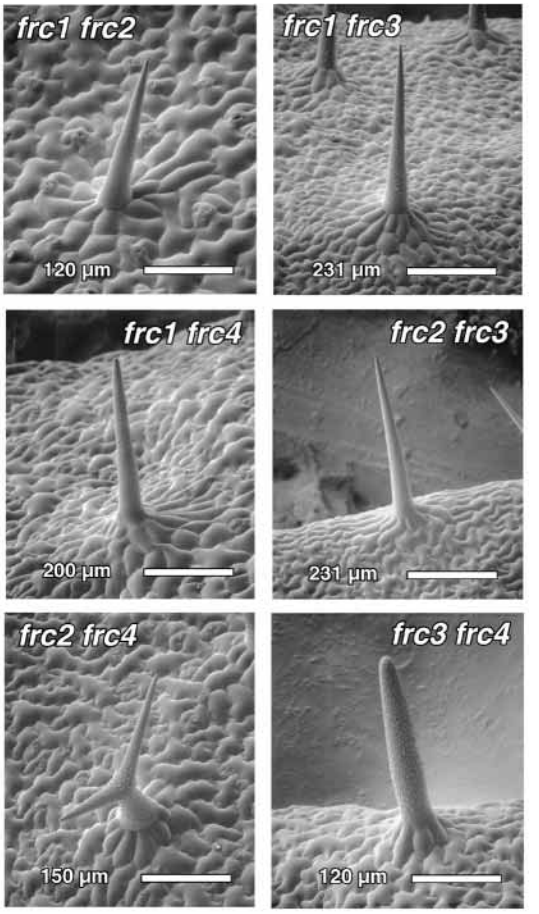


Fig. 3. Scanning electron micrographs of mature leaf trichomes of *frc frc* double mutants.

approximately the same proportions of two-branched and unbranched trichomes as *gl3* single mutants (Table 3; Fig. 2). This result suggests that *GL3*, *FRC2* and *FRC4* act in the same pathway to control trichome branch number (see Discussion).

frc nok and *frc try*

Plants homozygous for the *nok* mutation produce trichomes that have more branches than trichomes on wild-type plants (Folkers et al., 1997). Wild-type plants normally produce trichomes with three or four branches (very rarely five branches), whereas trichomes on *nok* mutants have up to eight branches (Folkers, et al., 1997; Tables 2, 3; Fig. 2). Thus, the *NOK* gene may act as a negative regulator of trichome branching. In addition to the effect on trichome branching, *nok* mutants also fail to complete trichome maturation; the trichomes on *nok* plants lack the wild-type number of papillae and appear glassy. To determine if any of the *FRC* genes might be negatively regulated by *NOK*, we constructed all the *frc nok* double mutants. The *frc1-1 nok-9310-11, frc2-1 nok-9310-11* and *frc3-1 nok* 9310-11 double mutants had more trichome branches than any of the *frc* single mutants, but fewer branches than in *nok* single mutants (Table 3; Fig. 2). This intermediate number of trichome branches demonstrates an additive effect of these *frc* mutations in combination with the *nok*-9310-11 mutation.

In contrast to the *frc1*, *frc2* and *frc3* mutations, the *frc4-1*

mutation was completely epistatic to the *nok-9310-11* mutation with respect to trichome branch number. Approximately 92% of the trichomes on *frc4-1 nok-9310-11* double mutants had two branches compared with 98% two-branched trichomes on *frc4-1* single mutants (Table 3). However, the trichomes on all the *frc nok* double mutants have the same glassy phenotype as *nok-9310-11* single mutants. This result shows that the effects of *nok* on maturation are genetically separable from the effects on branching.

Mutations in *TRY* also have pleiotropic effects on trichome development: in addition to an increase in branch number, trichomes on *try* mutants develop in clusters and have an increased amount of nuclear DNA (Hülskamp et al., 1994). The results of the *frc try* double mutant analysis were similar to those of the *frc nok* double mutants. The *frc1-1 try-240* and *frc3-1 try-240* double mutants generally had fewer trichome branches than observed on *try-240* single mutants, but more than observed on either *frc1-1* or *frc3-1* single mutants (Table 3; Fig. 2). For example, only 0.9% of the trichomes on *frc1-1* plants have three branches compared with 20% on *frc1*-1 *try*-240 double mutants, and 41.6% of the trichomes on *try*-240 single mutants have five branches compared with 0% of the trichomes on *frc1*-1 *try*-240 double mutants (Table 3). These results show an additive effect on trichome branching of either the *frc1* or *frc3* mutation in combination with the *try* mutation.

Like the *frc4-1 nok-9310-11* double mutant, the *frc4-1 try-240* double mutant produces mostly (92.3%) two-branched trichomes (Table 3; Fig. 2). Thus, the *frc4-1* mutation is epistatic to the *try-240* mutation with respect to trichome branch number. The *frc2-1* mutation was weakly epistatic to the *try-240* mutation; close to 40% of the trichomes on the *frc2-1 try-240* double mutant had two branches compared with approximately 60% of the trichomes on *frc2-1* plants (Table 3). All the *frc try* double mutants produced clusters of trichomes (Fig. 2) at the same frequency as *try* single mutants (data not shown).

frc an

Plants homozygous for the *an* mutation mostly produce trichomes with two branches instead of the wild-type number of three or four branches. All the *frc an* double mutants produced predominantly (82-100%) unbranched trichomes (Table 3; Fig. 2). Thus, the *frc* mutations have an additive negative effect on trichome branch number when in combination with the *an* mutation.

frc sta and *frc zwi*

Mutations in the *STA* gene lead to trichomes with mostly two branches (Table 9 and Fig. 9). The *sta* mutation was found to be epistatic to only the *frc2* mutations; the *frc2-1 sta-23* double mutants produced two- and three-branched trichomes in roughly the same proportions as in *sta-23* single mutants (Table 3). The *frc1* and *frc3* mutations had an additive effect in combination with the *sta-23* mutation. For example, both *frc3-1* and *sta-23* single mutants produced no unbranched trichomes, but nearly 60% of the trichomes on the *frc3-1 sta-23* double mutant were unbranched (Table 3).

We were unable to isolate a fertile *frc4-1 sta-23* double mutant – both single mutants were small, slow growing plants with decreased fertility, and the putative *frc4-1 sta-23* double mutant was severely stunted and infertile. However, we were able to identify several infertile, putative double mutants from which we estimated that they produced approximately 80% unbranched trichomes and 20% two-branched trichomes. Thus, *sta-23* had an additive effect in combination with *frc4-1*.

The results for the *frc zwi* double mutants were similar to those for the *frc sta* double mutants; *zwi* was epistatic only to *frc2* (Table 3; Fig. 2). Whereas *zwi-9311-11* single mutants produced approximately 60% two-branched trichomes, *frc1-1 zwi-9311-11*, *frc3-1 zwi-9311-11* and *frc4-1 zwi-9311-11* double mutants had more than 93% unbranched trichomes (Table 3).

DISCUSSION

During a screen for mutants with altered trichome branch number, we discovered seven mutations that define four new genes involved in the control of trichome branch number. The discovery of these new mutants allowed us to further test a previously published model for the control of trichome branch number (Folkers et al., 1997). We characterized the trichome phenotypes of these new mutants, and determined the genetic relationships of the new genes to each other and to previously identified genes known to control trichome branch number. We found that the phenotypes of the single and double mutants deviated significantly from those predicted by the model presented by Folkers et al. (1997) which suggested a fundamentally different view of the mechanisms by which trichome branch number is controlled.

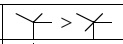
The *FURCA* genes act as positive regulators of trichome branch formation

Four new mutants (*frc1*, *frc2*, *frc3* and *frc4*) that show a reduction in the number of trichome branches were described in this report. Because all of these mutations lead to fewer than the normal number of trichome branches, these results demonstrate that the products of the wild-type *FRC* genes play positive roles in trichome branch initiation.

We attempted to determine whether primary or secondary branch initiation was affected in the *frc* mutants according to the criteria set by Folkers et al. (1997). However, the distinction between effects on primary or secondary branch initiation was ambiguous in our mutants, and we were unable to draw a clear distinction between them for the *frc* mutants. This issue is addressed in detail below.

A new model for the control of trichome branch number

None of the mutations examined in this study is epistatic to all others; this result suggests that parallel, partially redundant pathways exist for the control of trichome branch initiation. To construct a model for the control of trichome branch number we used the following rationale. If one branch mutation is epistatic to another then the two genes function in the same pathway; however, if the effects of two branch mutations are additive in the double mutant then the two genes function in separate pathways. Generally, this interpretation is applied to complete loss-of-function (null) alleles. None of the *FRC* genes have been cloned yet; therefore, we cannot determine if the mutant *frc* alleles are null alleles. However, because most induced mutations in *Arabidopsis* are loss-of-function

Col wildtype		
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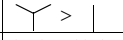
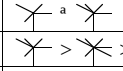
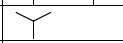
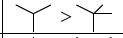
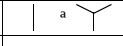
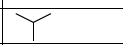
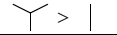
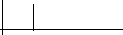
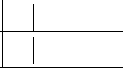
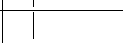
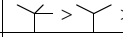
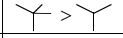
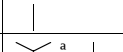
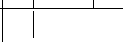
gl3		
nok-9310-11		
try-240		
an-496		
sta-23		
zwi-9311-11		
frc1-1		
frc1-1 frc2-1		additive
frc1-1 frc3-1		additive
frc1-1 frc4-1		additive
frc1-1 sti-9507-1		add/EPI
frc1-1 gl3		additive
frc1-1 nok-9310-11		additive
frc1-1 try-240		additive
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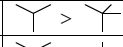
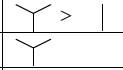
frc1-1 sta-23		additive
frc1-1 zwi-9311-11		additive
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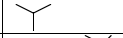
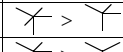
frc2-3 frc3-1		additive
frc2-1 frc4-1		EPISTATIC
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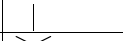
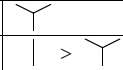
frc2-1 gl3		EPISTATIC
frc2-1 nok-9310-11		additive
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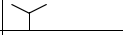
frc2-1 an-496		additive
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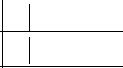
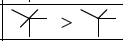
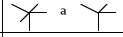
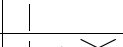
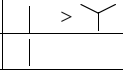
frc2-1 zwi-9311-11		EPISTATIC
frc3-1		
frc3-1 frc4-1		additive
frc3-1 sti		add/EPI
frc3-1 gl3		additive
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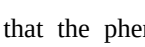
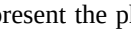
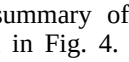
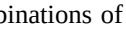
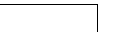
frc3-1 try-240		additive
frc3-1 an-496		additive
frc3-1 sta-23		additive
frc3-1 zwi-9311-11		additive

frc4-1		
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frc4-1 an-496		additive
frc4-1 zwi-9311-11		additive
*try-EM1 sti-EMU**		EPISTATIC
*try-EM1 an-EM1**		EPISTATIC
*try-EM1 zwi-EM1**		EPISTATIC
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*try-EM1 nok-122**	8-10 branches	additive
*try-EM1 gl3**		weakly additive
*nok-122 sti-EMU**		additive
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*nok-122 zwi-EM1**		EPISTATIC
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*nok-122 gl3**		WEAKLY EPISTATIC
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*gl3 zwi-EM1**		additive
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*zwi-EM1 an-EM1**		additive
*zwi-EM1 sta-23**		additive
*an-EM1 sta-23**		additive

Fig. 4. Summary of the phenotypes of the double mutants. I represents an unbranched trichome; Y represents a two-branched trichome; ⅄ represents a three-branched trichome, and so on. The interpretation of the interaction for each double mutant combination is given in the third column. The data for the double mutant combinations markedwith a '*' were taken from Folkers et al. (1997). A '>' between two trichomes means that the proportion of trichomes of the first type is greater than the proportion of trichomes of the second type. A '≈' symbol between two trichomes means that both types of trichomes are present in approximately equal proportions.

mutations, we will assume that the phenotypes of the *frc* mutants used in this study represent the phenotypes of strong loss-of-function alleles. A summary of the results of our genetic analysis is presented in Fig. 4. Applying the above rationale to the pairwise combinations of double mutants, we have developed a model for trichome branch initiation (Fig. 5). With this model, it is possible to predict the phenotypes of all the single and double mutants in this study as well as all the single and double mutants in the study conducted by Folkers et al. (1997). It is important to note that the model represents the genetic interactions between the genes controlling trichome branch initiation and not a developmental pathway per se.

The model shows that trichome branch initiation is controlled by parallel, partially redundant pathways. Our double mutant analyses did not support the hypothesis that primary and secondary branching are genetically separate events. If primary and secondary branching is controlled by different sets of genes, then the mutations that produce two-

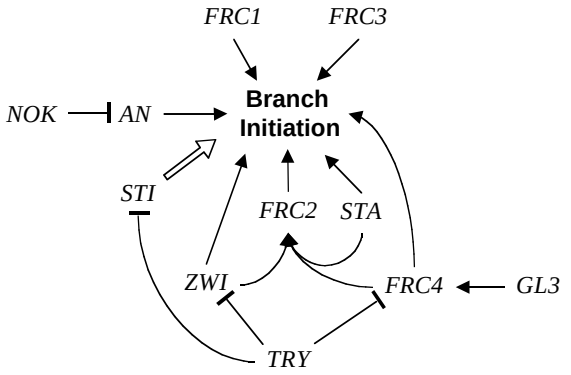

Fig. 5. A model for the genetic control of trichome branch number in *Arabidopsis*. Arrows indicate positive roles, blunt-ended lines indicate negative roles. The open arrow indicates the greater requirement for STI function during branching than the other branch number genes. The three-tailed arrow (under *FRC2*) indicates that each of the three genes (*ZWI*, *STA* and *FRC4*) must be present for *FRC2* function.

branched trichomes should fall into one of two classes: those that affect primary branching (like *STA*) and those that affect secondary branching (like *AN*). In double mutants containing combinations of primary branch mutations and secondary branch mutations, no trichome branches should be produced (as seen in *an sta* double mutants). Similarly, if primary and secondary branching are genetically distinct, then double mutant combinations of only primary branch mutations should produce branched trichomes. If double mutant combinations of two primary branch mutations produced unbranched trichomes, then this would indicate that both mutations affect both branching events, and thus are not genetically distinct. We found that all double mutant combinations of *frc1*, *frc3* and *frc4* produced predominantly unbranched trichomes (Table 3). Likewise, we found that all double mutant combinations of *an*, *frc3* and *frc4* (Table 3) produced unbranched trichomes. Therefore, we conclude that one or more of these genes must participate in *both* branching events even though the single mutants produce two-branched trichomes. Although, it is still possible that some components of branch initiation may be specific for one or the other of the branching events, the hypothesis that branch initiation is controlled by parallel, partially redundant pathways is sufficient to explain the observed phenotypes of the single and double mutants. Redundancy in pathways (both developmental and biochemical) is common in biology (Normanly and Bartel, 1999; Pickett and Meeks-Wagner, 1995; Thomas, 1993; Thomas et al., 1993). This makes sense, because redundancy in biological pathways increases the reliability of the pathway (McAdams and Arkin, 1999).

Cell expansion is a complex process involving the cytoskeleton, cell wall degradation and synthesis enzymes and the proteins that are needed to target them to the site of expansion. Thus, it is likely that trichome branch formation involves multiple proteins acting together at the site of branch initiation, and that such a multi-protein complex could be used for both branching events. With this in mind, one may expect that the genetic analysis of branch initiation would identify many genes acting at the same level in the genetic pathway (such as *ZWI*, *AN*, *FRC1*, etc.).

The relationship of *STI* to the *FRC* genes

We found that the *sti* mutation appeared to be epistatic to all the *frc* mutations. However, because *sti* single mutants produce exclusively unbranched trichomes, we were unable to distinguish additive from epistatic interactions. Therefore, it was difficult to determine whether *STI* acts upstream or downstream of the other branch number genes, or in which pathways STI functions. Several possibilities exist. First, *STI* may act upstream of several of the other branch number genes. For example, the *STI* product could be a transcription factor that regulates several of the branch number genes. Thus, *sti* mutations would phenotypically resemble the double (or multiple) mutants of those genes STI regulates. Second, *STI* may function downstream of several of the other branch number genes. Again, STI would need to be regulated by more than one of the other branch number genes because all the single mutants produce two-branched trichomes, and *sti* mutants produce unbranched trichomes. A third possibility is that *STI* lies on a pathway independent from the other branch number genes but is absolutely required for trichome branch initiation. Previously, *nok* mutations were shown to rescue branch initiation in a *sti* mutant background (Folkers et al., 1997). This result suggests that *NOK* and *STI* are on separate pathways. We have provisionally placed *STI* downstream from *TRY* in a pathway independent from the other branch number genes. This placement was chosen in part because *STI* is epistatic to *TRY* (Folkers et al., 1997). Because *nok sti* double mutants can initiate trichome branches (Folkers et al., 1997 and D. G. O., unpublished results), this result suggests that an increase in AN function (due to the *nok* mutation) can partially substitute for decreased STI function.

FRC2 function is partially redundant to both *FRC4* and *ZWI* function

At present, the *frc2* mutations have a less severe effect on trichome branch number than any of the other trichome branch number mutations (see Tables 2 and 3). One explanation for this effect is that *FRC2* function is redundant with some other branch number regulator. Both *zwi* and *frc4* mutations are epistatic to *frc2* mutations which suggests that *FRC2* is in the same pathway as *ZWI* and *FRC4*. However, the effect of the *zwi* mutation is additive in combination with the *frc4* mutation which suggests that *ZWI* and *FRC4* function in separate pathways. The most parsimonious resolution is to place *FRC2* downstream of, but redundant to, both *FRC4* and *ZWI*. In this position, one would predict that *frc2* mutations would be less severe than either *frc4* or *zwi* mutations (which is the case), and that both *frc4* and *zwi* mutations would be epistatic to *frc2* mutations (which is also the case). This hypothesis is supported by other *frc2* double mutants; the effects of *frc2* mutations were additive in combination with the other branch number reduction mutations (except *sta*; see below). Therefore, we conclude that the function supplied by *FRC2* is redundant to both *FRC4* and *ZWI*.

Relationship between *GL3* and the *FRC* genes

The *gl3* mutation has a number of effects on trichome development including trichome clustering, reduction in trichome branching, reduction in trichome endoreplication, and failure of some trichomes to expand out from the surface of the leaf. Trichome clustering is thought to be the result of the

inability of the nascent trichome to inhibit its neighbors in the equivalence group from also adopting the trichome cell fate. This 'lateral inhibition' occurs early during the trichome cell fate determination process. Likewise, expansion of the trichome out from the plane of the leaf blade precedes trichome branch initiation. Therefore, it is likely that *GL3* acts upstream of the *FRC* genes.

In a previous study of trichome branching (Folkers et al., 1997), mutations in *GL3* were shown to further reduce trichome branching in combination with *an*, *sta*, *nok*, and *zwi* mutations. These additive effects of *gl3* were interpreted as indicating that a minimum cell size (or level of endoreplication) was required before trichome branching could be initiated. However, we found that *gl3* was epistatic to both *frc2* and *frc4*, but additive when in combination with either *frc1* or *frc3* mutations. The simplest explanation for our results is that *GL3* functions in the same pathway as *FRC4* and *FRC2*, but in a pathway separate from *FRC1* and *FRC3*. In addition, in all the double mutant combinations of *frc* with *gl3*, the trichome clustering phenotype of *gl3* is still observed (Fig. 2 and data not shown). Therefore, we propose that *GL3* controls several independent processes during trichome development, two of which are endoreplication and trichome branch initiation. Additional support of our hypothesis comes from the recent identification of *GL3* as a member of the DRAT family of transcription activators (Payne et al., 1999). It is possible that the *FRC4* gene is a target of *GL3* along with genes that control the endoreplication cycle in trichomes. Thus, the processes of trichome branch initiation and endoreplication may be coupled, but one need not be dependent upon the other.

Similarly, the *try* mutation also has pleiotropic effects on trichome development. These effects include increased trichome endoreplication, trichome clustering, and an increased number of trichome branches. It was proposed that the increase in trichome branch number is due to the increased cell growth brought about by the extra rounds of endoreplication that occur in *try* mutants (Folkers et al., 1997; Perazza et al., 1999). As proposed for *GL3*, the data are consistent with the hypothesis that *TRY* regulates several independent processes.

The relationship of *STA* to the *FRC* genes

Our double mutant analysis has shown that the *sta* mutation has additive effects in combination with all the *frc* mutations except *frc2*. These results suggest that *STA* functions in the same pathway as *FRC2*, but in a separate pathway from the other *FRC* genes. Because the *sta* mutation is more severe than the *frc2* mutation (Tables 2 and 3), we hypothesize that *STA* function may be partially redundant to *FRC2* function.

The relationship between the negative regulators of trichome branching (*NOK* and *TRY*) and the *FRC* genes

Mutations in either *TRY* or *NOK* produce trichomes with more than the wild-type number of branches (see Fig. 2; Tables 2 and 3). This phenotype suggests that both *NOK* and *TRY* act as negative regulators of trichome branching. We found that *frc4* mutations were epistatic to both *nok* and *try* mutations. Folkers et al. (1997) previously reported that both *an* and *zwi* mutations were epistatic to both *try* and *nok* mutations. However, *AN* and *ZWI* do not function in a linear pathway because *an zwi* double mutants show an additive effect (Folkers et al., 1997; Fig. 4). When both the results of Folkers et al. (1997) and our results are considered together, the simplest explanation is that *TRY* and/or *NOK* negatively regulate *FRC4*, *AN* and *ZWI*. However, if this were the case, then in a *nok an* double mutant there should be an increase in *ZWI* function due to the loss of negative regulation by *nok*. But *nok* mutations do not rescue the reduction in branch number of *an* mutants; therefore, increased *ZWI* function cannot compensate for the loss of *AN* function. Therefore, we conclude that increased levels of *ZWI* and *AN* cannot substitute for one another in branch initiation: both must be active for branching to occur. This hypothesis is supported by *an zwi* double mutants which produce unbranched trichomes (Folkers et al., 1997; D. G. O., unpublished results). This same line of reasoning applies to the relationships between *NOK* and *FRC4* as well as the relationships between *TRY* and *FRC4*, *ZWI*, and *AN*. Therefore, we conclude that the *FRC4*, *AN* and *ZWI* products function together in trichome branch initiation, and that an increase in activity of one product cannot compensate for the loss of another. In addition, because the results from the epistasis analysis suggest that *NOK* is on a pathway separate from *FRC2*, *NOK* need only regulate AN to account for all the phenotypes of the *nok* double mutants.

Given that *NOK* and *FRC2* function in separate pathways, then the rescue of the *frc2* phenotype by *nok* mutations suggests that increased AN function (due to loss of negative regulation by *nok*) can compensate for loss of FRC2 function. The most likely explanation for this result is that FRC2 and AN perform similar functions, either as members of the same gene family, or as physically distinct proteins engaged in the same function.

We also found that the two-branched phenotype of both the *frc1* and *frc3* mutations can be suppressed by either *try* or *nok* mutations (see Fig. 2 and Table 3). These results suggest that *FRC1* and *FRC3* are not regulated by either *NOK* or *TRY*, but function in a pathway distinct from *NOK* and *TRY*. We conclude that an increase in *FRC4*, *ZWI*, or *AN* function can compensate for a decrease in *FRC3* or *FRC1* function. Similarly, we predict that an increase in either *FRC1* or *FRC3* function can compensate for a decrease in *ZWI*, *AN*, or *FRC4* function. Several other negative regulators of trichome branching have been identified recently (Perazza et al., 1999), and one or more of these may function to negatively regulate *FRC1* and/or *FRC3*.

In conclusion, we have isolated seven mutations that identify four new genes (*FRC1-4*) controlling trichome branch number in *Arabidopsis*. Based on genetic interactions among the trichome branch number mutations we have tested an earlier model of trichome branching. We have proposed a new model for trichome branch initiation in which parallel, partially redundant pathways regulate branch formation. In addition, we provide evidence that cell growth (or endoreplication) may be controlled independently from trichome branch initiation. Cloning of the *FRC* genes and biochemical analysis of the products will help unravel their role in the localized cell expansion events that give trichomes their distinctive shape.

The authors gratefully acknowledge M. Hülskamp for supplying *Arabidopsis* trichome mutants and Jolanta Nunley for the scanning

electron micrographs. The authors also thank John Larkin, Ed Stephenson, Janis O'Donnell, Sujatha Krishnakumar and Kevin Redding for stimulating discussions and critical reading of the manuscript. The authors also thank Mary Pollock for careful editing of the manuscript. The authors also gratefully acknowledge support from the NIH (R29 GM 5-30753). D. L. was supported in part by the University of Alabama SARTC and a Graduate Council Research Fellowship. The authors thank the *Arabidopsis* stock center for supplying seed of classical marker strains.

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