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

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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),...
showing the markers. To determine the genetic map position of the relative to classical phenotypic markers, the original parents. In addition, double mutants were backcrossed to mutants were confirmed by complementation tests with each of the

...1), cs85 (ANUGSTAFOLIA (AN), ZWICHEL (ZW1), 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 (at1-1), cs35 (cer3-1), cs30 (bp-1), cs34 (cer4-1), cs38 (cer7-1), cs85 (it4-1), cs75 (msl-1), cs137 (cer3-1) and Ler (er). Plants showing the frc phenotype were selected from the subsequent F2 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 F1 plants self. Plants displaying only one of the parental phenotypes were collected from the F2 population and allowed to self. Putative double mutants (plants with a novel phenotype) were selected from the F2 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 F2 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 F1 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 F2 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 |
| sti-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 | Krishnakumar and Oppenheimer (1999) |
| zwi-9311-11 | Fast neutrons | RLD | Krishnakumar and Oppenheimer (1999) |
| zwi-912 | EMS | RLD | Krishnakumar 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) |
Phenotypic analysis of the frc mutants

To determine if any of the frc mutations affect trichome number, we counted the number of trichomes 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.

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.
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

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

Table 3. Number of trichome branches on frc double
mutants

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<th>4</th>
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*% of trichomes having the indicated number of branch points (1 branch
point indicates a trichome with two branches).
†Total number of trichomes counted.

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

Fig. 3. Scanning electron micrographs of mature leaf trichomes of frc
frc double mutants.
mutation was completely epistatic to the nok-9310-11 mutation with respect to trichome branch number. Approximately 92% of the trichomes on frc-1 nok-9310-11 double mutants had two branches compared with 98% two-branched trichomes on frc-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 mutations 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
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-
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). Similarly, 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.
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