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Essential role of a kinesin-like protein in *Arabidopsis* trichome morphogenesis

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ABSTRACT **Little is known about how cell shape is controlled. We are using the morphogenesis of trichomes (plant hairs) on the plant *Arabidopsis thaliana* as a model to study how cell shape is controlled. Wild-type *Arabidopsis* trichomes are large, single epidermal cells with a stalk and three or four branches, whereas in *zwichel* (*zwi*) mutants the trichomes have a shortened stalk and only two branches. To further understand the role of the *ZWI* gene in trichome morphogenesis we have cloned the wild-type *ZWICHEL* (*ZWI*) gene by T-DNA tagging, and report here that it encodes a member of the kinesin superfamily of microtubule motor proteins. Kinesin proteins transport diverse cellular materials in a directional manner along microtubules. Kinesin-like proteins are characterized by a highly conserved "head" region that comprises the motor domain, and a nonconserved "tail" region that is thought to participate in recognition and binding of the appropriate cargo.**

Plant cell expansion is central to plant development. During plant cell differentiation, the relative rates of expansion of different regions of the cell wall determine the final shape of the cell. For most cell types the functional significance of cell shape is evident. For example, the special shape of stomatal guard cells permits the opening and closing of stomata, the shape of spongy mesophyll cells allows for efficient gas exchange, and the broadly expanded but thin shape of leaf epidermal cells increases the surface area of a leaf and allows penetration of light to the underlying photosynthetic mesophyll cells.

Plant cell expansion results from a complex interplay of diverse processes. These processes involve the loosening of the cell wall, the maintenance of turgor pressure which is needed to drive cell expansion, and the deposition of new cell wall material (reviewed in refs. 1–4). In addition, the cytoskeleton appears to play a role in directing the deposition of new wall material, thereby controlling the eventual shape of an expanding cell. Although progress has been made in defining the regulatory circuits controlling the cell fate decisions of specific plant cells such as trichomes (leaf hairs) and root hairs, the identities of the structural genes responsible for the final cell architecture remain elusive.

We are using a combined molecular and genetic approach to study the development of the shape of the trichomes on the plant *Arabidopsis*. The use of *Arabidopsis* trichomes as a model to understand the genetic control of plant cell differentiation has been well documented (5–10). In wild-type *Arabidopsis*, trichomes develop from isodiametric protodermal cells. Evidence suggests that cell–cell communication leads to the selection of trichome precursors (11). Once selected, the precursors undergo a defined cell expansion program that results in the formation of a large cell with a stalk and several branches (see Fig. 1). Genetic screens for plants with altered trichome shape have resulted in the identification of over 20 genes involved in trichome morphogenesis (6). One of these mutations, *zwichel* (*zwi*), results in a trichome with a greatly reduced stalk and only two branches. To further understand the role of the *ZWI* gene in controlling the final shape of the trichome cell, we cloned the *ZWI* gene by T-DNA tagging. We report here that the *ZWI* gene encodes a member of the kinesin-like family of microtubule motor proteins previously identified by its ability to bind calmodulin (12).

MATERIALS AND METHODS

Scanning Electron Microscopy. Plants were grown in soil under constant illumination. Shoot apices were pulled apart to expose the developing leaf primordia, quickly mounted on scanning electron microscopy stubs, and submerged in liquid nitrogen for 45 sec. Samples were transferred to a Bio-Rad E7400 Cryotrans System cryostage and etched for 10 min at −65°C, cooled to −180°C, sputter-coated with gold, and viewed at 10 kV using a Hitachi S-2500 scanning electron microscope.

Plant Transformation. Genomic fragments were subcloned into pBIN19 (13) and used to transform *zwi* mutant explants (RLD ecotype) as described (14). Regenerating explants were scored for the presence of wild-type trichome morphology. The ability to restore wild-type trichomes to mutant explants indicated the presence of a functional *ZWI* gene on the transforming DNA.

DNA Sequencing. Genomic fragments were subcloned into pMOB and subjected to random TN1000 insertions (15). Sequencing of double-stranded templates was performed by the Iowa State University Nucleic Acid Facility. DNA sequence analysis and database searches were done using the University of Wisconsin Genetics Computer Group software package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, Madison, WI).

Preparation of RNA and PCR Reactions. Plant tissue was harvested and frozen in liquid N_2. Total RNA was isolated essentially as described by Berry *et al*. (16). Random hexamers were used to prime cDNA synthesis from total RNA according to instructions included with the Superscript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD). The specific PCR reaction parameters were as follows: "Hot start," fol-

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Abbreviations: RI, Recombinant Inbred; KCBP, kinesin-like, calmodulin-binding protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF002678).

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lowed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min.

Genetic Mapping of the *ZWI* Locus. Recombinant Inbred (RI) lines (17) were used to map the *ZWI* locus. The RI lines were obtained from the *Arabidopsis* Stock Center (Ohio State University). Southern blots of DNA from 60 RI lines digested with *Xba*I were prepared using standard procedures (18). Probes representing approximately 35 kbp of the *ZWI* locus were labeled with digoxigenin using the Genius labeling kit (Life Technologies) according to manufacturer's recommendations. *ZWI* locus probes were hybridized to filters that contained the RI line DNA and the results were sent to the Nottingham Stock Center for analysis. The map position of the *ZWI* locus was calculated using the Kosambi mapping function.

RESULTS

Scanning Electron Microscopy Analysis of the *zwichel* Phenotype. To understand the processes involved in plant cell morphogenesis, we have isolated a variety of *Arabidopsis* mutants with altered trichome shape (D.G.O., unpublished work). Eight independently isolated mutations were found to be allelic to the recessive *zwi*chel (*zwi*) mutation described by Hülskamp *et al.* (6). To determine the stage in trichome development when the *zwi* mutants are affected, we used scanning electron microscopy to examine developing trichomes on wild-type and mutant plants (Fig. 1).

Wild-type trichome development begins when a protodermal cell expands out from the plane of the leaf blade. The growth dynamics of the developing trichome can be thought of as an iterative process of regulated cell expansion. The location and number of growth foci determines the branching pattern of a developing trichome. The initial or primary expansion focus produces a region that will comprise the trichome stalk. The production of a branch begins with the initiation of an additional focus of cell expansion near the top of the growing stalk (Fig. 1*C*). This is defined as a secondary focus of cell expansion. After the appearance of the secondary focus, the primary growth focus extends parallel to the surface of the leaf and forms a branch whose tip is often directed toward the

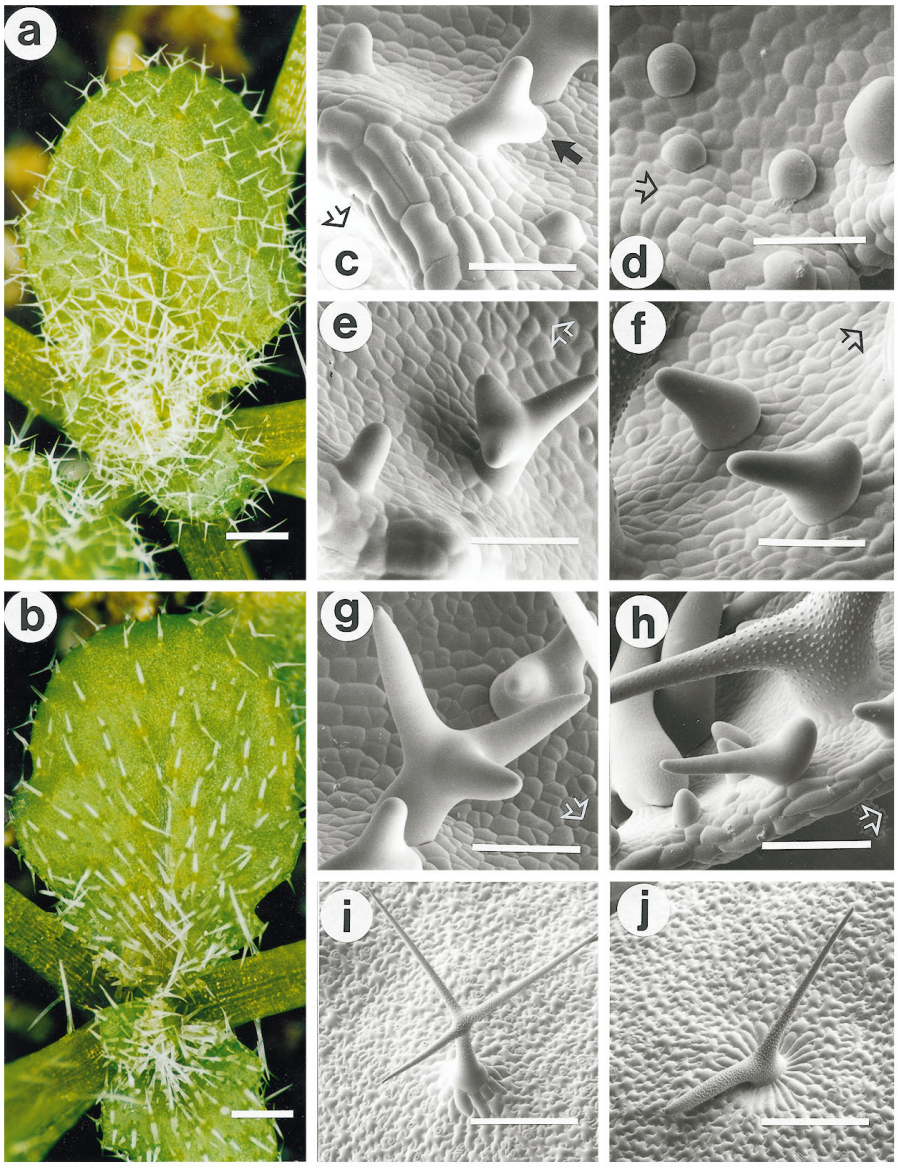

FIG. 1. Development of wild-type and mutant trichomes. (*A* and *B*) Light micrographs of wild-type (*A*) and *zwi*-3 mutant (ecotype Columbia) (*B*) plants showing the distribution and shape of the trichomes. (*C–J*) Scanning electron micrographs of wild-type (*C*, *E*, *G*, and *I*) and *zwi*-3 mutant (*D*, *F*, *H*, and *J*) trichomes at different stages of development. Open arrow indicates distal tip of the leaf blade. Closed arrow in *C* indicates the second focus of cell expansion, which produces the first branch. [Scale bars = 1 mm (*A* and *B*), 43 μm (*C–G*), 86 μm (*H*), and 250 μm (*I* and *J*).]

petiole of the leaf. The primary and secondary branches are oriented 180 degrees relative to each other if no additional branching occurs. However, in most *Arabidopsis* ecotypes, a third focus of cell expansion produces a third branch, and the three resulting branches are spaced approximately 120 degrees apart (Fig. 1 *E* and *G*). The emerging trichome cell continues to expand in both height and diameter, and during the final stage of morphogenesis the cell wall thickens and acquires a papillate surface (Fig. 1*I*).

The defect in mutants homozygous for strong *zwi* alleles is apparent at a very early stage of trichome morphogenesis. In *zwi* mutants, the trichomes initiate normally (Fig. 1*D*), but a stalk is not formed. Instead, a secondary focus of cell expansion appears to develop at the base of the cell (Fig. 1*F*). In some genetic backgrounds such as Wassilewskija, strong *zwi* alleles minimize secondary branch expansion, which results in a trichome with only a primary branch containing a fine tip and a small swelling at the trichome base (not shown). In the Col ecotype, strong alleles usually produce trichomes with two branches. However, the branch produced by the secondary focus of expansion is close to the leaf surface and develops a blunt tip (Fig. 1 *H* and *J*). The development of a third branch is suppressed. The orientation of the branches is striking (Fig. 1*B*). The two branches are oriented 180 degrees apart, and most often oriented approximately parallel to the long axis of the leaf. As found in wild type, the branch produced by the original focus of expansion possesses a finely pointed tip, which is usually directed toward the base of the leaf (Fig. 1*J*).

Identification of a T-DNA-Tagged *zwi* Allele and Isolation of the *ZWI* Gene. A T-DNA-transformed population of *Arabidopsis* plants (19) was screened for *zwi* mutants. Four lines segregated for plants that displayed a short-stalk phenotype and crosses to a *zwi* tester line confirmed that these were *zwi* mutants. Linkage was established between the T-DNA insert and the *zwi* mutation by following the cosegregation of the kanamycin resistance marker on the T-DNA and the *zwi* mutation. Two of the four lines, *zwi*-4673 and *zwi*-5002, showed linkage between the T-DNA insert and the *zwi* mutation. Plasmid rescue of genomic DNA flanking the T-DNA insertion sites was performed as described in ref. 20. The rescued plant DNA fragment was used as a probe to screen a wild-type *Arabidopsis* (ecotype Columbia) genomic library. Three λ clones were isolated that overlap and cover approximately 35 kb of genomic DNA. Several different restriction fragments from the λ clones were used to transform *zwi* mutant explants (Fig. 2). Regenerating transformants were scored for the presence of wild-type trichomes. One of the fragments restored the wild-type trichome shape to the mutant regenerating explants (Fig. 3), demonstrating that the transferred DNA contained the *ZWI* locus.

Approximately 12 kb of genomic DNA was sequenced. Open reading frames from this region were used in a BLAST (21) search (March 6, 1994) of GenBank sequences. One open reading frame showed significant sequence similarity to proteins of the kinesin superfamily (Fig. 4*A*) (26, 27). The sequence similarity was restricted to the microtubule- and ATP-binding regions of the highly conserved motor domain. A second open reading frame, beginning approximately 3.8 kb upstream of the putative motor domain, showed 33% amino acid identity with a domain from the tail region of an unconventional myosin from *Acanthamoeba* (28) (Fig. 4*B*).

To identify the coding sequence of the *ZWI* gene, total RNA was isolated from dissected *Arabidopsis* shoot apices and used in reverse transcription–PCR reactions to amplify overlapping cDNA fragments. Intron/exon junctions were identified by comparing the DNA sequences of the amplified cDNA fragments and the genomic DNA. Anchored PCR was used to determine the 3′ end of the mRNA. The *ZWI* coding sequence is interrupted by 20 introns ranging in size from 70–320 bp.

The predicted *ZWI* protein has 1259 amino acid residues and a calculated molecular weight of 143,000 (Fig. 4*C*). The kinesin-like motor domain is located in the C-terminal region of the protein, between amino acid positions 885 and 1202. The N-terminal domain that is similar to the tail region of an *Acanthamoeba* myosin is located between amino acid residues 104 and 234.

During the completion of this study, Reddy *et al.* (12) reported the sequence of a full-length cDNA that encodes a calmodulin-binding protein with a kinesin-like motor domain. Comparison of this DNA sequence with the *ZWI* cDNA sequence revealed that the cDNA isolated by Reddy *et al*. (12) could be encoded by the *ZWI* gene. The coding sequences are 99.6% identical. There are 5 amino acid differences between the predicted *ZWI* protein and the KCBP (kinesin-like, calmodulin-binding protein) predicted by Reddy *et al*. (12). The most significant difference is at amino acid position 118, where the *ZWI* protein contains an aspartic acid residue, and the KCBP contains an isoleucine residue and an insertion of a proline and another isoleucine residue. However, Southern blot hybridization analysis [Reddy *et al.* (12) and D.G.O., data not shown) indicates that the *ZWI*/KCBP gene is present as a

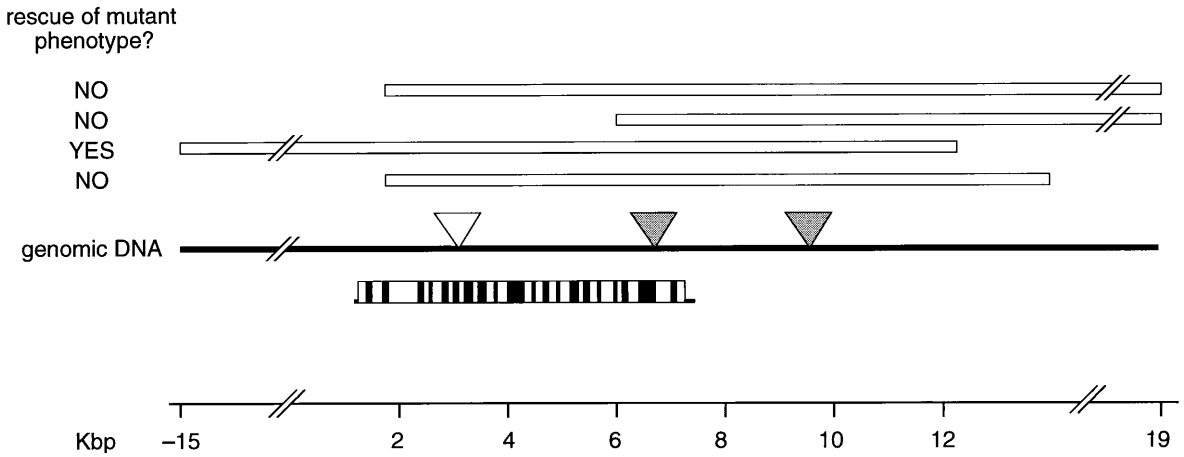

FIG. 2. Map of the *ZWI* locus. Open boxes represent the regions of the locus used in mutant rescue (complementation) experiments. The ability of the region to rescue the mutant phenotype is indicated on the left. At least four independent transformants were scored for each fragment. The shaded triangles represent the location of the T-DNA insertions. The open triangle represents the approximate location of the fast neutron-induced insertion. The diagram shown below the genomic region indicates the positions of the introns and exons. Exons are represented by open boxes, introns by solid boxes, and nontranslated regions by a solid line.

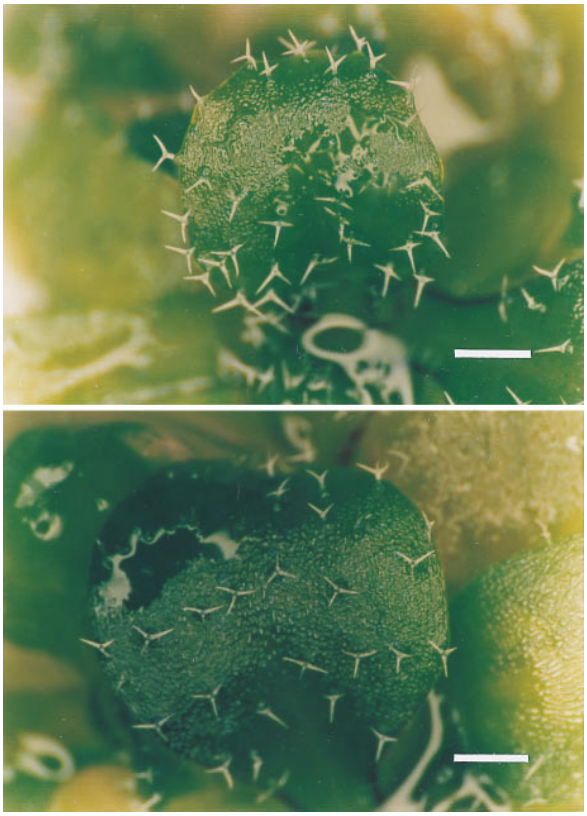

FIG. 3. Complementation of mutant phenotype by transformation. Light micrograph of two independent regenerating transformants showing wild-type trichome morphology. These explants were transformed by *Agrobacterium* strain AGL1 carrying the subclone indicated in Fig. 2. (Scale bars = 1 mm.)

single copy. Thus, the *ZWI* and KCBP sequences are most likely wild-type alleles.

Because we could not detect *ZWI* mRNA by Northern blot analysis using total RNA, a PCR-based approach was used to examine the expression of the *ZWI* gene. When total RNA from roots, seedlings, stems, and flowers of wild-type *Arabidopsis* plants was used in an reverse transcription–PCR reaction, *ZWI* expression was detected in each of these tissues (data not shown).

Characterization of *zwi* Alleles. To confirm that the kinesin-like protein is encoded by the *ZWI* gene, we characterized two T-DNA insertional alleles (ecotype Wassilewskija) and a third allele induced by fast neutron mutagenesis (ecotype RLD). All of these *zwi* mutations are recessive. The T-DNA insertion in the *zwi*-5002 mutant is located at position 6732, between the putative ATP-binding region and the putative microtubule-binding regions in the kinesin-like motor domain. Translation of mRNA from the insertion mutant would produce a truncated *ZWI* protein due to an in-frame stop codon 16 amino acids into the inserted sequence. Thus, this insertion most likely would result in a *ZWI* product that completely lacked motor function. The T-DNA insertion site in *zwi*-4673 is located at nucleotide position 9531, which is approximately 2,090 bp downstream from the putative polyadenylylation signal (Fig. 2). Although this insertion does not interrupt the *ZWI* coding sequence, it may disrupt the function of regulatory sequences required for proper *ZWI* expression. A *zwi* allele generated by fast neutron mutagenesis (*zwi*-9310–7), contains an insertion of approximately 500 bp of DNA of unknown origin between position 3000 and position 3450 (Fig. 2). An insertion of this size would most likely lead to a nonfunctional *ZWI* protein, which is consistent with the strong *zwi* phenotype of this allele.

The map position of the *ZWI* locus was determined using 60 RI lines from Lister and Dean (17). The calculated map position for the *ZWI* locus is on the lower arm of chromosome five, located 11.7 centimorgans distal to restriction fragment length polymorphism marker m555. This places the *ZWI* locus 7 centimorgans beyond the distal-most marker currently on the chromosome 5 map. Thus, the *ZWI* locus defines a new distal limit to *Arabidopsis* chromosome 5.

DISCUSSION

The molecular complementation of the *zwi* mutant and the molecular characterization of three mutant *zwi* alleles allows us to conclude that we have cloned the *ZWI* gene. Sequence analysis revealed that *ZWI* encodes a member of the kinesin-like family of motor proteins that had been previously identified by its ability to bind calmodulin (12). Members of the kinesin superfamily of motor proteins function to move specific cellular components along microtubules. Some members direct movement toward the plus end of microtubules, whereas other members are known to transport cargo toward the minus end (26). A family of kinesin-like protein genes has been characterized in *Arabidopsis* (22). One member of this family, KatAp, which has the motor domain located near the C terminus of the protein, is likely to be a minus end-directed motor (29). Recently, ZWI, which also has a C-terminal motor domain, has been shown to move along microtubules toward the minus end (30).

The structure of the ZWI kinesin-like protein has two unique features that distinguish it from the other known *Arabidopsis* kinesin-like proteins. First, ZWI has a region with similarity to a Class IV myosin found in *Acanthamoeba*. The function of Class IV myosins is unknown, and the role of the myosin region with similarity to ZWI is unclear. There is, however, evidence in yeast that a kinesin-like protein may share function with a myosin. Lillie and Brown (31) reported that the kinesin-like protein, SMY1, can act as a suppressor of a mutation in the MYO2 gene, suggesting that a kinesin-like protein can functionally substitute for a putative actin-based motor.

A second unique feature of ZWI is that it contains a calmodulin-binding site. Reddy *et al.* (12) isolated a gene nearly identical to *ZWI* called *KCBP* by screening a cDNA library with a biotinylated calmodulin probe. The 5 amino acid sequence differences that are observed between ZWI and KCBP may be due to allelic variation within the Col ecotype, which was the source for both the cDNA characterized by Reddy *et al.* (12) and the genomic clone described in this report. Their analysis indicated that a 23 amino acid sequence located close to the C terminus (which is completely conserved in both ZWI and KCBP) is a Ca^{2+}-dependent, calmodulin-binding domain. The presence of a calmodulin-binding domain within the ZWI protein strongly suggests that calcium and calmodulin regulate ZWI function.

ZWI mRNA was detected in seedlings, stems, and flowers of wild-type *Arabidopsis* plants. Similar results were obtained by Reddy *et al.* (12). Although the *ZWI* gene is expressed in tissues other than developing leaves, in all of the *zwi* mutants examined no phenotypic defects other than in trichome development were observed. This may be because another gene product may substitute for *ZWI* function in these other tissues. Alternately, trichome development may be uniquely sensitive to the loss of *ZWI* even in the presence of motor proteins that can compensate for the loss of *ZWI* in other tissues. It is also possible that the amino-terminal region of ZWI, which is intact in the mutant alleles described in this report, can carry out essential functions in nontrichome cells, but that trichome development requires both the amino- and carboxyl-terminal domains.

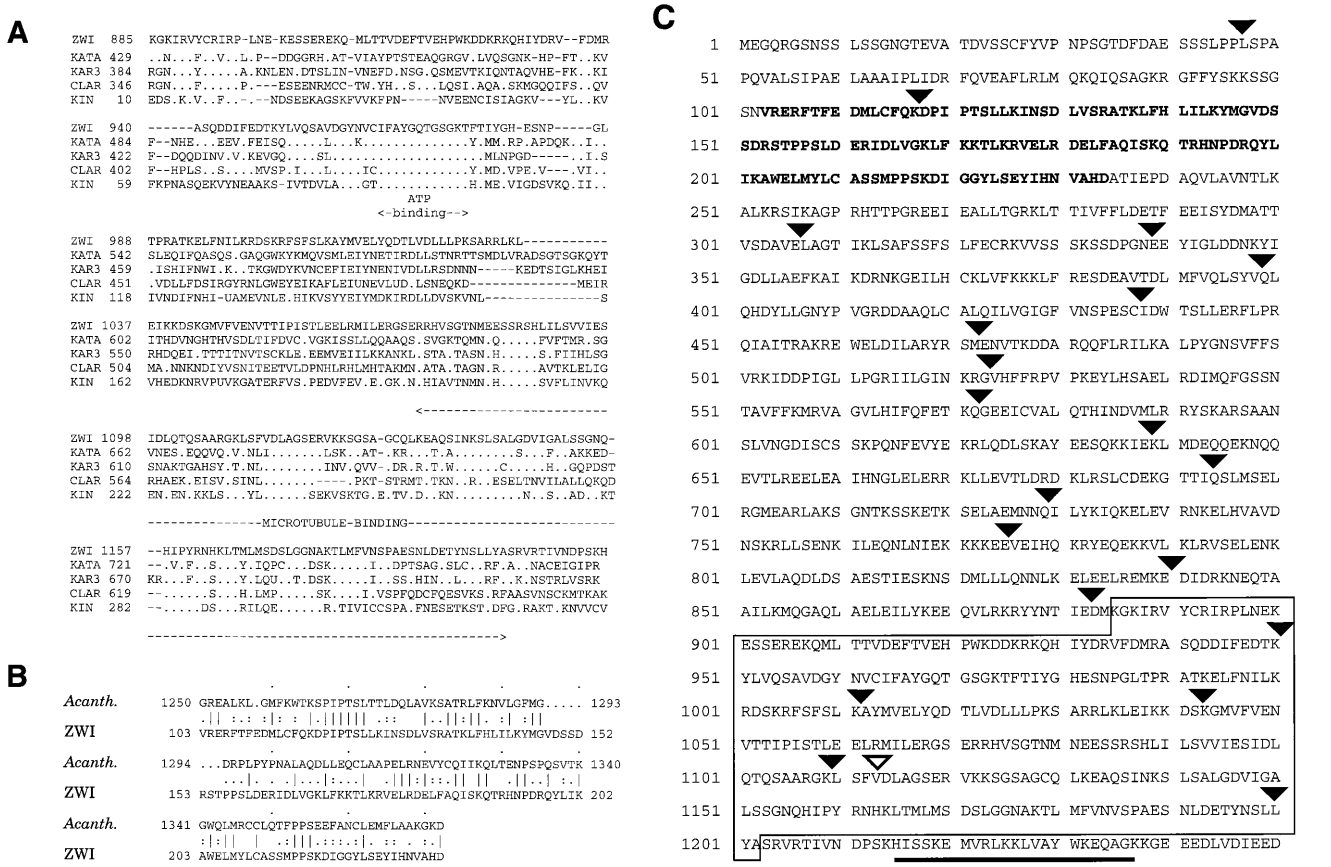

FIG. 4. ZWI protein comparisons. (*A*) Comparison of the putative motor domain of the ZWI protein with the motor domains of other kinesin-like proteins. Dots represent amino acids identical to the ZWI sequence; dashes represent gaps that have been introduced to improve the alignment. The putative ATP-binding and microtubule-binding regions are indicated. KATA, *Arabidopsis* kinesin-like protein (22); KAR3, *Saccharomyces* kinesin-like protein (23); CLAR, *Drosophila ncd*$^{+}$ (24); KIN, *Drosophila* kinesin heavy chain (25). (*B*) Comparison of the region of similarity between the Class IV *Acanthamoeba* myosin and ZWI. Dots in the protein sequence represent gaps that have been introduced to give optimal alignment. Vertical lines indicate amino acids that are identical, and colons represent similar amino acids. The numbers indicate amino acid positions. (*C*) Deduced amino acid sequence of the ZWI protein. Solid triangles show the positions of the introns. The open triangle shows the position of one of the T-DNA insertions. (The other T-DNA insertion occurs outside of the coding sequence.) The region with homology to the *Acanthamoeba* myosin is shown in boldface type, the kinesin-like motor domain is boxed, and the calmodulin-binding region is underlined.

Reddy *et al*. (32) have isolated a related *KCBP*-like gene from potato, which they named PKCBP. PKCBP shows 80% and 40% identity to ZWI in the calmodulin-binding and motor domains, respectively. Their analysis indicated that PKCBP is encoded by a single gene and is expressed in leaves, roots, and flowers. Furthermore, they demonstrated that PKCBP binds to microtubules. It will be interesting to determine if PKCBP plays a role in trichome development in divergent plant species.

One of the T-DNA insertions characterized in this study, *zwi*-4673, was located more than 2 kbp downstream of the putative polyadenylylation site of the *ZWI* gene. Because the phenotype of the *zwi*-4673 mutant is strong, we believe that this insertion prevents the proper expression of the *ZWI* gene by affecting the function of regulatory sequences located downstream of the gene. This was shown to be the case for the *GL1* gene, where the T-DNA insertion site was also located 3′ of the coding sequences and shown to disrupt regulatory sequences required for *GL1* function (33). It is likely that only minimal 5′ sequences are required for the regulation of *ZWI*. This is because 489 bases upstream of the *ZWI* start codon another open reading frame ends, which has a high degree of similarity to amino acid hydralases found in *Caenorhabditis elegans* and *Pseudomonas* (data not shown). The probable limits of the 3′ end are defined by two expressed tags ATTS44194 and ATTS2657, which are 3.5 kbp and 5.4 kbp, respectively, downstream of the insertion in *zwi*-4673.

The defects in trichome morphogenesis in *zwi* mutants indicate that wild-type *ZWI* affects several aspects of trichome expansion. Defects in the placement of secondary branches on the developing trichome suggest that *ZWI* is required for elongation of the stalk and/or the correct placement of branch initiation. However, the position of the secondary growth focus in *zwi* mutants is correctly placed on the circumference of the developing trichome stalk. This results in a trichome with the branches oriented approximately 180 degrees apart and essentially parallel to the proximal–distal axis of the leaf. The morphology of the secondary branch is altered in *zwi* mutants, and *zwi* mutants do not initiate tertiary branches. It is not known if the blunt tip of the secondary branch or the loss of additional branching of *zwi* trichomes is a direct effect caused by the loss of *ZWI* function or a secondary effect caused by the abnormal timing or position of prior branching events.

The discovery that *ZWI* encodes a kinesin-like motor protein that is most likely regulated by Ca^{2+} and calmodulin ties several cellular processes to trichome morphogenesis. The analysis of the role of *ZWI* in trichome morphogenesis will provide information on how the complex interactions between Ca^{2+} gradients, the cytoskeleton, and the cell expansion machinery control cell shape.

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