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Extragenic suppressors of the *Arabidopsis zwi*-3 mutation identify new genes that function in trichome branch formation and pollen tube growth

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SUMMARY

The plant cytoskeleton plays a pivotal role in determining the direction of cell wall expansion, and ultimately the cell's final shape. However, the mechanisms by which localized expansion events are initiated remain obscure. Mutational analysis of the trichome (plant hair) morphogenic pathway in *Arabidopsis* has identified at least eight genes that determine trichome branch number. One of these genes, *ZWICHEL* (*ZWI*), encodes a novel member of the kinesin superfamily of motor proteins. Mutations in the *ZWI* gene cause a reduction in the number of trichome branches. To identify additional genes involved in trichome branch initiation, we screened for extragenic suppressors of the *zwi*-3 mutation and isolated three suppressors that rescued the branch number defect of *zwi*-3. These suppressors define three genes, named *suz*, for *suppressor of zwichel*-3. All of the suppressors were shown to be allele specific. One of the suppressors, *suz2*, also rescued the trichome branch number defect of another branch mutant, *furca1*-2. Plants homozygous for *suz2* have more than the wild-type number of trichome branches. This suggests that *SUZ2* is a negative regulator of trichome branching and may interact with *ZWI* and *FURCA1*. The *suz1* and *suz3* mutants display no obvious phenotype in the absence of the *zwi*-3 mutation. The *suz1 zwi*-3 double mutants also exhibited a male-sterile phenotype due to a defect in pollen tube germination and growth, whereas both the *suz1* and the *zwi*-3 single mutants are fertile. The synthetic male sterility of the *suz1 zwi*-3 double mutants suggests a role for *SUZ1* and *ZWI* in pollen germination and pollen tube growth. DNA sequence analysis of the *zwi*-3 mutation indicated that only the tail domain of the zwi-3 protein would be expressed. Thus, the *suz* mutations show allele-specific suppression of a kinesin mutant that lacks the motor domain.

Key words: *Arabidopsis thaliana*, Suppressor, *ZWICHEL*, Kinesin, Cell shape, mRNA splicing, Male-sterile plant

INTRODUCTION

The final shape of a plant cell is the result of precisely guided cell wall expansion. The plant cytoskeleton is known to play a pivotal role in determining the direction of cell wall expansion, in part by controlling the orientation of the cellulose microfibrils in the cell wall (Fowler and Quatrano, 1997; Giddings and Staehelin, 1991; Nicol and Höfte, 1998). However, the molecular mechanisms that control localized expansion events remain obscure.

The differentiation of *Arabidopsis* trichomes is well-suited to address questions of plant cell morphogenesis. *Arabidopsis* trichomes are large (approximately 300-500 μm tall), single cells that develop on most of the aerial parts of the plant. Wild-type trichomes have a genetically defined shape consisting of a stalk topped by three or four branches. The trichome developmental pathway has been studied by mutational analysis and many mutations have been identified that affect the final shape of the trichome cell (Folkers et al., 1997; Hülskamp et al., 1994; Marks et al., 1991; Oppenheimer, 1998; Oppenheimer et al., 1993). One of these mutations, *zwichel* (*zwi*), results in a trichome that has only two branches and a shorter than normal stalk (Folkers et al., 1997; Oppenheimer et al., 1997). The *ZWI* gene was cloned by T-DNA tagging and shown to encode a kinesin-like calmodulin-binding protein (named KCBP), a novel member of the kinesin family of microtubule (MT) motor proteins that is regulated by calmodulin (Narasimhulu et al., 1997; Oppenheimer et al., 1997; Reddy et al., 1996; Song et al., 1997).

Kinesin-like proteins (KLPs) have been identified in essentially all phyla of eukaryotes (see Barton and Goldstein, 1996 and Hirokawa, 1998 for reviews). KLPs function in intracellular organelle transport as well as mitotic and meiotic spindle movement and stabilization. Generally, KLPs are composed of three domains: a force-generating head domain that binds to microtubules, a coiled-coil stalk region involved in dimer formation, and a cargo-binding tail domain. KLPs can be grouped into two classes based on their direction of movement along microtubules.

The N-type KLPs move toward the plus end of MTs and have their motor domain located at the N terminus of the protein. N-type KLPs function in organelle movement along

MTs (Hirokawa, 1998). The C-type KLPs move toward the minus end of MTs (Hirokawa, 1998). In all the C-type KLPs identified to date, the motor domain is located at the C terminus of the protein. Members of the minus-end directed family of KLPs participate in MT cross-linking, and they are postulated to be involved in mitotic and meiotic spindle stabilization and MT movement (Barton and Goldstein, 1996).

Like other C-type KLPs, KCBP has a C-terminally located motor domain, and its minus-end directed movement along microtubules has been confirmed in vitro (Song et al., 1997). An antibody to the calmodulin-binding domain has been used to localize KCBP to the preprophase band, the phragmoplast, and the mitotic spindle in cultured plant cells (Bowser and Reddy, 1997). In addition, MT cosedimentation assays using different KCBP regions defined a MT binding domain in the KCBP tail domain (Narasimhulu and Reddy, 1998). These results suggest that KCBP, like other C-type KLPs, may play a role in MT stabilization and/or MT movements; however, the proteins with which KCBP interacts remain unknown.

A genetic analysis of trichome branching was first undertaken by Folkers et al. (1997). Based on a phenotypic analysis of single and double mutants defective in the regulation of trichome branch number, Folkers et al. (1997) concluded that *ZWI* may be involved in establishing branching competence in the nascent trichome cell. Suppressor screens have been used successfully in model systems such as yeast, *Drosophila*, *C. elegans* and *Arabidopsis* to identify genes whose products either physically interact with, or bypass the requirement for, a particular protein (for example, Adams et al., 1989; Maine and Kimble, 1993; Pepper and Chory, 1997). To understand the role of *ZWI* during trichome branching, we screened for suppressors of the strong *zwi*-3 allele. We hypothesized that mutations that restored trichome branch number to *zwi*-3 mutants may define negative regulators of trichome branching, or identify genes whose products interact with ZWI.

In this report, we describe the isolation and characterization of three extragenic suppressors of the *zwi*-3 mutation. These suppressors, named *suz* for ***su**ppressor of **z**wi*-3, are recessive, and segregate as single loci. Two of the *suz* mutants, *suz1* and *suz3*, have no obvious phenotype other than the suppression of *zwi*-3. The *suz2* mutants, however, show an increase in the number of trichome branches. In addition, *suz1* mutants display a synthetic male-sterile phenotype in the presence of the *zwi*-3 mutation. We also report the DNA sequence of the *zwi*-3 allele and discuss possible mechanisms of suppression of this allele. The genes identified in our screen for suppressors of *zwi*-3 may encode proteins that interact with ZWI during trichome branch initiation.

MATERIALS AND METHODS

Plant strains and growth conditions

Plants were grown in a mixture of vermiculite and Metro-mix 360 (Grace/Sierra, Milpitas, CA) at 22°C under constant illumination from soft white fluorescent bulbs. Plants were watered with either Peters 20-10-20 general purpose fertilizer (pH 5.8) or a complete nutrient solution (Feldmann and Marks, 1987). All plants used in this study were of the Columbia-0 ecotype (Col) of *Arabidopsis thaliana* unless otherwise noted. Genetic nomenclature follows the accepted guidelines for *A. thaliana* (Meinke, 1995). The *zwi*-3 allele (Col ecotype, EMS-induced) was generously supplied by M. David Marks (University of Minnesota). The *zwi*-9311-11 allele (Rschew [RLD] ecotype) was isolated from a population of fast-neutron mutagenized seed obtained from Lehle Seeds (Round Rock, TX). The *zwi*-W2 allele (RLD ecotype) is an EMS-induced, weak allele of *zwi* that was generated in our laboratory. The *furca1-2* (*frc1-2*, Col ecotype) mutation is an EMS-induced, recessive mutation that was generated in our laboratory.

Mutagenesis and screens for suppressors of *zwi*-3

Approximately 18,000 *zwi*-3 seeds were mutagenized by imbibition of 0.2% EMS (Sigma, St. Louis, MO) for 18 hours, followed by extensive washing with sterile, distilled H_2O. Of the original mutagenized seed, approximately 10,000 M_1 plants survived. The M_2 seeds were collected in pools representing approximately 150 M_1 plants. The seeds from each pool were germinated on soil as described above, and screened for modifications of the *zwi*-3 trichome phenotype. Approximately 20,000 M_2 seeds were screened, which represented 1,500 M_1 plants.

DNA sequencing

Genomic DNA was isolated from Col *zwi*-3 mutants essentially as previously described (Edwards et al., 1991). PCR was used to generate fragments for sequencing. The PCR products were purified using QIAquick PCR Purification columns (Qiagen Inc., Valencia, CA), and sequenced by the Iowa State University Nucleic Acid Facility on an ABI automated sequencer. DNA sequences were analyzed using the University of Wisconsin Genetics Computer Group software package (Program Manual for the Wisconsin Package, Version 9.1, Genetics Computer Group, Madison, WI).

Genetic analysis

Standard *Arabidopsis* genetic methods (Malmberg, 1993) were used unless otherwise noted. All the *zwi*-3 *suppressor* (*suz*) double mutants had significantly more trichome branches than *zwi*-3 single mutants; however, the double mutants retained the short stalk phenotype characteristic of *zwi*-3 single mutants. Thus, the *zwi*-3 *suz* double mutants were easily distinguishable from both wild-type plants and *zwi*-3 single mutants. To distinguish revertants from true extragenic suppressors, linkage of each suppressor mutation to the *zwi*-3 mutation was determined. Putative *zwi*-3 *suz* double mutants were crossed to wild-type Col plants, and the F_1 seed was selfed. Segregation of *zwi*-3 and wild-type phenotypes in the F_2 population indicated that the suppressor mutation was genetically separable from *zwi*-3 demonstrating that the suppressor phenotype was not due to reversion of the *zwi*-3 allele.

To determine the phenotype of the suppressor mutations in the absence of the *zwi*-3 mutation, the *zwi*-3 *suz* double mutants were crossed to wild-type Col plants, and the subsequent F_2 population was examined for new phenotypes. To confirm the presence of the suppressor mutation, putative suppressor mutants were crossed to *zwi*-3 plants, and the subsequent F_2 population was examined for the segregation of the suppressed *zwi*-3 phenotype (trichomes with a short stalk and more than two branches).

To identify plants homozygous for the *suz1* mutation, *suz1 zwi*-3 plants were backcrossed to wild-type Col plants, and several phenotypically wild-type plants were selected from the subsequent F_2 population. Phenotypically wild-type, F_2 plants that were heterozygous for the *zwi*-3 mutation were identified by examining the F_3 self-progeny of each wild-type F_2 plant for the segregation of the *zwi*-3 phenotype; heterozygous *zwi*-3 plants were discarded. The 8 remaining phenotypically wild-type F_2 plants were crossed to *zwi*-3 plants, and the seeds from at least 8 individual F_3 plants were collected. Each F_4 population was examined for the segregation of plants displaying the *suz1 zwi*-3 phenotype. A plant was considered homozygous for the *suz1* mutation if all of its F_4 families segregated for the *suz1 zwi*-3 phenotype.

Plants homozygous for *suz2* were selected from the F_2 progeny of a cross between the *suz2 zwi*-3 double mutant and a wild-type Col plant. The *suz2* homozygotes were identified by their trichome phenotype (more branches than normal). Their genotype was confirmed by their ability to suppress the *zwi*-3 mutation.

To identify plants homozygous for the *suz3* mutation, *suz3 zwi*-3 double mutants were backcrossed to wild-type Col plants, and phenotypically wild-type plants were selected from the subsequent F_2 population. Plants of the genotype *suz3*/*suz3 zwi*-3/*ZWI* were identified by the segregation of double mutants and wild-type plants (but not *zwi*-3 mutants) in the F_3 progeny. Phenotypically wild-type plants were selected from the self progeny of the *suz3*/*suz3 zwi*-3/*ZWI* plants. Plants that did not produce *suz3 zwi*-3 double mutants in their progeny were identified as *suz3* homozygotes.

Allelism of the *suz* mutations was tested by two independent methods. First, pairwise crosses between the *suz zwi*-3 double mutants were performed, and complementation of the *suz zwi* phenotype was scored. Second, the genetic map position of each of the *suz* mutations was determined.

The *suz2 frc1*-2 double mutants were constructed by crossing *suz2* plants to *frc1*-2 plants, and selecting phenotypically *suz2* plants from the subsequent F_2 population. The *suz2* plants were allowed to self, and the *suz2 frc1*-2 double mutant was selected from the F_3 population of those *suz2* lines that were heterozygous for *frc1*-2. The F_3 *suz2 frc1*-2 double mutants were crossed to *frc1*-2 plants and the observation that all the F_4 progeny showed the *frc1*-2 phenotype confirmed that the F_3 double mutants were homozygous for the *frc1*-2 mutation.

To determine the allele specificity of the *suz* mutations, *suz1*, *suz2*, and *suz3* mutants were crossed to *zwi*-9311-11 mutants and to *zwi*-W2 mutants. The F_1 progeny were selfed and the F_2 populations were examined for the presence of plants displaying the suppressed *zwi* phenotype. In addition, for crosses of the *suz* mutants to *zwi*-W2, plants showing the *zwi*-W2 phenotype in the F_2 population were collected and selfed. The F_3 progeny were examined for segregation of phenotypically wild-type plants that would be present if the weak allele was completely suppressed.

Phenotypic analysis of the suppressor mutants

Scanning electron microscopy (SEM) of wild-type, *zwi*-3, and *suz zwi*-3 trichomes was carried out as previously described (Oppenheimer et al., 1997). Trichome branches were counted on trichomes from the first leaf pair of both wild-type and mutant plants. The first leaf pair of at least five plants of each mutant were counted.

To observe the root hair phenotype of the *suz zwi*-3 mutants, seeds from *zwi*-3, double mutants, and wild-type plants were sown on plates containing agar-solidified Murashige and Skoog medium. The plates were incubated in a vertical position to allow the roots to grow along the surface of the agar. After 14 days, roots were observed under 40× magnification using a dissecting microscope.

Molecular mapping methods

To generate mapping populations, the *suz1 zwi*-3 and *suz3 zwi*-3 double mutants were crossed to plants of the Landsberg *erecta* (Ler) ecotype, and the *suz2 zwi*-3 double mutant was crossed to RLD plants. The F_1 plants were selfed, and DNA was isolated (Edwards et al., 1991) from F_2 plants that displayed the *suz zwi* double mutant phenotype. PCR was carried out using primers that identified simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994), or cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993). The recombination frequency between the *suz* mutations and the molecular markers were used to determine the approximate genetic map position of the *suz* loci.

Pollen germination assays

Microscope slides were coated with molten pollen germination medium (1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.01% boric acid, 18% sucrose, 0.5% agarose, pH 6). Pollen grains from freshly opened anthers were placed on the microscope slides approximately 5 mm from freshly placed stigmatic surfaces of two carpels with pollen-covered stigmas. The stigmas and pollen were covered with 5 μl of pollen germination medium without agarose. The slides were incubated at room temperature (22-25°C) overnight in a humid chamber. Pollen grains were observed under light microscopy and pollen germination frequency was determined as the fraction of germinated pollen grains of the total number of pollen grains in a field.

RT-PCR conditions

RNA was isolated from flowers as previously described (Berry et al., 1985). Synthesis of cDNA was carried out using 2 μg of total RNA and Superscript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. The primer STL 17 (5′ TTGGGACTCTTCATAAGC 3′) was used for first strand synthesis; this primer is located in exon 10 of the *ZWI* gene (Oppenheimer et al., 1997). PCR was carried out on cDNA using primers STL 29 (5′ ATACTACACTGGAAACTGG 3′, in exon 5) and STL 30 (5′ GTGCAGATATTCTTTAGG 3′, in exon 9) in an Idaho Technology (Idaho Falls, ID) thermocycler. PCR conditions were as follows: 94°C for 45 seconds for the initial denaturation; 35 cycles (slope 6) of 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 40 seconds. PCR products were sequenced using primer STL 37 (5′ CATGACTATTTGCTAG 3′).

RESULTS

Isolation and phenotypic characterization of suppressors of *zwi*-3

In a screen of approximately 20,000 M_2 seedlings, we identified five plants that showed suppression of the *zwi*-3 trichome branch number defect. We named these putative suppressors *suz*. Three of these suppressors, *suz1*, *suz2* and *suz3*, are the focus of this report.

To determine the extent of rescue of the *zwi*-3 trichome branch number defect by the *suz* mutations, trichome branches were counted for each of the *suz zwi*-3 double mutants. The results of the trichome branch number counts are shown in Table 1. Trichomes on wild-type Col plants have three or four branches (two or three branch points) whereas trichomes on *zwi*-3 mutants have exclusively two branches. The *suz* mutations suppress the *zwi*-3 branch number defect; *suz zwi*-3 double mutants produced a significant number of three-branched trichomes (Table 1). These results suggest that the

Table 1. Number of branches on wild-type and mutant trichomes

	Number of trichome branch points*				
Genotype	1	2	3	4	Total‡
Col wild-type	0	96.6	3.4	0	206
zwi-3	100	0	0	0	352
suz1 zwi-3	50	40.5	9.5	0	302
suz2 zwi-3	30.2	62.3	7.5	0	298
suz3 zwi-3	64.9	31.3	3.8	0	273
suz2	0	28.1	60.9	11	621

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

‡Total number of trichomes counted on both leaves of the first leaf pair of at least five plants

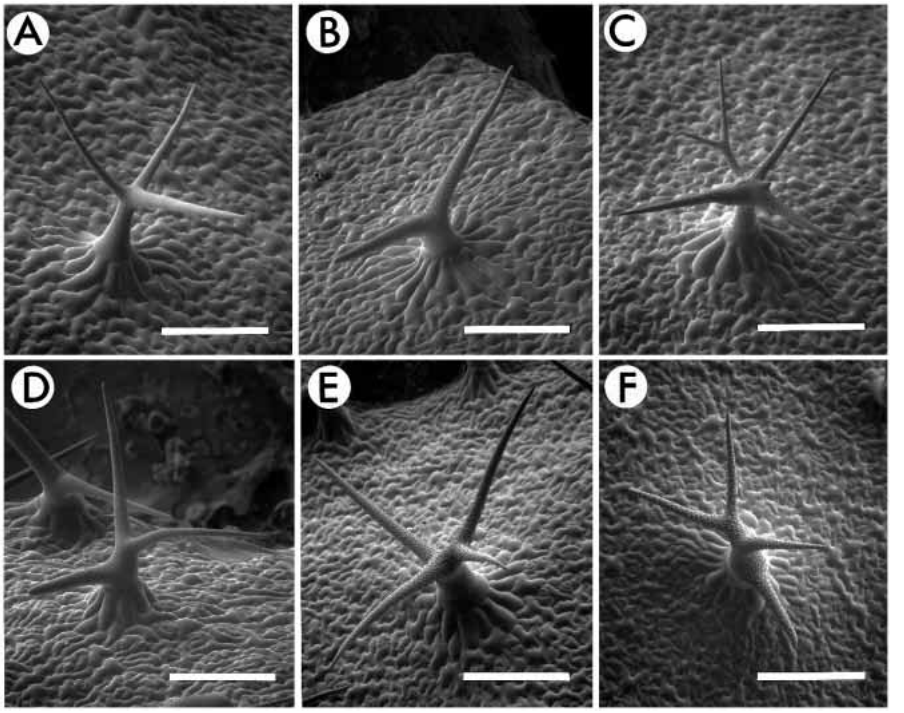

Fig. 1. Suppression of the *zwi*-3 branch number defect by *suz* mutations. Scanning electron micrographs of leaf trichomes. (A) Mature wild-type trichome. (B) Mature *zwi*-3 trichome. (C) Mature *suz2* trichome. (D) Mature *suz1 zwi*-3 trichome. (E) Mature *suz2 zwi*-3 trichome. (F) Mature *suz3 zwi*-3 trichome. Scale bars (A,E) 200 μm; (B) 270 μm; (C,D,F) 230 μm.

suz mutations either restore partial *ZWI* function or bypass the requirement for *ZWI* in trichome branch formation.

SEM was used to examine the trichomes on the *suz zwi*-3 double mutants. As shown in Fig. 1, the trichomes of *suz zwi*-3 double mutants generally have the wild-type number of branches, although the branches are not always the correct size or shape. One or more of the branches may be twisted, bent, or shorter than their wild-type counterparts.

Trichomes on *zwi*-3 mutants not only have a reduced number of branches, but also have a much shorter stalk (Folkers et al., 1997; Hülskamp et al., 1994; Oppenheimer et al., 1997); see Fig. 1. SEM of developing *zwi* trichomes has shown that the short stalk is the result of improper positioning of the first branch during trichome development (Oppenheimer et al., 1997). The short stalk defect is unaffected by any of the *suz* mutations, which suggests that either specification of trichome branch position is more sensitive to (partially) restored ZWI function, or that control of branch position and branch number are separate functions of ZWI.

Several mutations are known to affect the fate or the morphology of root epidermal cells, and trichome morphology (Galway et al., 1994; Masucci et al., 1996). Therefore, we examined the roots of *zwi*-3, *suz1 zwi*-3, *suz2 zwi*-3 and *suz3 zwi*-3 mutants to determine if any of these mutations affects root hair morphology. The morphology of the root hairs as observed under 40× magnification is not affected in any of these mutants (data not shown; see Materials and Methods).

Table 2. Segregation of phenotypes in the F_2 of *suz zwi* double mutants crossed to Col wild-type

	F_2 phenotypes (Obs./exp.)				
Cross	Wild-type	*zwi*	*suz zwi*	Total	χ^{2*}
suz1 zwi-3×Col wt	154/164	51/41.1	14/13.7	219	2.58
suz2 zwi-3×Col wt	327/320	78/80.1	22/26.7	427	1.03
suz3 zwi-3×Col wt	169/166	37/41.4	15/13.8	221	0.64

*χ^2 values were calculated based on three phenotypic classes (2 degrees of freedom). The 95% confidence limit is 5.99 for 2 degrees of freedom. Abbreviations: Obs./exp., observed/expected.

Genetic characterization of the *suz* mutants

Each of the *suz zwi*-3 plants were crossed to wild-type Col plants. In each case, wild-type and *zwi*-3 plants segregated in the subsequent F_2 population (Table 2). This demonstrates that the *suz zwi*-3 phenotypes are due to extragenic suppression of the *zwi*-3 mutation and not reversion of the *zwi*-3 allele.

To determine if the *suz* mutations were dominant or recessive, each of the *suz zwi*-3 plants were backcrossed to *zwi*-3 plants. Analysis of the phenotypes of the F_1 and F_2 plants showed that the *suz1* and the *suz2* mutations are completely recessive, but that the *suz3* mutation is weakly dominant (Table 3, and data not shown). Each *suz* mutation segregated as a single locus.

Table 3. Number of trichome branches on *suz zwi* trans-heterozygotes

	Number of trichome branch points*		
Genotype	1	2	Total‡
suz1/SUZ1 suz2/SUZ2 zwi-3/*zwi*-3	99.7	0.3	304
suz1/SUZ1 suz3/SUZ3 zwi-3/*zwi*-3	96.3	3.7	463
suz2/SUZ2 suz3/SUZ3 zwi-3/*zwi*-3	98.4	1.6	706
suz3/SUZ3 zwi-3/*zwi*-3	97.7	2.3	400
suz2/SUZ2 zwi-3/*zwi*-3	100	0	362
suz1/SUZ1 zwi-3/*zwi*-3	100	0	199

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

‡Total number of trichomes counted on both leaves of the first leaf pair of five plants.

Allelism of the *suz* mutations was determined by pairwise crossing of the *suz zwi*-3 double mutants, and phenotypic analysis of the F_1 plants. All of the F_1 progeny from the cross of *suz1 zwi*-3 to *suz2 zwi*-3 showed only the *zwi*-3 phenotype indicating that *suz2* is not allelic to *suz1*. The F_1 progeny of crosses of *suz3 zwi*-3 to either *suz1 zwi*-3 or *suz2 zwi*-3 showed a significantly higher number of three-branched trichomes than *zwi*-3 mutants even though the number was far smaller than that seen on either *suz1 zwi*-3 or *suz3 zwi*-3 mutants (Tables 1 and 3). The failure of the *suz3* mutation to fully complement the *suz1* or the *suz2* mutation is likely to be the result of the weakly dominant *suz3* mutation.

As a second independent test of allelism, we determined the genetic map positions of the *suz* loci. Each *suz* locus maps to a different region of the *Arabidopsis* genome. *SUZ1* is located on chromosome III, approximately 20 cM from SSLP marker nga162. *SUZ2* is located on chromosome IV, approximately 9 cM from CAPS marker AG1. Although the exact location of *SUZ3* was not determined, it is not linked to nga162, AG1, or *ZWI*. Thus, the *suz* mutations are not allelic and each defines a separate gene.

To determine the phenotype of the *suz* mutations in the absence of the *zwi*-3 mutation, the *suz zwi*-3 double mutants were crossed to wild-type Col plants, and *suz* homozygotes were identified (see Materials and Methods). Plants homozygous for either *suz1* or *suz3* are indistinguishable from wild-type plants (data not shown). Only the *suz2* mutation presents an obvious phenotype in the absence of the *zwi*-3 mutation: the trichomes on *suz2* plants have more branches than the trichomes on wild-type plants (Table 1). No other phenotype associated with the *suz2* mutation was observed. A scanning electron micrograph of trichomes on a *suz2* plant is shown in Fig. 1C. The observation of extra branches on trichomes of *suz2* mutants suggests that the normal function of *SUZ2* is to negatively regulate trichome branching. Thus, *suz2* mutants may bypass the requirement of *ZWI* for secondary branch formation in the suz2 *zwi*-3 double mutants.

The *suz2* mutation can suppress the trichome branch number defect of another trichome branch mutant

Because the *suz2* mutation has an obvious phenotype in the absence of the *zwi*-3 mutation, we were able to test the potential of *suz2* to suppress another branch number mutant, *frc1*-2 (D. Luo and D. G. O., unpublished data). The trichomes on *frc1*-2 mutants generally have two branches (Table 4). We constructed the *suz2 frc1*-2 double mutant, and examined the trichome branch number. As shown in Table 4, the *suz2* mutation suppresses the trichome branch number defect caused by the *frc1*-2 mutation. Along with the suppression of *zwi*-3, this result suggests that *suz2* mutation can bypass the requirement for two different gene products (KCBP and FRC1) in trichome branching, and hence may act as a bypass suppressor.

The *suz* mutations are allele-specific suppressors of *zwi*-3

If the *suz2* mutation can bypass the requirement for ZWI in trichome branching, then *suz2* should suppress the trichome defects of other *zwi* alleles – not only *zwi*-3. To test the allele specificity of the suppression of *zwi* mutations by the *suz2* mutation, *suz2* plants were crossed to a weak *zwi* allele (*zwi*-W2) and another strong *zwi* allele (*zwi*-9311-11). Surprisingly, *suz2* fails to suppress the phenotype of the other *zwi* mutations. Out of several hundred F_2 plants from each of the crosses, no plants displaying the suppressed *zwi* phenotype (trichomes with a short stalk and three or four branches) were observed although *zwi* and *suz2* plants segregated as expected (data not shown).

However, if the suppression of the weak phenotype of *zwi*-W2 was complete, the *suz2 zwi*-W2 double mutants would have a wild-type phenotype. To rule out the possibility that we had misidentified the *zwi*-W2 *suz2* double mutants because they might have a completely wild-type phenotype, plants showing the *zwi*-W2 phenotype were collected from the F_2 populations, allowed to self, and F_3 populations from nine *zwi*-W2 F_2 plants were examined for segregation of phenotypically wild-type plants. None of the F_3 populations segregated phenotypically wild-type plants (data not shown). Therefore, *suz2* cannot suppress *zwi*-W2. This result demonstrates that *suz2* is an allele-specific suppressor of the *zwi*-3 mutation. Furthermore, the inability of *suz2* to suppress either the *zwi*-W2 allele or the *zwi*-9311-11 allele indicates that *suz2* cannot bypass the requirement for *ZWI* function, and hence does not function as a bypass suppressor.

The *suz1* and *suz3* mutations also were tested for allele specificity of suppression by crosses to *zwi*-W2 and *zwi*-9311-11 plants, and observation of the subsequent F_2 populations for plants with the suppressed *zwi* phenotype. No plants displaying the suppressed *zwi* phenotype were seen in the F_2 populations (several hundred plants per population) although *zwi* plants segregated as expected (data not shown). In addition, nine phenotypically *zwi*-W2 plants were selected from the F_2 populations (from the *suz1* and *suz3* crossed to *zwi*-W2) and allowed to self. No phenotypically wild-type plants were observed in the subsequent F_3 populations. This result indicates that *suz1* and *suz3* are allele-specific suppressors of *zwi*.

Table 4. Suppression of the *frc1*-2 phenotype by *suz2*

	Number of trichome branch points*						
	0	1	2	3	4	5	Total‡
suz2	0	0	28.1	60.9	11	<1	621
frc1-2	1.4	74.8	23.8	0	0	0	794
suz2 frc1-2	0	15.3	84.7	0	0	0	691

*% of trichomes having the indicated number of branch points (0 branch points indicates a trichome with no branches i.e., a spike).

‡Total number of trichomes counted on both leaves of the first leaf pair of five plants.

The *suz1 zwi*-3 double mutant has a synthetic phenotype

In addition to the trichome branch number phenotype, the *suz1 zwi*-3 double mutants exhibited a fertility defect. Under our plant growth conditions, an individual *Arabidopsis* plants produce an estimated 20,000 to 50,000 seed. When grown under identical conditions, the *suz1 zwi*-3 double mutants produce 0-10 seeds per plant. Both the *suz1* and the *zwi*-3 single mutants are completely fertile, indicating that the reduced fertility of the *suz1 zwi*-3 double mutant is due to a synthetic gene interaction between *suz1* and *zwi*-3. To

determine the sex specificity of the fertility defect, we performed reciprocal outcrosses of the *suz1 zwi*-3 mutants to wild-type Col plants. Crosses failed to produce seeds when the *suz1 zwi*-3 mutant was used as the male parent, but not when the *suz1 zwi*-3 mutant was used as the female parent (data not shown). This result shows that the reduced seed set of *suz1 zwi*-3 plants was due to male sterility.

The synthetic male sterility of the *suz1 zwi*-3 double mutant is due to a pollen germination or a pollen tube growth defect

To investigate the nature of the *suz1 zwi*-3 male sterility, we examined developing *suz1 zwi*-3 anthers and pollen by SEM. We observed no obvious difference between the morphology of anthers and pollen from *suz1 zwi*-3 mutants and wild-type Col plants (Fig. 2). We then examined the ability of *suz1 zwi*-3 pollen to germinate in vitro. The frequency of *suz1 zwi*-3 pollen germination was reduced compared to the frequency of wild-type Col pollen germination (Table 5, and Fig. 3). When *suz1 zwi*-3 pollen did germinate, the pollen tubes were much shorter and somewhat aberrantly shaped. Occasionally, forked pollen tubes were observed. In addition, the longer pollen tubes of *suz1 zwi*-3 mutants always contained numerous spherical bodies (arrows in Fig. 3D) that were not observed in wild-type or *zwi*-3 pollen tubes. The inability of the *suz1 zwi*-3 pollen to develop normal pollen tubes suggests a role for both SUZ1 and ZWI in both pollen germination and pollen tube growth.

Molecular analysis of the *zwi*-3 allele

The genetic analysis of *zwi*-3 was initiated prior to the isolation of the *ZWI* gene. To further understand the nature of the suppression of the *zwi*-3 mutation, we sequenced the *zwi*-3 allele. A comparison of the *zwi*-3 DNA sequence to the wild-type Col sequence revealed a G → A transition at the conserved +5 position relative to the 5′ splice donor site of intron 8 in *zwi*-3 (Fig. 4A). This G → A mutation may reduce the efficiency of splicing at this donor site. To determine if correct splicing of intron 8 is reduced in *zwi*-3 mutants, we amplified *zwi-3* mRNA using RT-PCR and sequenced the resulting cDNA. The analysis of the sequence shows that in *zwi*-3 mutants, intron 8 is spliced using a novel 5′ splice donor, 5 bases upstream from the normal splice donor (Fig. 4B). The use of this splice site causes exon 9 to be out-of-frame. As a result, the zwi-3 protein is truncated at amino acid position 522 following the addition of 7 new amino acids because of the altered reading frame. When translated, the *zwi*-3 mRNA should produce a truncated ZWI protein containing most of the putative cargo-binding domain, but lacking the coiled-coil dimerization motif and motor domain.

To examine the mechanism of *suz1* suppression of *zwi*-3, we sequenced mRNA isolated from *suz1 zwi*-3, *suz2 zwi*-3, and *suz3 zwi*-3 double mutants by using RT-PCR. The sequence of the *zwi*-3 mRNA at the exon 8/9 junction shows that the incorrect splice site was used by all of the *suz zwi*-3 double mutants (data not shown). These results suggest that suppression of the branch defect of *zwi*-3 by the *suz* mutations is not due to suppression of incorrect splicing of the *zwi*-3 mRNA.

Table 5. Frequency of in vitro pollen germination of wild-type and *suz1 zwi*-3 mutants

	Germinated	Not germinated	Total
Wild-type	218 (73.9%)	77 (26.1%)	295
suz1 zwi-3	25 (4.7%)	511 (95.3%)	536

DISCUSSION

Suppression of the *zwi*-3 mutation

To understand the role of ZWI during trichome branch initiation, we carried out a screen for suppressors of the *zwi*-3 mutation. During the course of this mutant screen, the *ZWI* gene was cloned by T-DNA tagging (Oppenheimer et al., 1997). This enabled us to determine the molecular lesion responsible for the *zwi*-3 mutation. DNA sequence analysis of the *zwi*-3 allele shows that the *zwi*-3 phenotype is due to a G → A transition at the +5 position of the donor splice site of intron 8. Sequence analysis of the *zwi*-3 cDNA shows that this mutation results in the use of a new 5′ donor site located 5 bases upstream of the wild-type splice site. When translated, the *zwi*-3 cDNA should produce a severely truncated ZWI protein (KCBP) lacking the coiled-coil and motor domains. Previous study of a T-DNA-induced *zwi* mutation (*zwi*-5002) has shown that the C-terminal motor domain is required for ZWI function during trichome branch formation (Oppenheimer et al., 1997). Therefore it is not surprising that *zwi*-3 mutants display a strong *zwi* phenotype.

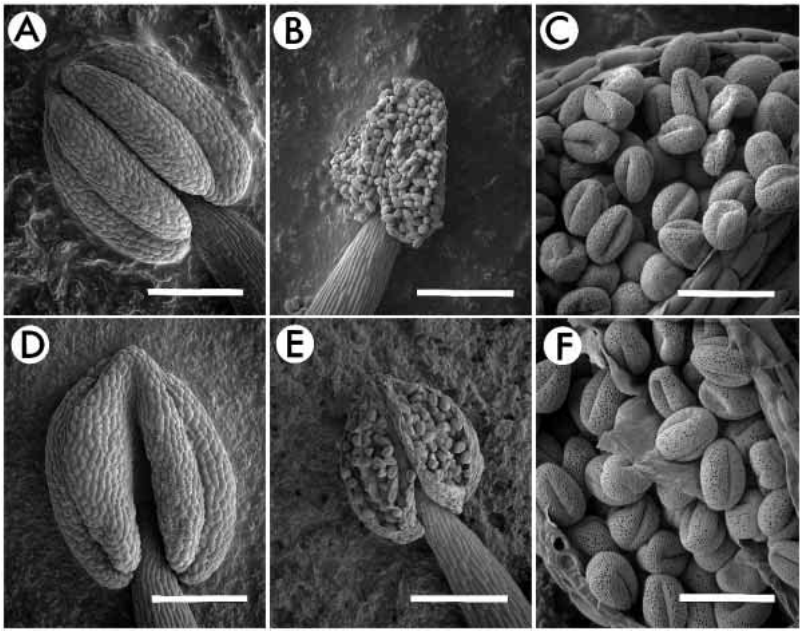

Fig. 2. Comparison of wild-type and *suz1 zwi*-3 anthers and pollen. Scanning electron micrographs of mature anthers and pollen. (A) Wild-type anther, pre-anthesis. (B) Mature wild-type anther from an opened flower. (C) Wild-type pollen from a mature wild-type anther. (D) Pre-anthesis *suz1 zwi*-3 anther. (E) Mature *suz1 zwi*-3 anther from an opened flower. (F) *suz1 zwi*-3 pollen. Scale bars, (A,B,D,E) 200 μm; (C,F) 43 μm.

All the suppressors of the *zwi*-3 mutation isolated in our screen were recessive and extragenic. Extragenic suppressors generally fall into three groups that can be distinguished by their allele and gene specificity (Guarente, 1993, for review; Hartman and Roth, 1973). The first group of suppressors are gene specific but allele nonspecific; the suppressor will suppress different alleles of a specific gene, but not mutant alleles of other genes. The second group includes suppressors that are gene nonspecific, but allele specific. These types of suppressors are usually called informational suppressors and include suppressors of nonsense mutations (tRNA suppressors) and suppressors of certain transposon mutations. The third group of suppressors are gene and allele specific; only particular alleles of one gene are suppressed by the suppressor mutation. This type of suppression is called interactional suppression because it often results from the physical interaction of the suppressor with its target (the product of the suppressed allele). Mechanistically, allele-specific suppression can be accomplished by a lock and key model where original contact between the suppressor and its target is restored, or by formation of new contact sites (Adams et al., 1989; Phizicky and Fields, 1995; Sandrock et al., 1997).

The observation that all the *suz* mutations act as allele-specific suppressors of *zwi*-3 suggests that they may be interactional suppressors. This result implies that the wild-type products of the *ZWI* and *SUZ* genes interact. We have not tested whether *suz1* or *suz3* are gene-specific suppressors, because of the difficulty in following the segregation of mutations that produce no observable phenotype. In the case where gene specificity of suppression was examined, the *suz2* mutation suppressed the *frc1*-2 mutation.

There are several models that can explain the suppression of *zwi*-3 by the *suz* mutations. An obvious model is that *suz* mutants can overcome the splicing defect of *zwi*-3 and restore normal *ZWI* function. This mechanism of suppression would be akin to informational suppression described above; however, we were unable to detect properly spliced *zwi* mRNA in *suz zwi*-3 double mutant plants. These results suggest that the *suz* mutations cannot restore normal splicing to the *zwi*-3 mRNA, but that another mechanism must be employed to rescue the trichome branch number defect of *zwi*-3 mutants. It is formally possible, however, that an undetectable amount of *zwi*-3 mRNA is spliced properly in *suz zwi*-3 mutants, and that this produces enough full-length KCBP protein to partially rescue the trichome branching defect of *zwi*-3.

A second model for the suppression of *zwi*-3 by the *suz* mutations supposes that KCBP participates in either microtubule crosslinking or movement during trichome branch initiation. The *zwi*-3 mutation destroys the microtubule crosslinking function of KCBP by eliminating the motor domain and the coiled-coil domain (which is thought to be involved in dimerization). The *SUZ* genes may encode MT binding proteins, and the mutant *suz* products may interact with zwi-3 to partially restore the MT crosslinking function and hence trichome branch initiation.

A third model supposes that the *SUZ* genes may encode other kinesins, and the *suz* mutations may allow the *zwi*-3 product to interact with the *suz*-encoded kinesin. The interaction may restore enough motor function to the zwi-3 tail domain to suppress the trichome branching defect. This type of allele-specific interaction may be similar to the allele-specific interactions of certain suppressors of actin mutations in yeast (Adams et al., 1989).

The above models for the suppression of *zwi*-3 by the *suz* mutations involve the interaction of the truncated *zwi*-3 product with some other protein. It is possible, however, that the truncated KCBP encoded by the *zwi*-3 mRNA is unstable and does not accumulate. This hypothesis will be tested once an antibody that specifically recognizes the KCBP tail domain is

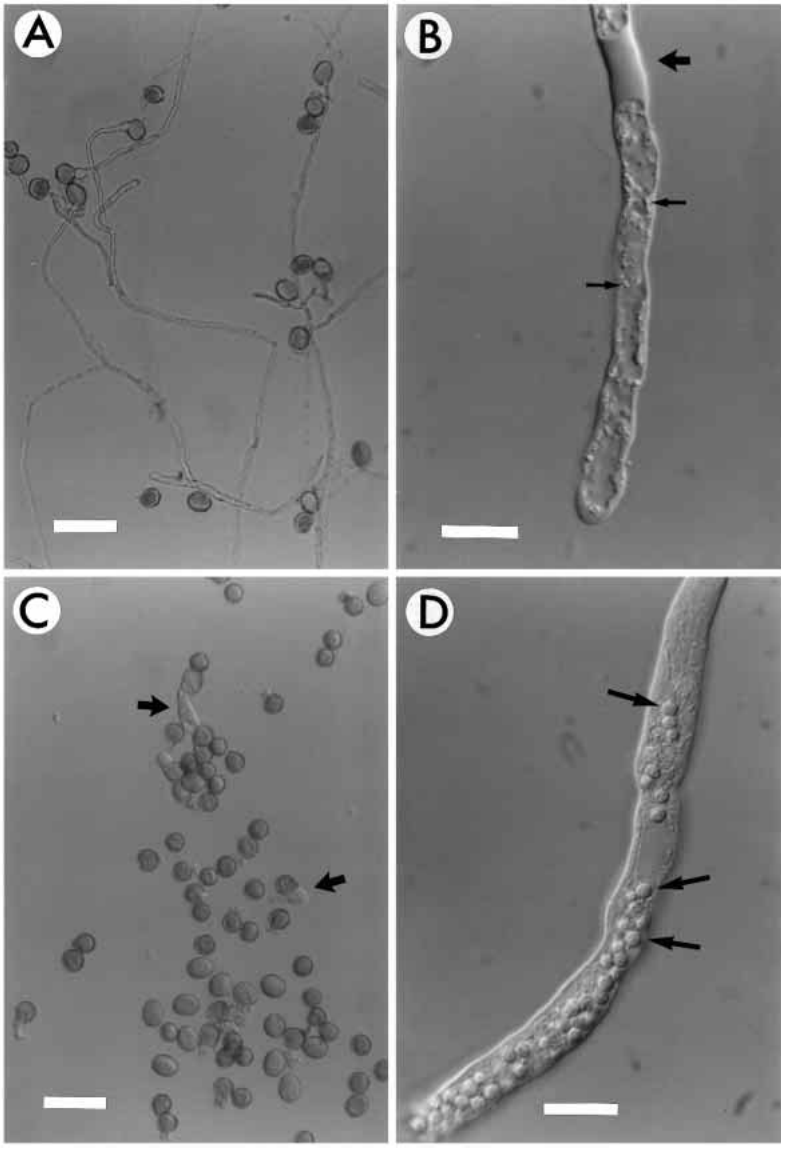

Fig. 3. Comparison of wild-type and *suz1 zwi*-3 pollen germination. Light micrographs of in vitro-germinated pollen. (A) Wild-type pollen with long pollen tubes. (B) Wild-type pollen tube showing numerous small vesicles (small arrows) and a callose plug (large arrow). (C) *suz1 zwi*-3 pollen showing only a few short pollen tubes (arrows). (D) *suz1 zwi*-3 pollen tube showing numerous spherical bodies (arrows). Scale bar equals 100 μm in (A,C) and 20 μm in (B,D).

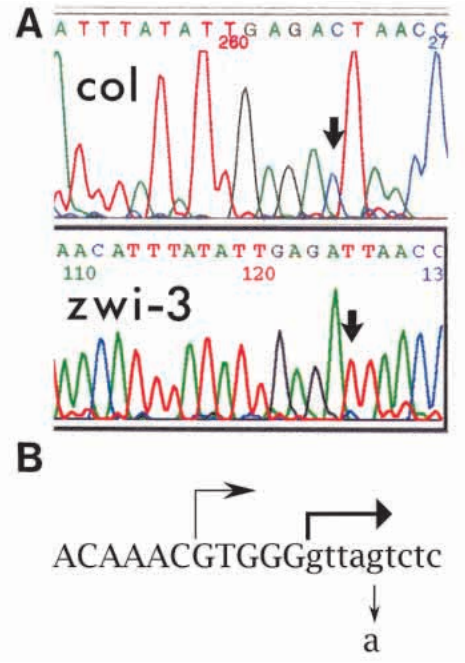

Fig. 4. The zwi-3 mutation is a G → A transition within intron 8. (A) DNA sequence data (non-coding strand) from wild-type Col and *zwi*-3 alleles. The arrows indicate the +5 position of intron 8 of the *ZWI* genomic sequence. (B) DNA sequence of the *ZWI* intron 8 splice donor site. The upper case letters correspond to coding sequence, and the lower case letters correspond to intron 8 sequence in the Col wild-type *ZWI* allele. The thick horizontal arrow shows the intron 8 splice donor site used in the Col wild-type *ZWI* allele, and the thin horizontal arrow shows the intron 8 splice donor site used in the *zwi*-3 allele. The vertical arrow shows the G → A transition found in the *zwi*-3 allele.

available. If the *zwi*-3 product does not accumutate in cells, the following model explains the allele-specific suppression of the *zwi*-3 mutation. In this alternative model, KCBP and another KLP overlap in function and can bind to the same site in the cell. Normally, this other KLP is prevented from binding to the KCBP-binding site by interaction with the *SUZ* product (the *SUZ* product acts as a negative regulator of the other KLP). The *suz* mutation prevents this interaction, thus allowing the other KLP to bind to the KCBP binding site and suppress the *zwi*-3 mutation. If other alleles of *zwi* produce a nonfunctional product that is still capable of binding to the KCBP binding site, then binding of the other KLP is prevented and suppression of the *zwi* mutation cannot occur.

The *SUZ1* and *SUZ3* loci could encode KLPs with functions similar to those of *ZWI*. The absence of any obvious phenotypes displayed by *suz1* and *suz3* single mutants suggests that the *SUZ1* and *SUZ3* functions are redundant. ZWI is expressed in cells other than trichomes, yet *zwi* mutants display no obvious phenotype in these other cell types. In addition, other kinesin-like proteins have been found in *Arabidopsis* (Liu et al., 1996; Mitsui et al., 1994; Mitsui et al., 1993). Thus, it is likely that multiple kinesins with similar functions are present in a single cell type. The *suz1* and *suz3* mutations could result in mutant products that are able to partially substitute for *ZWI* function in trichomes. Similarly, *SUZ1* and *SUZ3* could encode the cargo-associated receptors to which ZWI putatively binds. The *suz1* and *suz3* mutations could result in the binding of the *suz* products to other kinesin-like proteins that have functions similar to *ZWI*.

Assuming that the *zwi*-3 allele produces a truncated protein, we hypothesize that FRC1, the SUZ proteins, and KCBP are members of a multiprotein complex that functions in trichome branch initiation. The products of the *suz* alleles restore partial function of the complex through interactions with zwi-3. In addition, interactions of suz2 with frc1-2 can restore function of the complex. Allele-specific, synthetic interactions between *zwi* and *frc1* mutations have been identified (S. K., D. Luo, and D. G. O., unpublished data) which suggests that FRC1 and KCBP interact.

Control of branch position and branch number by *ZWI*

Currently, all of the suppressors of the *zwi*-3 allele only partially suppress the *zwi* phenotype – the branch number defect is suppressed, but the short stalk defect is not. Even *suz2*, which rescues the branch number defect of a mutation in another branch number gene (*FRC1*), does not rescue the trichome stalk defect of *zwi*-3 mutants. The failure of the suppressors to rescue the stalk defect may be due to the inability of the suppressors to completely rescue full ZWI function. For example, none of the *suz* mutations isolated in this screen completely suppressed the trichome branch number defect of *zwi*-3 mutants (Table 1). A minimum threshold of *ZWI* function may be necessary to produce a wild-type trichome stalk, and the amount of suppression provided by the *suz* mutations may not be sufficient for this function. Stalk height may be regulated separately from branch initiation, and these two processes may require different levels of KCBP function, or different functions of the KCBP protein. Also, the branch-position function of KCBP may be regulated by a different set of interactions; the SUZ proteins may play no role in branch position.

Alternatively, control of trichome branch position may require KCBP motor function which is lacking in *zwi*-3 mutants. Branch initiation may not require motor function, but only a functional tail domain. Tail domain function may be rescued by the *suz* mutations and hence branch initiation can occur although branch site positioning may still be defective. This hypothesis is supported by the observation that most of the three- and four-branched trichomes on *suz zwi* plants have asymmetrically arranged branches compared to wild-type (see Fig. 1). In addition, recently we identified three mutants (which define three new genes) that affect trichome branch position but not trichome branch number (S. K. and D. G. O., unpublished observations). Furthermore, analyses of the trichome phenotypes of weak *zwi* alleles crossed into different ecotypes has indicated that trichome stalk height is controlled, in part, by genetic background (M. A. Pollock and D. G. O., unpublished observations). Therefore, we favor the hypothesis that the determination of trichome branch position and branch number are separate processes, and that both require ZWI function.

SUZ2 acts as a negative regulator of trichome branching

Trichomes on *suz2* mutants have more branches than wild-type trichomes which suggests that the normal function of the *SUZ2* gene is to suppress trichome branching. This function is similar to that of the *NOECK* (*NOK*), and *TRIPTYCHON* (*TRY*) genes

(Folkers et al., 1997). Mutations in *zwi* are epistatic to these two mutations since *nok* and *try* cannot suppress *zwi* mutations. Similarly, *suz2* is unable to suppress the *zwi*-9311-11 and the *zwi*-W2 mutations. Because *zwi* mutations are epistatic to *suz2*, *nok* and *try* mutations, *ZWI* may be under negative regulation by *SUZ2*, *NOK*, or *TRY*. There is also biochemical evidence that ZWI is under negative regulation. Binding of Ca^{2+}/calmodulin to KCBP in vitro has been shown to inhibit binding of the motor to microtubules (Deavours et al., 1998; Song et al., 1997). It is possible that *suz2* may be a hypermorphic allele of a positive regulator of branching. This possibility is remote, however, because most hypermorphic alleles are dominant and *suz2* is recessive.

The role of *SUZ1* and *ZWI* in pollen germination and pollen tube growth

Previous studies have shown that MTs are not required for pollen tube tip growth per se (Franke et al., 1972), but there is evidence that MTs may contribute to the maintenance of pollen tube polarity (Joos et al., 1994). MT motor proteins, including kinesin-like proteins, have been identified in pollen tubes (Cai et al., 1996; Liu and Palevitz, 1996), but their functions are unknown. The surprising synthetic male-sterility phenotype of *suz1 zwi*-3 double mutants provides genetic evidence for a role for SUZ1 and KCBP in both pollen germination and pollen tube growth. The fact that *suz1 zwi*-3 double homozygotes can be isolated suggests that the *SUZ1* and *ZWI* products are not required in the gametophyte. Thus, SUZ1 and KCBP function must be required prior to meiosis even though the defect is not observable until pollen germination. The functions of *SUZ1* and *ZWI* during pollen development must overlap with the functions of other proteins because both *suz1* and *zwi* mutants do not show fertility defects. The requirement of KCBP during pollen development is consistent with the expression pattern of the *ZWI* gene; *ZWI* mRNA has been detected in flowers (Oppenheimer et al., 1997; Reddy et al., 1996).

In this study, we found that large spherical bodies, presumably vesicles, accumulate in the few *suz1 zwi*-3 pollen tubes that elongate. Thus, SUZ1 and/or KCBP could have functions in vesicle movement or targeting during pollen tube growth. Alternatively, normal vesicle movement could be blocked or slowed due to defects in cell expansion during pollen germination and continued pollen tube growth. We postulate that SUZ1 and KCBP act to prepare the pollen cytoskeleton for polarized cell elongation and that they have a parallel role in trichomes during branch initiation. Presumably expansion in these two distinct cases would require unique subsets of additional components which could explain why the interaction of mutant suz1 and zwi-3 proteins has a positive effect in trichomes (suppression of the branch defects), but a negative effect in pollen tubes.

In conclusion, the allele specificity of the suppression of the *zwi*-3 mutation suggests that interactional suppression is the mechanism for the partial rescue of the branch phenotype of *zwi*-3 mutants. This hypothesis is supported by the synthetic male sterility seen in *suz1 zwi*-3 double mutants. In addition, this synthetic interaction uncovers a role for both *SUZ1* and *ZWI* in pollen germination and pollen tube growth. These events, like trichome branch initiation, require reorientation of the cortical microtubules to prepare the cell for the change in the direction of cell expansion. Thus, we believe that it is likely that the *suz* mutations identify genes whose products interact with KCBP. Once the *SUZ* genes are cloned, the hypothesis that the *SUZ* products physically interact with KCBP can be tested directly.

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