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Trichome Cell Growth in *Arabidopsis thaliana* Can Be Derepressed by Mutations in at Least Five Genes

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ABSTRACT

Leaf trichomes in Arabidopsis are unicellular epidermal hairs with a branched morphology. They undergo successive endoreduplication rounds early during cell morphogenesis. Mutations affecting trichome nuclear DNA content, such as *triptychon* or *glabra3*, alter trichome branching. We isolated new mutants with supernumerary trichome branches, which fall into three unlinked complementation groups: *KAKTUS* and the novel loci, *POLYCHOME* and *RASTAFARI.* They map to chromosomes IV, II, and V, respectively. The trichomes of these mutants presented an increased DNA content, although to a variable extent. The *spindly-5* mutant, which displays a constitutive gibberellin response, also produces overbranched trichomes containing more nuclear DNA. We analyzed genetic interactions using double mutants and propose that two independent pathways, defined by *SPINDLY* and *TRIPTYCHON*, act to limit trichome growth. *KAKTUS* and *POLYCHOME* might have redundant actions mediating gibberellin control via *SPINDLY.* The overall leaf polysomaty was not notably affected by these mutations, suggesting that they affect the control of DNA synthesis in a tissue- or cell type-specific manner. Wild-type tetraploids also produce overbranched trichomes; they displayed a shifted polysomaty in trichomes and in the whole leaf, suggesting a developmental program controlling DNA increases via the counting of endoreduplication rounds.

TRICHOMES are epidermal hairs found on the aerial surfaces of nearly all plants; in different species, they adopt different shapes and play a variety of functions, including acting as glandular secretor organs and protecting against insect predators (Wagner 1991; Eisner *et al.* 1998). In the diploid plant, *Arabidopsis thaliana*, trichomes are single and very large cells; they are regularly spaced on leaves, as the outcome of nonrandom cell fate decisions (Larkin *et al.* 1996). Trichome initiation is an early event in leaf organogenesis, taking place while neighbor epidermal cells keep on dividing (Hülskamp *et al.* 1994) and ending on expanding leaves with a basipetal gradient (Hülskamp *et al.* 1994; Larkin *et al.* 1996; Szymanski *et al.* 1998). The incipient trichome cell first enlarges slightly within the protoderm plane and then protrudes largely out of the epidermis plane, forming a stalk. Leaf trichomes adopt a stereotyped stellate shape: the stalk emerging out of the epidermis divides into branches, usually three (*e.g.*, Folkers *et al.* 1997). Stem and sepal trichomes, on the other hand, are predominantly unbranched (Marks and Feldmann 1989).

Arabidopsis is a highly polysomatic species: many cells, in rosette leaves and in hypocotyls in particular, contain nuclear DNA amounts more than four times higher than C, the haploid DNA content (Galbraith *et al.* 1991; Gendreau *et al.* 1997). These cells are generated by endoreduplication rounds: DNA first replicates as for a mitotic cycle, but there is no nuclear division, which yields a 2^{n+1}C nucleus after *n* successive rounds. In the leaf epidermis, the cell endoreduplication status is somehow linked to final differentiation. Stomata guard cells remain small and never endoreduplicate (Melaragno *et al.* 1993; Larkin *et al.* 1997). Epidermal pavement cells vary in ploidy from 2C to 16C and enlarge proportionally to their DNA content (Melaragno *et al.* 1993). Finally, trichome cells grow large and present very large nuclei: DNA contents ranging from 4C to 64C have been reported (Melaragno *et al.* 1993), and an average close to 32C suggests that trichomes undergo four successive endocycles (Hülskamp *et al.* 1994; Schnittger *et al.* 1998).

The dispensable nature of trichomes for the laboratory life of Arabidopsis has facilitated elaboration of a genetic system to analyze endoreduplication control. Mutants in more than 20 loci are known to affect trichomes and have been recovered in several screens us-

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ing ethyl methanesulfonate (EMS; Koornneef *et al.* 1983; Hülskamp *et al.* 1994; Folkers *et al.* 1997; Szymanski *et al.* 1998) or T-DNA insertions as a mutagen (Oppenheimer *et al.* 1991, 1997; Rerie *et al.* 1994). Mutants have been grouped into several phenotypic classes: (i) initiation mutants, (ii) mutants with a branch number up- or downregulated, (iii) mutants incapable of epidermal outgrowth, (iv) mutants presenting a distorted appearance, and (v) mutants affecting cell wall maturation (Hülskamp *et al.* 1994). The identified endoreduplication mutants belong to the first or to the second class.

Trichome initiation is impaired by mutations affecting the *TRANSPARENT TESTA GLABRA* or the *GLABROUS1* genes. While the *ttg* syndrome is pleiotropic, affecting also seed coat and root epidermis (Koornneef 1981; Galway *et al.* 1994), the *gl1* phenotype affects only trichomes (Koornneef *et al.* 1983). *GL1* encodes a Myb-like protein, probably a transcription factor (Oppenheimer *et al.* 1991). No trichome precursor cells (large flat cells with a larger nuclei) can be recognized in the epidermis of strong *gl1* and *ttg* mutants, suggesting that endoreduplications are required for trichome initiation (Hülskamp *et al.* 1994). Interestingly, the leaky *gl1-2* allele allows some residual initiation, but impairs a full morphogenesis in many emerging trichomes (Esch *et al.* 1994).

Trichome branch number is altered together with DNA content in several mutants. In the *glabrous3* mutants (*gl3*), trichomes are smaller, producing at most two branches (Koornneef *et al.* 1982); their nucleus is smaller too, with a 16C content strongly suggesting that the last endoreduplication is abolished (Hülskamp *et al.* 1994). In addition, the *gl3* mutations lead to a decrease of trichome initiation (Koornneef *et al.* 1982). In the *triptychon* mutants (*try*), trichomes are overdeveloped, producing four to six branches, and contain a larger nucleus. These trichomes present an increased nuclear DNA content, suggesting that an extra endocycle takes place. Besides their semidominant effect on branch number, *try* mutations have a recessive effect on trichome patterning: initiation of trichomes by adjacent cells is derepressed (Hülskamp *et al.* 1994; Schnittger *et al.* 1998). The *kaktus-1* mutant (*kak-1*) also presents trichomes with supernumerary branches and a larger nucleus (Hülskamp *et al.* 1994). In this series of mutants, changes in trichome branch number appear to reflect changes in final cell volume.

Trichome branching can also be affected without apparent modification of the final cell volume or of the nucleus size: architecture mutants with a reduced branching define four loci (*angustifolia*, *stachel*, *stichel*, and *zwichel*; Hülskamp *et al.* 1994), while the *noek* mutant (*nok*) presents an increased branching (Folkers *et al.* 1997). *ZWICHEL* encodes a kinesin homologue, suggesting a regulatory role of the cytoskeleton in trichome morphogenesis (Oppenheimer *et al.* 1997). Genetic analysis indicates that these five genes define a pathway acting independently, or downstream from the endoreduplication pathway. In double mutants, the *gl3* mutation decreases trichome branching in the *an*, *sta*, *sti*, *zwi*, and *nok* mutant backgrounds, whereas the *try* mutation increases branching in the *nok*, but not in the *an*, *sti*, and *zwi* backgrounds. These data are consistent with the idea that the *GL3* and *TRY* genes control trichome branch number through cell volume, while "architectural" genes organize the number and the relative positions of branching points on the growing cell (Folkers *et al.* 1997).

Both initiation and morphogenesis of trichomes are under gibberellin hormone (GA) control. The expression of the *GL1* gene is positively regulated by GAs (Perazza *et al.* 1998). Plants grown on mild concentrations of GA biosynthesis inhibitors like paclobutrazol or uniconazol show a reduction in both trichome number (Chien and Sussex 1996) and trichome branching (Perazza *et al.* 1998); plants germinating at higher concentrations are glabrous. Leaves of *ga1-3* mutant plants, which are incapable of GA biosynthesis (Barendse *et al.* 1986; Sun *et al.* 1992), are nearly glabrous when grown in the absence of exogenous hormones; the rare *ga1-3* trichomes also remain underdeveloped, with at most two branches (Chien and Sussex 1996; Telfer *et al.* 1997; Perazza *et al.* 1998). The SPINDLY (SPY) protein has been involved as a repressor of GA signaling; it contains a tetratricopeptide repeat domain, proposed to mediate protein-protein interactions (Jacobsen *et al.* 1996), and a domain related to O-linked *N*-acetylglucosamine transferases (Robertson *et al.* 1998). Mutants at the *SPY* locus resemble wild-type plants with a GA overdose (Jacobsen and Olszewski 1993); they are also derepressed in both trichome initiation (Chien and Sussex 1996; Telfer *et al.* 1997) and trichome branch number (Perazza *et al.* 1998).

The implication of several unlinked mutations in the successive events of trichome initiation and of cell morphogenesis suggests that both processes are controlled by overlapping mechanisms, where gibberellins play an important role. In this work, we describe the production of overbranched trichomes by new mutants as well as by polyploid lines. The mutant lines define three genes, *KAKTUS* (*KAK*), *RASTAFARI* (*RFI*), and *POLYCHOME* (*PYM*). Analysis of the genetic interactions in double mutants give new insights on how trichome growth is controlled in Arabidopsis. Overbranched trichomes contained more nuclear DNA than wild type, and our results give new evidence for a link between processes controlling endoreduplications and cell morphogenesis in trichomes.

MATERIALS AND METHODS

Arabidopsis stocks: The original tetraploid line derived from Columbia (Col) ecotype (4n Col) was isolated from a

population mutagenized using *Agrobacterium tumefaciens* T-DNA and *in vitro* regeneration from roots, kindly provided by Csaba Koncz (Max-Planck Institut, Köln, Germany), in a screen for seedlings germinating on uniconazol; the trichome phenotype of this line was discovered fortuitously. Triploid plants are the F_1 plants from a $2n$ Col × $4n$ Col cross. These seeds are fully viable, despite endosperm genetic imbalance; they are fully fertile, but their F_2 offspring contain many abortive or strongly deformed plants (aneuploids). The tetraploid line derived from Landsberg *erecta* and used in Figures 1, 5, and 7 is a kind gift from Dr. Jeff Leung (CNRS, Gif-sur-Yvette, France). The *spy-5* mutant is in a L*er* background (Wilson and Sommerville 1995), as well as the *try* allele used in this work, *Try*-EM1, and the *kak-1* mutant (Hülskamp *et al.* 1994). The *spy-5* mutant was kindly provided to us by Dr. Nick Harberd (John Innes Center, Norwich, UK), and the *spy-3* mutant by Prof. Neil Olszewski (University of Minnesota, Minneapolis).

Mutant screening: Approximately 16,000 individual F_2 lines descending from L*er* seeds mutagenized with EMS according to Mayer *et al.* (1991) were screened for trichomes displaying supernumerary branches. We identified 15 new lines in which the majority of trichomes had four branches and at least a few trichomes had five branches. An additional line with a weaker phenotype was not included in this study. Of the 15 lines, 6 had no obvious increase in trichome body size (*i.e.*, more, but smaller branches), 2 had glassy trichomes, indicative of a more pleiotropic defect, and 1 presented a *try* phenotype. Of the 6 remaining lines, 1 (u408) presented a flow cytometry pattern typical of a tetraploid leaf. The other 5 lines, originally numbered 67, 99, 158, 16, and 150, are the *kak-2*, *kak-3*, *kak-4*, *pym*, and *rfi* mutants, respectively; they have been back crossed once to wild-type L*er* plants and stable F_3 families with a mutant phenotype obtained.

Plant growth and observation: Plants were grown in soil under long day conditions (16/8 hr) and observed under low magnification (×20–50) with a binocular lens. All visible trichome branches were counted, irrespective of their size; χ^2 tests were performed using Microsoft Excel5 software to compare distributions, and trichome populations were considered different when the associated probability was lower than 0.01. For example, in Figure 2, trichomes with 3:4:>4 branches were 101:127:34 for F_1 *try* × *pym vs.* 103:99:8 for F_1 *try* × L*er* ($P < 10^{-3}$).

For scanning electron microscopy, plants were vacuum infiltrated and fixed for 4 hr at room temperature with 4% glutaraldehyde in phosphate buffer, pH 7.0, 0.02% Tween-20. Third leaves were dissected, mounted on specimen stubs, dehydrated in an ethanol series, critical point dried, and sputter-coated with 200 Å gold-palladium.

Genetic mapping: Homozygous mutant plants (L*er* background) were crossed to Col plants and about 20 F_2 plants with a strong overbranched phenotype were selected for each cross. DNA was extracted either from single F_2 inflorescences or from F_3 pools and characterized by PCR using simple sequence length polymorphism (SSLP) markers (Bell and Ecker 1994) or on Southern blots using ARMS (Fabri and Schäffner 1994). Linkage data were recorded and analyzed using Map Manager 2.6.5 (http://mcbio.med.buffalo.edu/mapmgr.html; Manly 1993). For the *kak-2* × Col cross, we noted an excess of Col (*i.e.*, recombinant) chromatides on chromosome 1 at markers *m254*, *AthGeneA*, *m315* (strongest deviation, L*er*:Col = 7:31), and *nga111*; the *m283* locus on chromosome 2 also showed deviation (9:23). A Col excess was also noted in the *kak-2* × Col cross (14:30); other crosses were not assayed at the above loci.

Construction of double mutants: Homozygous mutants were crossed pairwise and the F_1 plants were allowed to self. For seven crosses (corresponding to the double mutants on Figure 4), F_2 plants fell into three classes of increasing phenotypic strength. The weakest class produced a majority of three branch trichomes, *i.e.*, a phenotype compatible with wild-type or single heterozygous genotypes. The second class produced overbranched trichomes resembling the homozygous parental lines. The third class produced very large trichomes bearing at least seven (*try* × *pym*, *try* × *rfi*, and *try* × *spy-5* crosses) or eight (other crosses) branches, termed maxichomes. The F_2 progeny from most crosses between unlinked mutations (*try* × *kak-1*, *try* × *kak-2*, *try* × *kak-3*, *try* × *kak-4*, *try* × *pym*, *pym* × *rfi*, *kak-2* × *pym*, *kak-3* × *pym*) contained a high proportion of plants bearing maxichomes, compatible with either 3/16 or 5/16. F_2 plants with maxichomes were selfed, and at least 12 plants were observed for each of the resulting F_3 progenies. The maxichome phenotype was transmitted to the F_3, either stable or in segregation with plants resembling simple mutants. Stable F_3 families, devoid of plants with a trichome branching equivalent to either parental simple mutant, identified the *pym rfi*, *kak-2 pym*, *try pym*, and *try spy-5* double homozygous mutants. For *try kak-2*, neither of the two harvested F_3 families was stable, but a stable F_4 family was obtained. The production of nested, adjacent trichomes was an additional stable trait in all *try* double mutants.

The *kak-3 rfi* double mutant was isolated as a stable F_3 family showing a synergetic effect on stems. The *kak-3* stems were nearly wild type with a majority of nonstellate (and very rare three-branch) trichomes. On *rfi* stems, most trichomes had two or three branches; nonstellate trichomes were rare, and four-branch trichomes were never observed. One F_3 progeny of the *kak-3* × *rfi* cross produced stem trichomes with four and five branches on all 12 plants observed, identifying the double homozygous. Leaf trichomes bearing eight or more branches were present (but rare) on nearly every *kak-3 rfi* plant; they were absent from *rfi* and *kak-3* leaves.

In the cross between the linked *try* and *rfi* mutations, 143 F_2 plants were observed: 9 plants were noted as wild type, 125 with a phenotype compatible with a *try*/+, *rfi*/+, *try* or *rfi* genotype, and 9 as maxichome bearing; phenotypic segregation in F_2 thus confirmed that these mutations are not allelic. None of the nine F_3 families harvested was stable for the maxichome trait, but stable F_4 families have been isolated.

Trichome DNA staining and measurements: Trichomes were isolated from the third leaf of 20- to 25-day-old soil grown plants under binocular lens using dissection forceps, fixed for 4 hr in 3.7% paraformaldehyde in phosphate buffer, pH 7, 0.02% Tween-20, and washed three times in fixative-free buffer. Nuclei were stained for 15 min at room temperature with fresh 4′,6-diamidinophenylindole (DAPI) solution (1 μg/ml) in phosphate buffer, pH 7, 0.02% Tween-20 containing 0.1 mg/ml *p*-phenylene diamine as antifading agent. After three washes in phosphate/Tween-20 buffer, samples were mounted between slide and coverslip in 50% glycerol containing 0.1 mg/ml *p*-phenylene diamine and visualized using epi-fluorescence microscopy. Microfluorometry was carried out using the SAMBA device (System for Analytical Microscopy in Biomedical Applications, UNILOG, Meylan, France). The hard- and software packages of the system were as described (Giroud 1986). Nuclear areas were recorded in parallel with fluorescence values, and the two parameters were found to be in good linear correlation.

To measure the DNA content of guard cells, whole leaves from wild-type tetraploids were processed as detached trichomes as used in whole mount. Fluorescence units for trichome nuclei were converted into C values by taking the mean fluorescence of $4n$ stomatal guard cell nuclei as 4C (an external standard representing the lowest C content on the leaf; Melaragno *et al.* 1993). Guard cells from diploid wild

type were processed in parallel. When used as an alternative, 2C external standard, the estimated contents for wild-type trichome were 43 ± 17C (Figure 5) and 48 ± 20C (Figure 6).

Flow cytometry: A fresh, fully expanded Arabidopsis leaf was chopped together with a 25-mm^2 piece of tomato leaf (internal standard). The chopping buffer (pH 7) contained 45 mm $MgCl_2$, 30 mm sodium citrate, 20 mm 4-morpholinepropane sulfonate, and 1 mg/ml Triton X-100, as described by Galbraith *et al.* (1983), and was supplemented with 5 mm sodium meta-bisulfite and 4 mg/liter DAPI. The nuclei were filtered through 30-μm nylon and analyzed on a cytometer (EPICS V, Coulter, Hialeah, FL) with laser excitation (100 mW) at 351 + 364 nm.

RESULTS

A screen for mutants with large, overbranched trichomes: Wild-type, diploid *A. thaliana* plants produce branched trichomes on rosette leaves. In many ecotypes, and in particular Landsberg *erecta* (L*er*), the three branch trichomes predominate (Figure 1); the stalk and the three branches regularly present a nearly tetrahedral geometry. Since an increase in trichome DNA content appears to lead to an increased branch number in a *try* mutant, we reasoned that other mutants presenting an overdevelopment of trichomes might be defective in endoreduplication control. We had previously observed that a reduction in GA biosynthesis can mimic the *gl3* phenotype (Perazza *et al.* 1998); this prompted us to ask whether an excess of GA signaling leads to the opposite effect. Examination of *spy-5* trichomes revealed overbranching, with a clear majority of four branch trichomes (Figures 1 and 2A). The same was true when comparing *spy-3* to its wild-type ecotype, Col-0 (D. Perazza, unpublished observation). Trichome overbranching had previously been reported for the *kak-1* mutant, which we included in this analysis (Hülskamp *et al.* 1994; Figures 1 and 2A). We then screened the offspring of mutagenized populations in search of plants bearing overdeveloped trichomes. A kanamycin-resistant line obtained by T-DNA mutagenesis produced supernumerary trichome branches, but turned out to be a tetraploid (see below). Five new EMS mutant lines derived from L*er* ecotype were isolated (materials and methods) and called *pym*, *rfi*, *kak-2*, *kak-3*, and *kak-4* (see evidence for allelism below). With respect to wild type, all mutants showed an increased branching of leaf trichomes, with the appearance of five- and sometimes six-branch trichomes on every plant (Figure 2A).

When comparing trichomes of equal branch number, the repertories of cell shapes produced by all single mutants appeared to overlap deeply. Branching points often appeared farther apart from each other than on wild-type trichomes, resulting in forked branches (Figure 1); this trend was more pronounced on five- and six-branch trichomes. The phenotype of single mutants showed some extent of variability in successive batches of soil-grown plants. In at least four independent measurements, the proportion of three-branch trichomes varied from 78 to 92% for L*er*, 7 to 40% for *pym*, 13 to 30% for *try*, and 2 to 8% for *rfi*. The hierarchical order of phenotypic strengths for branch number, L*er* < *pym* ≤ *kak-2*, *kak-3*, *try* < *rfi*, was nonetheless always respected when plants were grown side by side.

In addition to trichome overdevelopment, the *rfi* mutation also led to a small but consistent and significant increase in trichome clustering. On *rfi* plants, 2.5% trichomes were found within a nest, *i.e.*, 22 trichomes out of 884 had another trichome as neighbor cell *vs.* 0.1% for L*er* (2/1456) and 10.5% for *try* (43/411). Trichome clustering was not increased in *spy-5*, *pym*, and *kak* mutants. The number of trichomes per leaf was somewhat reduced in *try*, *pym*, and *rfi*, but not in the *kak* mutants (data not shown). The distribution of trichomes on the leaf blade appeared otherwise normal in all mutants. On stems, the majority of wild-type trichomes were non-

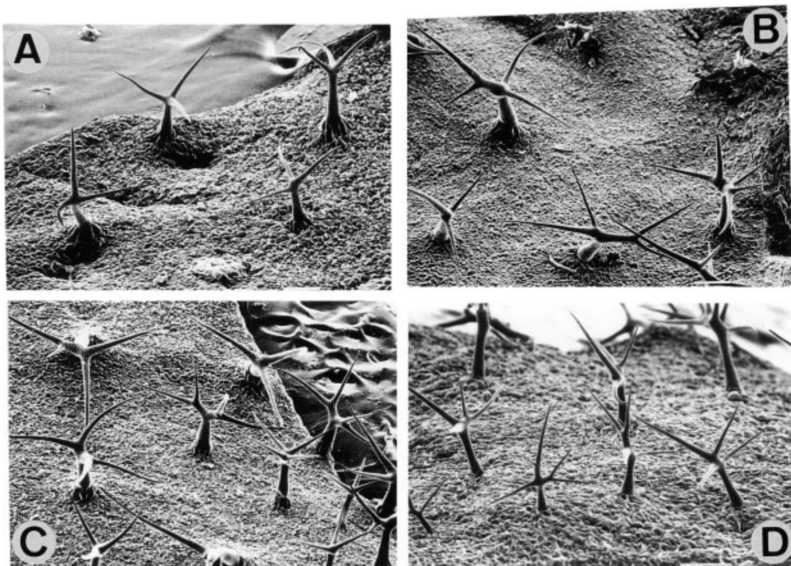


Figure 1.—Trichome overdevelopment in polyploid and mutant lines. Surfaces from rosette leaves were examined by scanning electron microscopy. (A) Diploid, wild-type L*er*, (B) tetraploid L*er* derivative, (C) *kak-1* mutant, and (D) *spy-5* mutant. Note four-branch trichomes in the last three cases. Bars, 100 μm.

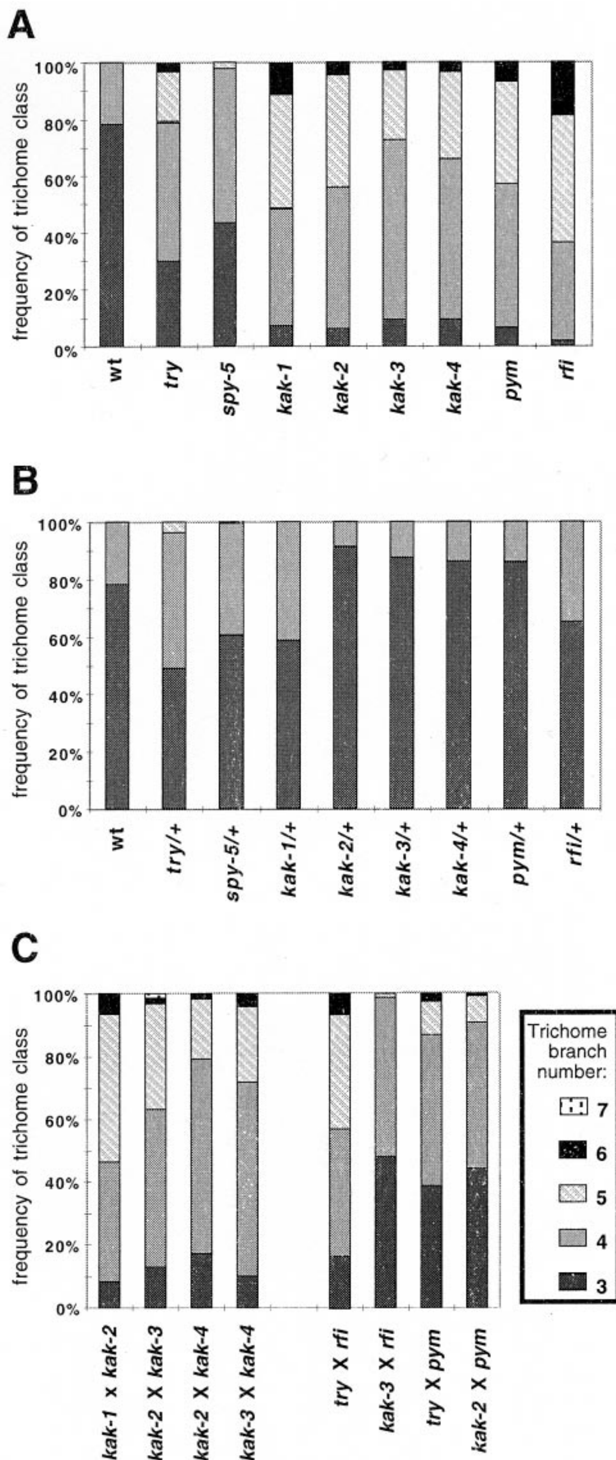


Figure 2.—Trichome branching in mutant lines. Branch number was counted for all trichomes of the third and fourth leaf blades. Trichome classes with increasing branch number are represented as a percentage of total trichomes (see inset symbol legend). (A) Homozygous plants; wt, wild-type L*er.* The total trichomes observed were between 193 and 294, corresponding to three to seven plants. (B) Heterozygous plants: F_1 plants from backcrosses to L*er.* Total trichome numbers were between 187 and 287, representing three to four plants. (C) *Trans*-heterozygous plants: F_1 plants resulting from the indicated crosses. Total trichome numbers were between 121 and 297, corresponding to two to four plants.

stellate (*i.e.*, unbranched), and only 10–20% had two branches. No major increase in trichome stem branching was observed for the *pym* and *spy-5* mutants. The *kak* mutants presented a very weak stellate phenotype on stems (data not shown). By contrast, nearly all stem trichomes from *try* and *rfi* mutants were stellate, presenting two or three branches, and with stalks typically shorter than on leaves.

To define the formal nature of their genetic defects, mutant lines were backcrossed to their wild-type ecotype, L*er.* Heterozygous F_1 plants produced by crosses with *kak-2*, *kak-3*, *kak-4*, and *pym* were wild-type in phenotype (Figure 2B). In addition, the F_2 populations derived from crosses of these four mutant lines with either L*er* or Col wild-type plants segregated for wild type and strongly overbranched phenotypes in a ratio close to 3:1 (data not shown). These data indicate the presence of a single, recessive Mendelian mutation for *kak-2*, *kak-3*, *kak-4*, and *pym.* By contrast, lines *try*, *spy-5*, *kak-1*, and *rfi* yielded F_1 plants with a weakly overbranched phenotype (Figure 2B). The semidominant nature of the *try* mutation on trichome branching is in agreement with Folkers *et al.* (1997). The *spy-5* mutation also showed semidominance in this test, in agreement with other germination and vegetative traits reported for *spy* alleles (Jacobsen and Olszewski 1993; Jacobsen *et al.* 1996). The F_2 populations derived from crosses of *rfi* and *kak-1* with either L*er* or Col wild-type plants segregated wild-type, weakly, and strongly overbranched individuals in ratios close to 1:2:1 (data not shown). The *rfi* and *kak-1* defects are therefore both due to a single, semidominant mutation.

Genetic mapping defines five complementation groups: Due to the fact that some mutations are semidominant, it may be misleading to class mutants into complementation groups on the sole phenotype of F_1 plants from crosses between homozygous mutants. As a first step to exclude possible allelic relationships, we therefore mapped the mutations, using DNA polymorphic markers (Figure 3). Mutants were crossed to wild-type plants of a different ecotype, Col, F_2 plants showing a strong overbranched phenotype were selected, and their DNA was analyzed (materials and methods). Whereas most markers yielded L*er* and Col chromatides in 1:1 ratio, we noted a skew in favor of Col in a few cases (materials and methods); most of these markers also show a Col excess in the Col × L*er* recombinant inbred (RI) population (Lister and Dean 1993), and were considered unlinked. We also found, as expected, markers showing a clear excess of L*er* chromatides, indicating linkage (Figure 3). The *pym* mutation showed linkage to the bottom of chromosome 2. The *rfi* mutation showed linkage to chromosome 5. The four *kak* mutations all showed tight linkage to the bottom of chromosome 4. To confirm linkage of these four mutations, we crossed *kak* mutants with each other and searched for wild-type recombinants in F_2 populations

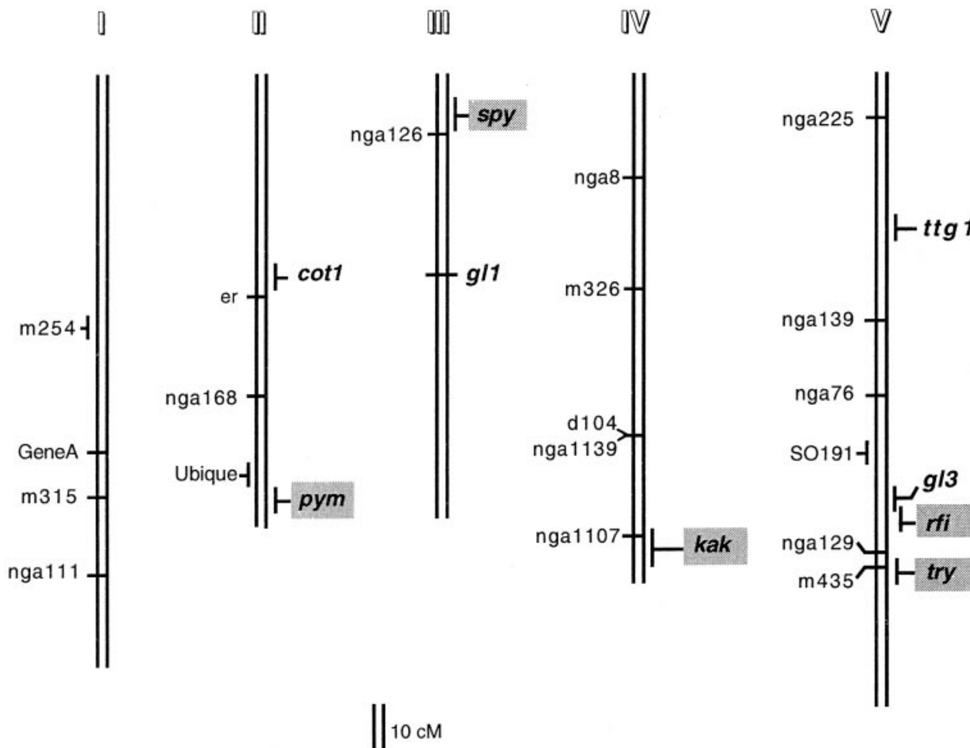


Figure 3.—Genetic map of loci affecting trichome initiation and cell size. Mutations used in this study are in gray boxes. The relevant codominant DNA markers are indicated on the left side of chromosomes. Vertical bars denote mutations not anchored on the RI map (http://genome-www3.stanford.edu/). Mapping references: Szymanski *et al.* (1998) for *cot1*, Jacobsen *et al.* (1996) for *spy*, Koornneef *et al.* (1983) for *gl1*, *ttg*, and *gl3*, M. Hülskamp (unpublished data) for *try*. Markers linked to the *kak*, *pym*, and *rfi* loci were identified by a Col:L*er* distribution skewed in favor of L*er* in mapping crosses with Col. For Col × *pym*, the observed recombinant/total chromatide ratios were 2/46 and 1/40 at the *nga168* and *AthUbique* loci. For Col × *rfi*, ratios were 12/50, 8/48, 6/46, and 15/48 at the *nga139*, *nga76*, *AthSo191*, and *nga129* loci, respectively. The *kak* mutations are tightly linked (see Table 1); for crosses Col × *kak-1*, Col × *kak-2*, Col × *kak-3*, and Col × *kak-4*, segregation data at the *nga1139* locus were, respectively 8/40, 10/44, 6/46, and 7/28 (Col/total); at the *nga1107* locus, they were 2/40, 0/44, 1/42, and 1/30. For Col × *kak-2*, ratios were 10/44 and 3/48 at the ARMS markers *m326* and *d104.*

(Table 1). No wild-type recombinants were found, indicating that these four mutations are very closely linked. Evidence for allelism was finally obtained in a complementation test. The phenotype of *trans*-heterozygous *kak* F_1 plants was similar to either homozygous parent (Figure 2, C and A), whereas *kak* × L*er* F_1 plants showed a phenotype much closer to wild type (Figure 2, B and C). These data thus indicate a lack of functional complementation within this group. Taken together, these results strongly suggest that the four chromosome 4 mutations all affect the same gene, *KAK.*

TABLE 1

The four *kak* mutations are tightly linked

Cross	Phenotype		
	kaktus[a]	Wild type[b]	r^{max} (cM)[c]
kak-1 × *kak-2*	221	0	0.7
kak-1 × *kak-3*	376	0	0.4
kak-1 × *kak-4*	158	0	0.9
kak-2 × *kak-4*	124	0	1.2
Total	879	0	0.2

[a] F_2 plants with a strong overbranched phenotype, *i.e.*, some five-branch trichomes, and a majority of four-branch trichomes.

[b] F_2 plants with a majority of three-branch trichomes, *i.e.*, a +/+ genotype. No plant with an intermediate phenotype compatible with a *kak-1*/+ genotype was observed.

[c] The maximum genetic interval separating both mutations, r^{max}, expressed in percentage recombination (*i.e.*, in centimorgans, the interval being small), was computed from the binomial law at the α = 95% confidence level using MapManager Calculator.

To summarize, our genetic screen allowed the definition and mapping of three loci, *KAK*, *PYM*, and *RFI*, where mutations result in a trichome overdevelopment. These phenotypes are strongly reminiscent of both *try* and *spy* plants.

Double mutants define distinct pathways regulating trichome cell growth: To define whether the five wild-type genes *SPY*, *TRY*, *KAK*, *PYM*, and *RFI* act in a single, linear pathway or in several parallel ones, double mutants were constructed to define their additive or epistatic interactions. Each of the 10 possible pairwise combinations between two different complementation groups was represented by at least one cross. In several cases, *trans*-heterozygous F_1 plants showed overbranched trichomes, as illustrated on Figure 2C for four crosses. Mutations *try* and *rfi* are both semidominant; the *trans*-heterozygous *try*/+ *rfi*/+ plants could not be distinguished from either homozygous parent (Figure 2, C and A). The three other crosses involved at least one (*kak-3* × *rfi*, *pym* × *try*) or even two recessive mutations (*kak-2* × *pym*). Again, the corresponding *trans*-heterozygous plants showed a stronger phenotype than plants heterozygous at a single locus (compare Figure

2, C and B). According to our mapping data (Figure 3), the mutations combined in these four crosses are not allelic. Indeed, in the last three cases, the phenotype of plants heterozygous at two distinct loci was less marked than that exhibited by the homozygous parents (Figure 2, A and C). These data therefore indicate that some cumulated haplo-insufficiencies can lead to a synthetic phenotypic enhancement.

In seven crosses (corresponding to the double mutants on Figure 4), some F_2 plants produced clearly larger trichomes bearing more than seven branches, hereafter referred to as maxichomes. Maxichomes from three different genotypes are illustrated on Figure 4. Branches ramified twice or more were frequent, resulting in tree-shaped cells. The relative size of branches was variable, and the extreme situation of a bump on a main branch could be found (Figure 4C): branch initiation is therefore maintained until late stages of mutant trichome cell growth. Maxichomes were never observed in the homozygous parental lines, so they must represent a synthetic phenotype. In the *try* × *spy-5* cross, about 1/16 of the F_2 plants showed such a maxichome phenotype (Table 2), indicating that this additive interaction requires both loci to be homozygous to be detected. Other crosses between unlinked mutations produced significantly more than 1/16 F_2 plants bearing maxichomes (see list in materials and methods; data not shown), suggesting that some genotypes with one homo- and one heterozygous mutant locus lead also to an additive phenotype.

Homozygous double mutants were isolated on the basis of their enhanced phenotype, which was stabilized in their offspring (materials and methods). Trichome branching was quantified for each double mutant and compared to parental single mutants. This revealed five strongly additive interactions (Figure 4D) and two weakly additive ones (Figure 4E). The *try* mutation was strongly additive in double mutants with *spy-5*, *pym*, or *kak-2* (Figure 4D). This was characterized by an increase in branch number in the double mutants higher than the sum of the increases observed in the parental single mutants. Maxichomes were also frequently observed in the F_2 progeny of *try* × *kak-1*, *try* × *kak-3*, and *try* × *kak-4* crosses (data not shown), indicating that a strong additivity is not due to specific interactions with a given *kak* allele. The simplest explanation to these strongly additive combinations is that *TRY* defines a repression route independent of *SPY*, *KAK*, and *PYM.* Besides increased trichome branching, we also noted that the four double mutants *try spy-5*, *try pym*, *try kak-2*, and *try rfi* frequently produced clustered trichomes (*e.g.*, see Figure 4, B and C). They also contained a small proportion of trichomes with one very basal branch emerging very low on the stalk, a feature also observed on the *try* simple mutant but on no other genotype. On both *try pym* and *try rfi* double mutants, we sometimes noted swellings affecting the upper part of the stalk or one main branch: the swollen part could be twice as wide as the stalk.

The quantitative effects of the *rfi* mutation on trichome branching varied sharply in three different genetic combinations. The *pym rfi* double mutant presented the highest score (Figure 4D), suggesting that *RFI* and *PYM* act in independent pathways. By contrast, the *try rfi* and *kak-3 rfi* double mutants only showed a partial additivity (Figure 4E). In the *try rfi* double mutant, the increase in trichome branching was clearly less than the added contributions of the two single mutations. A similar situation was observed for the *kak-3 rfi* double mutant: when *kak-3 rfi* was compared to *rfi*, the global increase in leaf trichome branching was extremely low, although an additive phenotype was also observed on *kak-3 rfi* stems (materials and methods). The *kak-2* × *rfi* cross produced F_2 plants similar to the *kak-3* × *rfi* cross (data not shown).

The *pym* mutation showed a strong additivity in double mutants with *try* and *rfi*, and also with *kak-2* (Figure 4D). Some of the plants observed in the F_2 progeny of *kak-4* × *pym* and *kak-3* × *pym* crosses were very similar to the *kak-2 pym* double mutant, suggesting that a strong additivity does not require a specific *kak* allele (data not shown). The simplest explanation for these additive phenotypes is that the wild-type *PYM* gene represses branch formation through a pathway independent of both *KAK* and *TRY.*

By contrast with the seven genetic combinations described above, where at least a qualitative increase could be found, three crosses with *spy-5* produced F_2 populations devoid of plants with an increased trichome branching: *spy-5* × *kak-2*, *spy-5* × *pym*, and *spy-5* × *rfi.* Table 2 shows that the complete absence of F_2 plants defining an additive class is highly significant for unlinked mutations. The simplest explanation for each of these three pairs of mutations is that they both act to block (or downregulate) the same genetic pathway.

Tetraploid lines produce overbranched trichomes: A Col tetraploid derivative was isolated during our initial screening for lines showing overbranched trichomes, as mentioned above. Among L*er* offspring, we also isolated a tetraploid line with a similar phenotype (materials and methods), suggesting that trichome overbranching is a general feature of genetically polyploid lines. Examination of another L*er* tetraploid derivative confirmed the prominent presence of four-branch trichomes (Figure 1B), *i.e.*, a leaf phenotype very close to *kak* and *pym* mutants. We quantified the increase in trichome branching with ploidy, which was clear in both L*er* and Col ecotypes (Figure 5A). Interestingly, triploid plants presented a trichome branching intermediate between diploids and tetraploids, with about as many trichomes bearing three branches as four. Arabidopsis stocks described as polyploid and available from the Ohio Arabidopsis Biological Resource Center (http://aims.cps.msu.edu/aims/) were scored for trichome

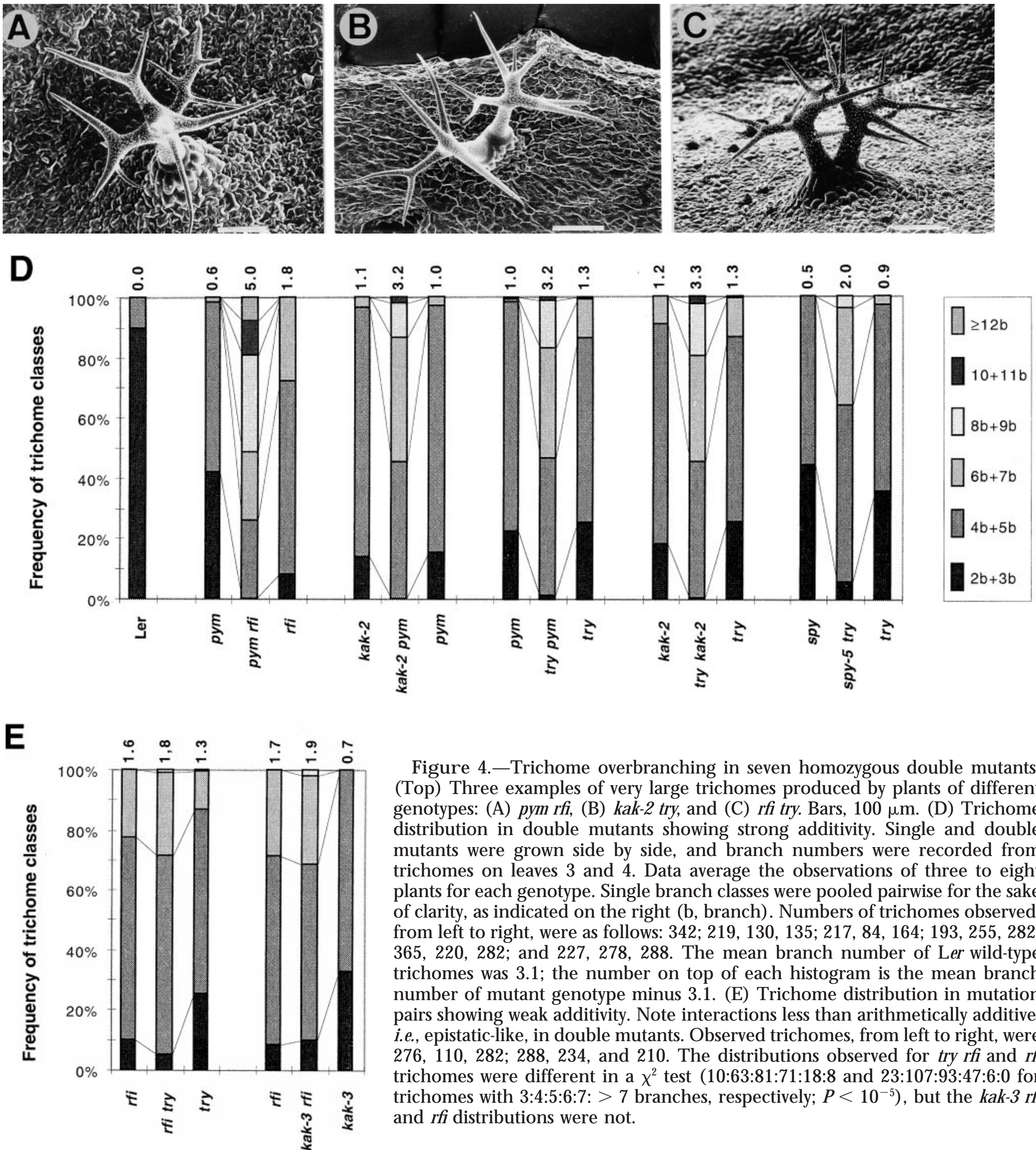


Figure 4.—Trichome overbranching in seven homozygous double mutants. (Top) Three examples of very large trichomes produced by plants of different genotypes: (A) *pym rfi*, (B) *kak-2 try*, and (C) *rfi try*. Bars, 100 μm. (D) Trichome distribution in double mutants showing strong additivity. Single and double mutants were grown side by side, and branch numbers were recorded from trichomes on leaves 3 and 4. Data average the observations of three to eight plants for each genotype. Single branch classes were pooled pairwise for the sake of clarity, as indicated on the right (b, branch). Numbers of trichomes observed, from left to right, were as follows: 342; 219, 130, 135; 217, 84, 164; 193, 255, 282; 365, 220, 282; and 227, 278, 288. The mean branch number of L*er* wild-type trichomes was 3.1; the number on top of each histogram is the mean branch number of mutant genotype minus 3.1. (E) Trichome distribution in mutation pairs showing weak additivity. Note interactions less than arithmetically additive, *i.e.*, epistatic-like, in double mutants. Observed trichomes, from left to right, were 276, 110, 282; 288, 234, and 210. The distributions observed for *try rfi* and *rfi* trichomes were different in a χ^2 test (10:63:81:71:18:8 and 23:107:93:47:6:0 for trichomes with 3:4:5:6:7: > 7 branches, respectively; $P < 10^{-5}$), but the *kak-3 rfi* and *rfi* distributions were not.

branching and assayed for their genomic content by flow cytometry. The two lines confirmed to be true tetraploids, CS3151 (another Col derivative) and CS3432, also produced mainly four- and five-branch trichomes (data not shown). Thus a general consequence of polyploidy is to raise the probability for a leaf trichome to make more than three branches.

As trichome cells are known to undergo endoreduplications, we studied the trichome nuclear DNA content in diploid, triploid, and tetraploid plants. DAPI-stained trichomes were examined by microfluorometry (Figure 5B). Trichome nuclei from wild-type diploid plants presented a broad distribution ranging from about 16C to about 64C. For triploid and tetraploid lines, trichome nuclei distributions were shifted to higher values, and the mean DNA fluorescence values

TABLE 2

Lack of additivity between *spy-5* and either *kak*, *pym*, or *rfi* mutations

Cross	Trichome phenotype: Plants without maxichomes[a]	Trichome phenotype: Plants with maxichomes[b]	Total F_2 plants	*P* value[c]
spy-5 × *try*	192	12	204	0.83
spy-5 × *kak-2*	200	0	200	$<3\ 10^{-4}$
spy-5 × *pym*	177	0	177	$<6\ 10^{-4}$
spy-5 × *rfi*	183	0	183	$<5\ 10^{-4}$

[a] F_2 plants showing a trichome branching on rosette leaves equivalent to or weaker than either homozygous parent.

[b] F_2 plants showing a trichome branching stronger than either homozygous parent (see text for a definition of maxichomes).

[c] Probability of obtaining a distortion equal to or worse than the one observed in a χ^2 test performed with the null hypothesis of a 15:1 distribution. In case of additivity between unlinked mutations, at least 1/16 of the F_2 population is expected to produce maxichomes.

increased with the initial ploidy level. In genetically polyploid plants, an increase in trichome branching is therefore correlated with an increased trichome DNA content.

More DNA in mutant trichomes with supernumerary branches: We then asked whether trichomes from mutant lines also contained an increase in nuclear DNA content. Figure 6 shows that a shift toward higher DNA contents was observed for all single mutants; the extent of this shift, however, varied among mutants. The *pym* mutant showed the strongest increase in DNA fluorescence. An intermediate increase was observed for the *spy-5*, *kak-1*, and *kak-2* mutants, as well as for the *kak-3* mutant (compared to L*er* in an independent experiment; data not shown). The increase in DNA content was the weakest, and clearly less than twofold for the *kak-4* and *rfi* mutants. Compared to wild type, the increases in mean trichome nuclear fluorescence were nonetheless significant: for *rfi vs.* L*er*, $P < 10^{-4}$ and for *kak-4 vs.* L*er*, $P < 10^{-3}$ in Student's *t*-tests. Trichome nuclear areas from mutants also showed a shift toward higher values, with mean values significantly higher than those for wild type (data not shown). The observed trend thus suggests that the Arabidopsis genome, as a part or as a whole, undergoes additional replication during development of overbranched trichomes.

Arabidopsis leaves are known to be highly polysomatic, containing only a minority of 2C cells and high proportions of cells with nuclear contents of 4C, 8C, and 16C (Galbraith *et al.* 1991). This raises the question whether the deregulation of DNA synthesis observed in mutants is specific to trichome cells or general. We analyzed by flow cytometry the nuclei of whole leaves from wild type and mutants. Figure 7 shows the histograms of nuclei distributions. Interestingly, no obvious difference was observed between the mutant and wild-type genotypes. These data suggest that endoreduplications in trichome cells and in the inner layers of the leaves are regulated by distinct mechanisms. On the other hand, for a tetraploid line, peaks of 4C, 8C, 16C, and 32C nuclei were present instead of the 2C, 4C, 8C, and 16C peaks for a wild-type diploid (Figure 7). Furthermore, the 2^{n+1}C peaks from tetraploid replaced the 2^nC peaks from diploid in an essentially quantitative manner. This result strongly suggests a mechanism regulating the number of endoreduplication rounds independently of the initial chromosome number in the inner leaf cell layers.

We finally examined whether an increased trichome branching corresponds to a further increase in DNA content in a double mutant situation. Trichomes from wild-type, *pym*, *rfi*, and *pym rfi* plants were stained with DAPI, and their nuclear fluorescence was recorded (Figure 8). As described above, trichome nuclei in either simple mutant were larger on average than in wild type. The double mutant exhibited a very broad distribution, with 8% nuclei having less than one time and 25% nuclei having more than four times the wild-type trichome mean DNA content (6 and 19 nuclei out of 76, respectively). This class of very large nuclei was absent in *rfi* and much less abundant in *pym* trichomes (8%, 6/85). The mean nuclear fluorescence was significantly higher for the double mutant than for either single (*i.e.*, *pym rfi vs. pym*: $P < 0.05$ in a Student's *t*-test), and both single mutants were higher than wild type. Therefore, at least for this double mutant, the presence of trichomes bearing more branches correlates with the presence of larger nuclei with a brighter fluorescence. These data are compatible with the idea that a fraction of the *pym rfi* maxichomes has undergone two more endoreduplication rounds than wild-type trichomes.

DISCUSSION

New large overbranched trichome mutants: Previous studies have identified three types of mutants producing

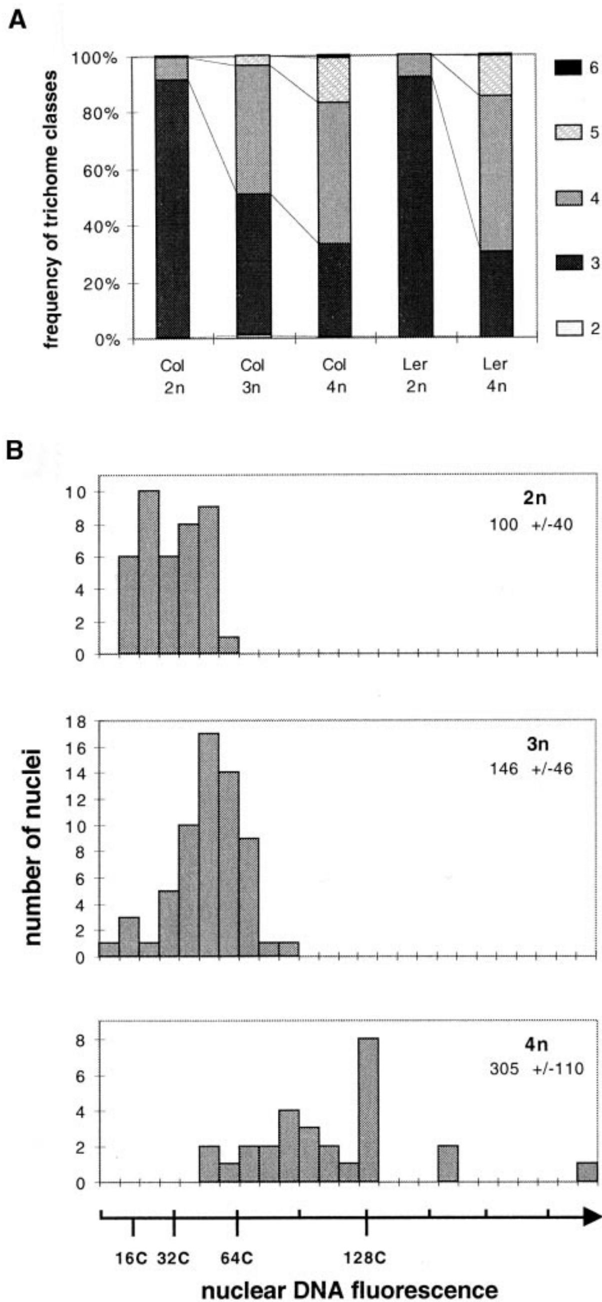
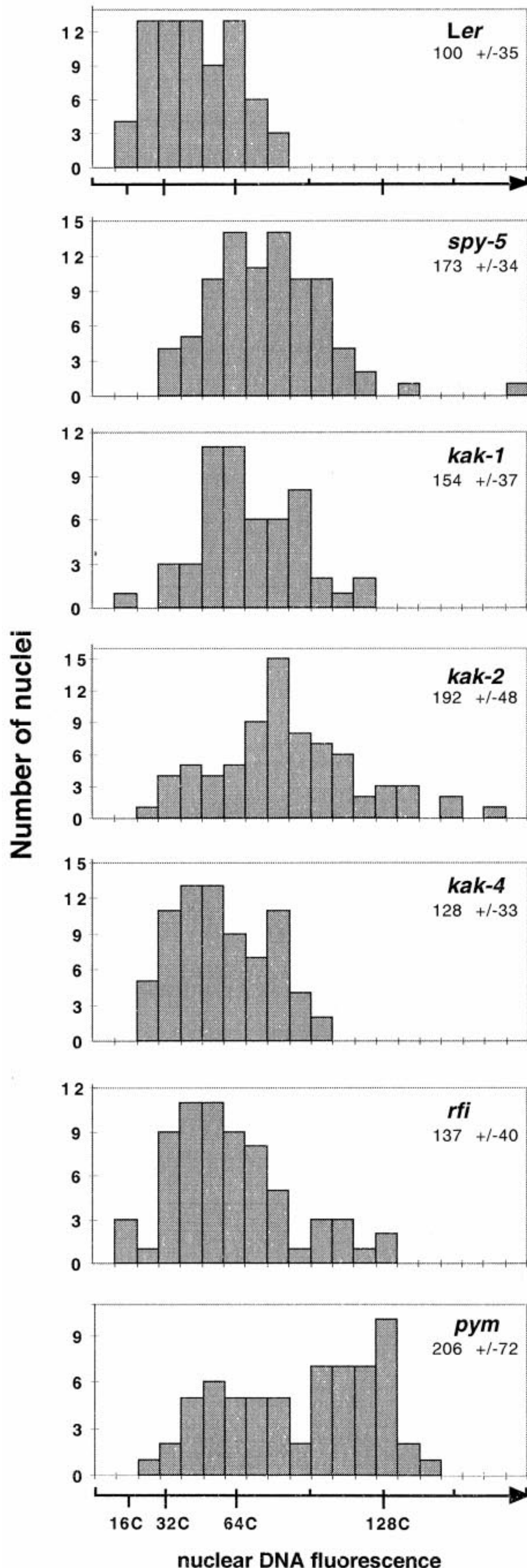


Figure 5.—Branch number and nuclear DNA content in trichomes from polyploid lines. (A) Distribution of trichomes into classes of increasing branch number. The number of branches of each class is indicated on the right. For each genotype, three to five plants were examined. Total trichomes observed were, from left to right: 472, 405, 596, 325, and 282; the differences in branching between lines presenting different ploidy levels were all significant when distributions were compared pairwise. (B) DNA fluorescence distribution of trichome nuclei stained with DAPI and quantified by image analysis. $2n$, wild-type L*er*; $3n$, triploid Col; $4n$, tetraploid Col derivative. Trichomes from expanded leaf blades were observed by epi-fluorescence microscopy and their fluorescence was recorded. Three-, random, and four-branch trichomes were selected, and 40, 62, and 28 nuclei analyzed, respectively, for $2n$, $3n$, and $4n$ lines. DNA fluorescence was quantified by image analysis, and the mean trichome nuclear content was estimated to be 36C for wild type by comparison to guard cell nuclei (see materials and methods). This was converted into 100 arbitrary units to express mean ± SD of nuclear fluorescence values (numbers below genotypes). Wild-type trichome DNA content was estimated to be 36 ± 14C (mean ± SD). When compared in pairwise Student's *t*-tests, the mean fluorescence values for genotypes of different ploidy levels were all significantly different (*e.g.*, $2n$ *vs.* $3n$, $P < 10^{-6}$).

large overbranched trichomes: *try*, *kak* (Hülskamp *et al.* 1994), and *spy* (Perazza *et al.* 1998). For the *try* mutant, the increase in trichome DNA content had been quantified, and it has been proposed that *TRY* represses a fifth and extra endoreduplication round (Hülskamp *et al.* 1994; Schnittger *et al.* 1998). With the aim of identifying new loci controlling endoreduplication, we screened an EMS-mutated population in search of plants producing overbranched trichomes. We isolated five new mutants, which define three genes distinct from *SPY* and *TRY.* Three of the new mutations (*kak-2*, *-3*, and *-4*) are allelic with *kak-1* and define the *KAK* gene, which maps at the bottom of chromosome 4. Whereas the three *kak* alleles are recessive, *kak-1* is semidominant; as the phenotype of all four lines appears similar in strength, *kak-1* may correspond to a slightly dominant negative defect. The *pym* and *rfi* mutants define two new independent loci, named *PYM* and *RFI.* The *pym* mutation is recessive and maps on chromosome 2, clearly farther south than *RTN* and *cot1*, two loci which control trichome initiation and map close together north of *er* (Larkin *et al.* 1996; Szymanski *et al.* 1998). The *rfi* mutation affects a locus on chromosome 5 distinct from *try*, which maps close to *m435* (M. Hülskamp, unpublished data). This mutation is semidominant and could be either a loss or a gain of function. In the first case, *RFI* defines a new (and fifth) gene limiting cell growth. On the other hand, *rfi* maps in the same area as *GL3*, a locus where recessive mutations lead to trichome underdevelopment (Koornneef *et al.* 1983); this raises the interesting possibility that *rfi* defines a gain-of-function allele of *GL3.*

A single mutant allele only has been recovered for each of the *RFI* and *PYM* genes, and it may be that mutations in more than five genes lead to a similar phenotype. Trichomes from the mutants described in this study differ from those produced by the *nok*-122 mutant, which also have supernumerary branches, but whose cell and nuclear size are similar to wild-type trichomes (Folkers *et al.* 1997). We did isolate some lines with a *nok*-like phenotype in our screen (materials and methods), but the corresponding trichomes were clearly smaller in cell size than *kak* trichomes. These mutants have therefore not been included in this study, and some of them have turned out to be allelic to *nok* (M. Hülskamp, unpublished results). We also recovered a new *try* allele, which was not included here (see mate-



rials and methods; Arp Schnittger, personal communication). The fact that we did not find *spy* alleles is most likely due to the fact that the *spy* phenotype is highly pleiotropic (Jacobsen and Olszewski 1993); such plants have not been selected since the screen initially aimed at mutants affected in trichome only.

***SPY* and *TRY* act by independent pathways:** The five genes *TRY, SPY, KAK, PYM,* and *RFI* act on trichome growth, as mutant trichomes bear extra branches and appear larger than wild type. Trichome growth derepression is never a fully dominant trait in the corresponding mutants; it is recessive in *pym* and three of the *kak* mutants. Other traits associated with *spy-5* and *try* mutations are also recessive (Wilson and Somerville 1995; Schnittger *et al.* 1998). With the possible exception of *RFI*, and despite the general lack of molecular proof for losses of function, it therefore seems that these genes all act formally as repressors of trichome morphogenesis.

The trichome overbranching phenotype provoked by single mutations described in this study was strongly enhanced in some double mutant combinations and weakly or not at all enhanced in others. A nonadditive phenotype in a double mutant shows the epistasy of one mutation over the other and indicates that the two corresponding genes act in a common, linear pathway. A weak, less than arithmetically additive phenotype can be assimilated with epistasy (*e.g.*, see Koornneef *et al.* 1998). Finally, a strongly additive phenotype is compatible with two explanations when the null or non-null status of the mutations combined is not known, which is the case for all mutations but *spy-5* (see below). The two mutations may affect two pathways that act independently from each other, in parallel. Alternatively, both mutations may be leaky (non-null) and provoke partial and cumulative losses of function along a single, linear genetic pathway. As discussed by Guarente (1993), if one mutation is a null, a linear pathway will be knocked out in the corresponding single mutant, and cannot work less than zero in a double.

A common feature to the mutations used in this study is that the phenotype they confer to single mutants could always be strongly enhanced in at least one double mutant combination (*e.g.*, *spy-5* was enhanced by *try*). This observation implies that no single mutation is sufficient to fully derepress trichome cell growth. The first

Figure 6.—DNA fluorescence distributions in mutant trichome nuclei. Trichomes with 3- and ≥4-branch trichomes were selected for wild type and mutants, respectively, DAPI-stained, and treated as for Figure 5B. Nuclei analyzed were 74 for wild-type L*er*, 85 for *spy-5*, 54 for *kak-1*, 75 for *kak-2*, 75 for *kak-4*, 67 for *rfi*, and 64 for *pym*. The C scale was set as for Figure 5B and the mean wild-type trichome DNA content was estimated to be 45 ± 18 C; this value was converted into 100 arbitrary units to express mean ± SD below each genotype.

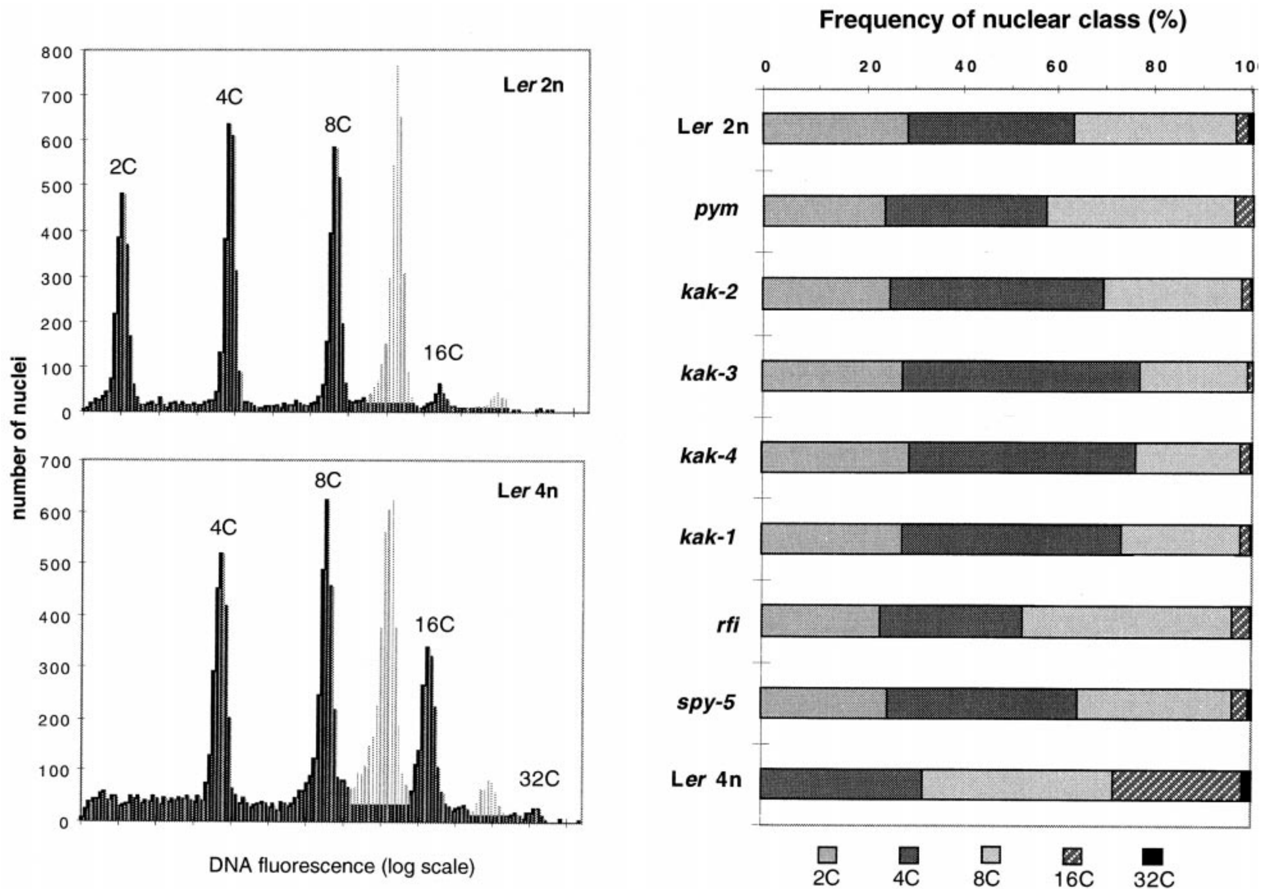


Figure 7.—Flow cytometry analysis of whole leaves. Nuclei were released from the third or fourth leaf blade of 25-day-old Arabidopsis plants. The Arabidopsis leaf was chopped with a razor blade together with a piece of tomato leaf (used as internal standard) in a DAPI-containing buffer and then filtered. The filtrate was then analyzed by flow cytometry (materials and methods). (Left) Distribution of nuclei on a log fluorescence scale, shown for two samples only. The peaks corresponding to tomato 2C and 4C nuclei are shaded gray, and those belonging to Arabidopsis are in black. Between the successive 2C, 4C, 8C, and 16C peaks, the numbers of intervening channels are the same, as are the coefficients of variation that correspond to precise doublings of the DNA content (the same was true for mutant genotypes). (Right) Quantification of the different nuclear classes for the indicated genotypes. The DNA contents of the corresponding classes are indicated below the histogram. The total number of Arabidopsis nuclei analyzed per genotype ranged from 2415 to 8775.

possible explanation is that all mutations are leaky. The *spy-5* mutation is a missense (Jacobsen *et al.* 1996) and is indeed non-null since it has a weaker phenotype than some other *spy* alleles; on the other hand, none of the 18 known *spy* alleles can be considered a definitive null mutation, since not one of them contains a long insertion or deletion in the coding sequence (N. Olszewski, personal communication). The idea that mutations nonallelic with *spy* are also leaky, however, poorly accounts for the fact that some double mutants show no additive phenotype. The alternative and more appealing explanation is that some redundancies (*i.e.*, parallel pathways) exist in the genetic network limiting trichome growth. This would explain why no single locus mutant with a maxichome phenotype has been recovered to date, despite several screens by us and others (see Introduction). Genetic redundancy can result from genes encoding homologous products, or from nonhomologous genes having acquired convergent functions (for review, see Pickett and Meeks-Wagner 1995).

A *SPINDLY*-dependent pathway can be defined from the epistatic relations of *spy-5* with *pym*, *kak*, and *rfi* in double mutants (Table 2). The *kak rfi* double mutants presented an extremely weak phenotypic additivity (Figure 4E), which further suggests that the *SPY*, *KAK*, and *RFI* genes act within a linear pathway. However, the *kak pym* and *pym rfi* double mutants presented a strongly additive phenotype (Figure 4D), and this suggests a redundancy between *PYM* and *KAK/RFI* to mediate *SPY* action. The recessive mutations *kak-2* and *pym* displayed nonallelic noncomplementation (Figure 2C), and a possible explanation is that *KAK* and *PYM* functions are largely overlapping. At present, we have no genetic evidence to order genes within the proposed *SPY/KAK/RFI* and *SPY/PYM* pathways. However, the highly pleiotropic phenotype of *spy* compared to *kak*, *rfi*, and *pym* mutants makes *SPY* likely to act upstream from *KAK*, *RFI*, and *PYM* genes. Whereas *SPY* modulates most gibberellin responses (Jacobsen and Olszewski 1993), we have noticed no alteration in germination, greening, or

flowering for the *kak*, *rfi*, and *pym* mutants (data not shown).

A *SPINDLY*-independent pathway is suggested primarily by the additive phenotype between *spy-5* and *try* (Table 2; Figure 4D). This proposal implies that *TRY* is not acting within the *SPY-PYM* linear pathway and is confirmed by the strongly additive *try pym* phenotype. Similarly, *TRY* is not cross-talking with *SPY/KAK* since the four *try kak* combinations also show strong additivity. On the other hand, only weak additivity was observed between *try* and *rfi*, which contrasts with the other *try* double mutants. This suggests that *TRY* could exert part (or all) of its effect through the *RFI* gene; it would be consistent with the occurrence of clustered trichomes on leaves and of highly branched trichomes on stems in *try* and *rfi* mutants only. As mentioned above, *RFI* also appears to interact with *KAK*, and this would mean that the *TRY* and *KAK* pathways are convergent, with *RFI* acting at (or downstream of) the convergence point.

Taken together, our data are compatible with a model in which the *TRY* and *SPY* genes limit trichome growth by distinct pathways. The *SPY*-dependent response may involve the parallel actions of *PYM* and *KAK*. The *TRY* and *KAK* pathways would converge on *RFI*.

Derepressed DNA synthesis in mutants: An increase in nuclear DNA content was observed in *pym*, *spy-5*, *kak*, and *rfi* trichomes. This increase was a plain doubling for *pym* but surprisingly less for the *spy*, *kak*, and *rfi* overbranched trichomes. A less than twofold increase in nuclear DNA fluorescence has also been reported

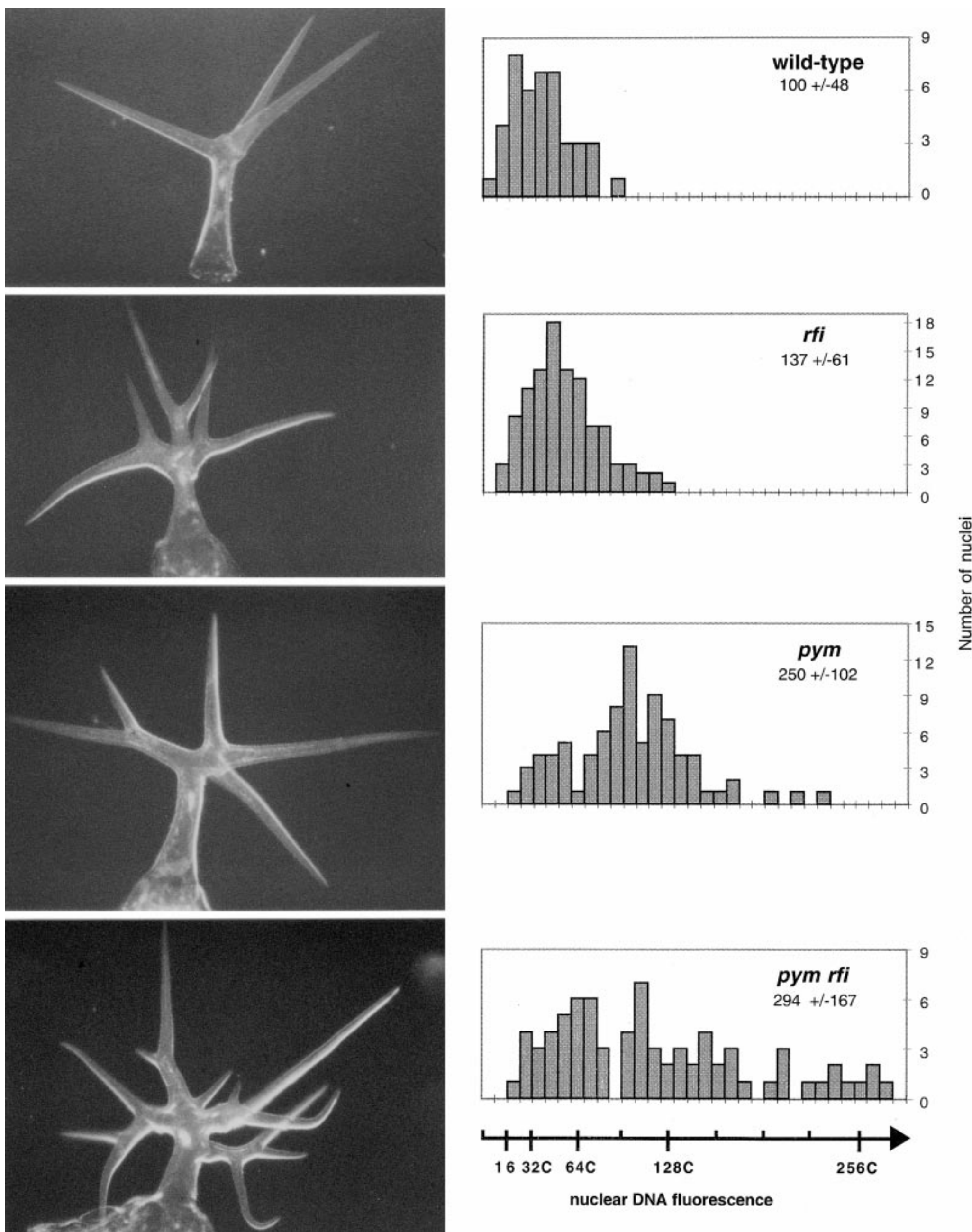


Figure 8.—Additive effects of *pym* and *rfi* mutations on trichome nuclear DNA content. (Left) DAPI-stained trichomes, observed by epi-fluorescence microscopy and counterilluminated by light transmission. Note the small size of nuclei from adjacent cells and the difference in size and brightness between wild-type and *pym rfi* trichome nuclei. (Right) Genotypes and the quantification of trichome nuclear fluorescence. The relative mean ± SD fluorescence values are indicated below genotypes. Trichomes with three and more than three branches were selected for wild-type (L*er*) and mutant genotypes, respectively, and 43, 103, 76, and 85 nuclei were analyzed.

for *try* trichomes (Schnittger *et al.* 1998) and contrasts with the 2C, 4C, and 8C modes obtained by flow cytometry of whole leaves, which fit to a 2^n geometric progression (Galbraith *et al.* 1991; this work). Like other authors, we have observed a broad range of fluorescence measurements for trichome nuclei in the wild type and even broader ranges in mutant trichomes with larger nuclei (Melaragno *et al.* 1993; Hülskamp *et al.* 1994; Schnittger *et al.* 1998; this work, Figures 2, 5, and 8). These dispersed distributions may reflect the unresolved presence of two discrete subpopulations (*e.g.*, a bulk of 32C and a minority of 64C nuclei on the wild-type leaf). Within this frame, it may be that the increased trichome DNA content in mutants reflects an upregulation in the number of endocycles. The alternative view that all parts of the Arabidopsis genome are not equally represented in wild-type or in mutant trichomes is also possible: the endocycles taking place in trichomes would be incomplete. In the germline cysts of Drosophila, a significant fraction of the wild-type genome becomes underrepresented in highly endoreduplicated nuclei. This fraction corresponds to satellite and ribosomal DNA, and the latter sequences replicate fully in a cyclin E mutant (Lilly and Spradling 1996). New technical approaches using molecular hybridization should help in answering the qualitative issues of DNA synthesis in trichomes.

Interestingly, mutations leading to an increased DNA content in trichomes did not lead to a general derepression of endoreduplications in all plant tissues, as indicated by leaf polysomaty distributions close to wild type (Figure 7). This suggests that nuclear DNA synthesis is triggered differently in trichomes and in the major tissues of the leaf blade (*i.e.*, in mesophyll cells). This may involve distinct mechanisms, or different threshold levels of a common mechanism in different cell types. Our flow cytometry histograms of bulk leaf tissue, however, may not detect changes in the polysomaty of a minor class like epidermal pavement cells. The issue of a cell-type or tissue-specific action of the mutated genes therefore remains open.

Gibberellin hormones have recently been shown to be involved in both trichome initiation and trichome branching control (see Introduction). We show here that *spy-5* trichomes contain nearly twice as much DNA as wild-type trichomes. So far, all processes affected by the *spy* mutations are known to be GA-controlled (Jacobsen and Olszewski 1993), and this strongly suggests that an extra round of endoreduplication is positively regulated by gibberellins. A role for GA hormones in the activation of the plant cell cycle has been shown in several cases (Jacobsen and Olszewski 1991; Sauter *et al.* 1995; Gendreau 1998). The action of GAs on trichome initiation is known to be dose dependent. This has been shown negatively, by growing wild-type plants on hormone biosynthesis inhibitors (Chien and Sussex 1996; Perazza *et al.* 1998). It has also been shown positively: trichome initiation can be gradually restored by exogenous GAs on the abaxial leaf epidermis of *ga1-3* plants (Telfer *et al.* 1997). Interestingly, the expression of the *GL1* gene, which is restricted to the epidermis in growing leaves, is strongest in committed trichome cells (Larkin *et al.* 1993). As the *GL1* mRNA level is positively regulated by GAs (Perazza *et al.* 1998), it might be that increasing levels of GA signaling through *GL1* are required for the successive trichome endocycles, which would normally stop after the fourth round or after a fifth round in a derepressed mutant background.

A patterning defect in *rfi* and *try* mutants: The *rfi* mutant presented an increase not only in trichome branching but also in trichome clustering: whereas wild-type trichomes are evenly spaced on the epidermis, *rfi* trichomes were found at a higher frequency with another trichome as neighbor cell. This pleiotropic phenotype has been noted earlier for the three *try* alleles (Schnittger *et al.* 1998), but was not apparent on *pym*, *spy-5*, or *kak* simple mutants. The clustering defect indicates an alteration in the process driving neighbor cells into different fates. This process appears to be related to the phenomenon of lateral inhibition described in some animal tissues: cells are initially equivalent, and a cell committing to a given fate actively lowers the probability that neighbor cells will do the same (Hülskamp *et al.* 1994; Larkin *et al.* 1996; reviewed in Larkin *et al.* 1997 and Hülskamp and Schnittger 1998). Since trichome cells undergo endoreduplications while neighbor cells divide, a possible explanation is that trichome patterning is governed through *TRY* and *RFI* by coordinated cell cycle decisions from neighbor cells. The cell specified to become a trichome would irreversibly exit the mitotic cycle and undergo endoreduplications, while the neighboring cells would be prevented from adopting the same fate and keep on dividing.

Leaf cells count the number of endocycles: Diploid, triploid, and tetraploid plants were found to produce trichomes containing increasing nuclear DNA amounts. This strongly suggests a developmental program in which the cell specified to become a trichome counts the number of endocycles, independently of its initial chromosome number. Flow cytometry analysis of whole leaves of diploid and tetraploid lines indicates that the ratios of leaf cells having undergone 0, 1, 2, or 3 endoreduplication cycles are also independent of the initial genome size. Our data rule out a feedback mechanism warning the nucleus about its genomic content past a critical threshold. An equivalent conclusion has been reached by comparing hypocotyl cells on diploid and tetraploid seedlings (Gendreau *et al.* 1998). Thus, the extent of polysomaty is controlled by an endocycle counting mechanism in all investigated cell types.

More DNA in larger cells: The increased trichome branch number in polyploid *vs.* diploid lines suggests a coupling between cell size and nuclear DNA content

in wild-type trichomes. Such a coupling has been documented in other Arabidopsis tissues and in many other eukaryotic organisms. Arabidopsis epidermal pavement cells, for instance, show a linear correlation between cell volume and nuclear DNA content (Melaragno *et al.* 1993). In the yeast *Saccharomyces cerevisiae*, diploid cells are larger than haploid cells (120 *vs.* 70 μm^3; Sherman 1991). Mutant *Schizosaccharomyces pombe* cells that undergo successive endoreduplication rounds become gigantic (Moreno and Nurse 1994; Nishitani and Nurse 1995; Kominami and Toda 1997), much like giant cells found in insect salivary glands with polytene chromosomes.

The *spy-5*, *kak*, *rfi*, and *pym* mutants showed an increase in both trichome nuclear content and branch number. A first possibility is that increases in DNA content are the consequences of an increase in cell volume. This is, however, quite unlikely because the opposite has been described in hypocotyl cells: when seedlings are allowed to germinate, endoreduplications take place before cell elongation (Gendreau *et al.* 1997). Further, DNA synthesis is known to be required for cell elongation, since aphidicolin, a DNA polymerase inhibitor, prevents hypocotyl elongation (Gendreau 1998). A second possibility is that the DNA content dictates the trichome cell size, and that the coupling between both parameters is maintained in mutants. Endoreduplications would allow successive increases in cell size, which would be reflected by the final branch number. This explanation may hold for a mutation like *pym*, whose consequences on both trichome branch number and trichome DNA content are similar to those of tetraploidy. This is, however, also unlikely to hold true for all mutations. The hierarchy in trichome branching observed for single mutants (*e.g.*, L*er* < *pym* < *rfi*, Figures 3 and 7) did not match strictly with the measured nuclear contents (L*er* < *rfi* < *pym*, Figures 5 and 8). That *rfi* brings about an increase in trichome nuclear DNA was also observed in a *pym* background; in the *pym rfi* double mutant, however, the increase was again more pronounced in trichome branching than in nuclear fluorescence. It might be that the DNA amounts are somehow underestimated in *rfi* trichomes. Alternatively, the major contribution of *rfi* to an increased branch number could be independent from its role in DNA synthesis: *rfi* would uncouple cell growth from nuclear DNA content. An opposite example of uncoupling may be provided by trichomes of transgenic plants overexpressing *GL1* (*GL1*^{oe}) that are mainly three-branched (Oppenheimer *et al.* 1991; Larkin *et al.* 1994; Szymanski *et al.* 1998), yet present a genetic content higher than that of wild type (Schnittger *et al.* 1998). Interestingly, uncoupling leading to 2C cells larger or smaller than wild-type cells has been reported for plants ectopically expressing cell cycle transgenes (Hemerly *et al.* 1995; Doerner *et al.* 1996).

A very recent article provides evidence for the involvement of *TRY* in cell cycle regulation during leaf development (Szymanski and Marks 1998). In a *try* mutant, leaves contained more (*i.e.*, smaller) epidermal pavement cells per area unit than in wild type; pavement cells also presented a decrease in average DNA content (contrasting with an increase in *try* trichomes). *GL1*^{oe} reduced nuclear polysomaty but not cell size in pavement cells, *i.e.*, led to an uncoupled cell growth; this article and Schnittger *et al.* (1998) report, however, opposite effects of *GL1*^{oe} on trichome DNA content.

We are undertaking the cloning of negative regulators of trichome growth to provide insights into the molecular mechanisms they govern. This should help clarify whether these genes play a role in GA signal transduction. It may also help in understanding the intricate links between endoreduplication and trichome morphogenesis control.

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