

TANGLED1: A Microtubule Binding Protein Required for the Spatial Control of Cytokinesis in Maize

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Abstract. Spatial control of cytokinesis in plant cells depends on guidance of the cytokinetic apparatus, the phragmoplast, to a cortical “division site” established before mitosis. Previously, we showed that the Tangled1 (Tan1) gene of maize is required for this process during maize leaf development (Cleary, A.L., and L.G. Smith. 1998. Plant Cell. 10:1875–1888.). Here, we show that the Tan1 gene is expressed in dividing cells and encodes a highly basic protein that can directly bind to microtubules (MTs). Moreover, proteins recognized by anti-TAN1 antibodies are preferentially associated with the MT-containing cytoskeletal structures that are misoriented in dividing cells of tan1 mutants. These results suggest that TAN1 protein participates in the orientation of cytoskeletal structures in dividing cells through an association with MTs.

Key words: cytokinesis • phragmoplast • preprophase band • microtubules • plant cytoskeleton

Introduction

Because plant cells are surrounded by walls that immobilize them within tissues, the orientations in which they divide during development constrain patterns of organ growth and are critical for establishing the cellular organization of plant tissues. Plant cells achieve cytokinesis through the action of a phragmoplast, a cytoskeletal structure composed of microtubules (MTs)¹ and F-actin, which directs the formation of a new cell wall between daughter nuclei after mitosis (Staehelin and Hepler, 1996). The phragmoplast arises initially in isolation from the parental wall and plasma membrane, and expands centrifugally as cytokinesis proceeds. The location at which the new cell wall will fuse with the parental wall is governed by an interaction between the expanding phragmoplast and a cortical “division site” established before mitosis (Gunning, 1982; Smith, 2001). This site is marked throughout prophase by a cortical band of MTs and actin filaments, the preprophase band (PPB), which probably plays an essential role in establishing the division site during prophase (Wick, 1991; Smith, 2001). Very little is known in molecular terms about the nature of the cortical division site or how the phragmoplast interacts with it during cytokinesis.

To better understand the spatial control of cytokinesis in plant cells, we have analyzed the function of a gene in maize, Tangled1 (Tan1), which is required for this process. Throughout the development of tan1 mutant leaves, the majority of cells in all tissue layers divide in abnormal orientations (Smith et al., 1996). Analysis of the cytoskeleton in dividing leaf cells of tan1 mutants showed that all the cytoskeletal structures involved in cell division are formed and appear structurally normal, but are not oriented normally within dividing cells. Abnormally oriented cell divisions can be attributed mainly to the failure of most phragmoplasts to be guided to the former PPB site (Cleary and Smith, 1998). Here, we present an initial molecular characterization of the Tan1 gene and its protein product. In combination with our previous analysis of the tan1 mutant phenotype, the results suggest a direct role for TAN1 in orienting cytoskeletal structures during cell division.

Materials and Methods

Plant Material

tan-Mu1 was isolated from a Mutator mutagenized population (Smith et al., 1996). tan1-py1 was obtained from the Maize Genetics Stock Center. The ethyl methanesulfonate–induced tan-gt1 allele was a gift from Sharon Kessler and Neelima Sinha (University of California at Davis, Davis, CA).

¹Abbreviations used in this paper: APC, adenomatous polyposis coli; MT, microtubule; PPB, preprophase band.
Nucleic Acid Isolation and Gel Blot Analysis

Genomic DNA isolation from leaf tissue and Southern blots was carried out according to standard protocols (Chen and Dellaporta, 1994; Ausubel et al., 2000). Blots were hybridized at 65°C in 0.25 M NaPO₄ pH 7.2, with 2% SDS and washed in 0.2× SSC with 0.2% SDS (high stringency) or at 54°C in 0.5 M NaPO₄, pH 7.2, with 7% SDS and washed at 54°C in 100 mM NaPO₄, pH 7.2 with 5% SDS (low stringency). Total RNA was extracted using Trizol reagent (GIBCO BRL) and enriched for poly A' RNA using the PolyATract mRNA isolation system (Promega). Northern blots were carried out as described by Luehrsen (1994). To demonstrate equal loading, Northern blots were stripped and reprobed with a 700-bp PstI-SacI fragment of the ubiquitin clone pSKUBI (Christensen et al., 1992), a gift from P. Quail (US Department of Agriculture Plant Gene Expression Center, Albany, CA).

Cloning and Sequence Analysis of Tangled

The 2.5-kb Mul-hybridizing SstI fragment cosegregating with the tanl phenotype was cloned from a size-selected library of SstI-digested genomic DNA from a homozygous mutant constructed in Lambda Zap (Stratagene). Full-length genomic and cDNA clones were isolated using the 600-bp Tanl fragment (see Fig. 1A) to screen a B73 genomic DNA library (a gift from Pioneer Hi-Bred, Johnston, IA) and a B73 vegetative shoot tip cDNA library (a gift from B. Veit and S. Hake, US Department of Agriculture Plant Gene Expression Center). The sequences of three different cDNA clones and one full-length genomic clone were assembled using MacVector software (v6.5). Sequencing of genomic PCR products amplified from the tanl-gtl allele revealed the presence of a point mutation near the end of exon 2. A combination of PCR and Southern blotting was used to identify a 6-kb insertion of unknown identity in the first intron of the tanl-gtl allele.

Protein and Antibody Production

Polyclonal rabbit antibodies were raised against a COOH-terminal TAN1 peptide (CGLKQRPGYSLTVRTVSSKISSR) coupled to keyhole limpet hemocyanin at Covance Research Products (Denver, PA) using their standard protocols. Antibodies were affinity-purified on peptide-coupled SulfoLink beads (Pierce Chemical Co.) as described by Harlow and Lane (1988). For the peptide competition experiments (see Fig. 5, O and P), 1.5 μg of affinity-purified COOH-terminal peptide antibody in 200 μl of PBS with 1 mg/ml BSA was absorbed with beads coupled to ~33 μg of peptide and used without further dilution for cell labeling experiments. mAbs were raised against the portion of TAN1 encoded by exons 1 and 2 (expressed as a glutathione S-transferase [GST] fusion protein from the pGEX-4T-2 vector [Amersham Pharmacia Biotech] and cleaved from glutathione S-transferase with thrombin protease) at Covance Research Products according to their standard protocols. The His-TAN1 fusion protein used for MT overlay assays was constructed by cloning a full-length TAN1 cDNA in frame with the histidine tag of pQE-30 (QIAGEN).

Protein Extraction and Analysis

Plant extracts were prepared by homogenizing fresh tissue with an Omni processor and centrifuged at various speeds for 10 min in an Eppendorf microcentrifuge at 4°C. Proteins were separated by SDS-PAGE as described by Ausubel et al. (2000). Western blots were carried out as described by Harlow and Lane (1988), using the affinity-purified COOH-terminal peptide antibody at 5–10 μg/ml or hybridoma TAN75 culture supernatant at 1:20, and alkaline phosphatase–conjugated anti–mouse Ig as described above. The MT overlay assay was based on the method of Saunders et al. (1996). Lea primordia ≤1 cm in length were removed from 2–3-wk-old seedlings, fixed as described in Cleary and Smith (1998), and squashed onto coverslips essentially as described in Wick et al. (1991). Alternately, tissue slices were plunge frozen in liquid nitrogen–cooled propane, freeze substituted, embedded in butyrimethylocrylate resin, sectioned at 3 μm, and incubated in acetone to remove the resin as described by Baskin et al. (1996). These methods gave very similar results. Coverslips/slides were blocked in PBS with 3% normal mouse serum (PBS/NMS), incubated with primary antibodies (anti-COOH peptide at 5–10 μg/ml TAN75 supernatant at 1:50 in PBS/NMS) for 2 h at room temperature, washed in PBS with 0.2% Triton X-100, incubated with appropriate FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), and washed again as above. For double labeling experiments, coverslips/slides were then blocked in PBS with 5% normal mouse serum (PBS/NMS) and incubated for 2 h in Cy3-conjugated anti–β-tubulin mAb Tub 2.1 (Sigma-Aldrich) diluted into PBS/NMS (incubations were always done in this order to ensure that FITC labeling coinciding with MTs could not result from cross-reaction of the FITC secondary antibodies with the tubulin antibody).

Online Supplemental Materials

Supplementary Materials including details of the relationship between TAN1 and vertebrate adenomatous polyposis coli (APC) proteins, and additional biochemical characterization of TAN proteins, can be found at http://www.jcb.org/cgi/content/full/152/1/231/DC1.

Results

Cloning of Tangled1 Using a Transposon Tag

The tan-Mul mutation arose in a stock with active Mutator transposons. A Mul-hybridizing 2.5-kb SstI fragment that cosegregated with the mutant phenotype was cloned and used as a probe to isolate three cDNAs and a full-length genomic clone. Sequencing of these clones revealed the gene structure illustrated in Fig. 1A. The Mul insertion in tan-Mul coincides approximately with the presumed start site of transcription, as estimated from the size of tanl mRNA (~1.5 kb, see below). Southern blot analysis and sequencing of genomic PCR products showed that the tanl-pyl mutant allele contains an ~6-kb insertion of unknown identity in the first intron of the gene, and the ethyl methanesulfonate–induced tanl-gtl allele contains a premature stop codon near the end of the second exon, which would truncate the TAN1 protein to <10 kD.

The 600-bp fragment shown in Fig. 1A hybridizes to the 2.5-kb SstI fragment in genomic DNA of homozygous mutant individuals, a 1.2-kb fragment in their homozygous wild-type siblings, and to both fragments in heterozygous siblings (Fig. 1B). This is the expected pattern of hybridization for a fragment of the Tanl gene, since the wild-type allele should be smaller than the tanl-Mul allele by the size of Mul (1.3 kb). Proof that the 600-bp fragment is a part of the Tanl gene came from the analysis of somatic revertant sectors. Because tanl mutant leaves have a rough crepe papery texture, large wild-type sectors in mutant leaves, such as that illustrated in Fig. 1D, are readily visible. Four such sectors showed the result illustrated in Fig. 1C: the 600-bp fragment hybridizes to a 2.5-kb SstI

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fragment in DNA from mutant tissue (Fig. 1 C, lane 1) and to both 2.5 and 1.2-kb fragments in DNA from the adjacent wild-type sector (Fig. 1 C, lane 2). Thus, appearance of the wild-type phenotype in this sector correlates with excision of \( \text{Mu1} \) from one of the two mutant alleles. At high stringency, the 600-bp \( \text{Tan1} \) probe hybridizes to a single fragment of maize genomic DNA digested with various restriction enzymes (Fig. 1 E, asterisks). At low stringency, this probe hybridizes to the same fragment (Fig. 1 F, asterisks) and to one additional fragment (Fig. 1 F, arrows).

**Tan1 Gene Expression Is Correlated with Cell Division**

Northern blot analysis using the 600-bp \( \text{Tan1} \) probe illustrated in Fig. 1 A was carried out to examine the pattern of \( \text{Tan1} \) gene expression. As illustrated in Fig. 2 A, this probe hybridizes to a single mRNA of \( \sim 1.5 \) kb in wild-type vegetative shoot tips consisting of leaf primordia and the bases of immature leaves, the shoot apical meristem, and unexpanded stem tissue (highly enriched in actively dividing cells). This mRNA is greatly reduced in \( \text{tan1-Mu1} \) mutants and to a lesser extent in \( \text{tan1-py1} \) mutants. In addition, a smaller transcript is observed in \( \text{tan-py1} \) mutants (and their wild-type siblings, mostly \( \text{tan-py1} \) heterozygotes), presumably a product of aberrant RNA splicing (Fig. 2 A, asterisks). \( \text{Tan1} \) mRNA accumulation is only slightly reduced in shoot tips of homozygous \( \text{tan-gt1} \) mutants, consistent with the presence of a premature stop codon in this allele. As shown in Fig. 2 B, compared with the 0–2-cm shoot segment enriched in dividing cells (Fig. 2 B, lane 1), \( \text{Tan1} \) mRNA is vastly reduced in the 3–5-cm shoot segment composed mainly of postmitotic expanding leaf cells (Fig. 2 B, lane 2), and in the 6–8-cm segment composed mainly of fully expanded leaf cells that are differentiating (Fig. 2 B, lane 3), as well as in mature (fully expanded and differentiated) leaf tissue (Fig. 2 B, lane 4). In addition, \( \text{Tan1} \) is strongly expressed in other tissues enriched in dividing cells: ear primordia (Fig. 2 B, lane 5), and embryos (Fig. 2, lane 6). Thus, \( \text{Tan1} \) gene expression correlates with cell division in the shoot.

**Tan1 Encodes a Highly Basic Protein with Microtubule Binding Activity**

The \( \text{Tan1} \) cDNA sequence (sequence data are available from GenBank/EMBL/DDBJ under accession no. AF305892) contains an open reading frame encoding a predicted protein of \( \sim 41 \) kD that is highly basic with a pI of 12.6. Analysis of this protein sequence reveals no strong homology with other proteins or motifs of known function, but a distant similarity was observed between 85% of the \( \text{TAN1} \) protein and the basic regions of vertebrate APC proteins (see supplemental material available at http://www.jcb.org/cgi/content/full/152/1/231/DC1). The basic region of APC has been shown to bind to tubulin in vitro and associate with MTs in vivo (Polakis, 1997), suggesting that \( \text{TAN1} \) might perhaps be an MT binding protein.

Antibodies were raised against various fragments of \( \text{TAN1} \) to provide tools for analysis of this protein. Fig. 3 A shows a Western blot of proteins extracted from wild-type...
fragments illustrated in Fig. 1 A were used to probe ~5 μg of poly A–selected RNA from vegetative shoot tips of mutants (mut) and their wild-type (wt) siblings in families segregating tan1-py1, tan1-Mu1, and tan1-gt1. *Aberrant transcripts seen only in tan1-py1 homozygotes and heterozygotes. (B) The same Tan1 fragment was used to probe poly A–selected mRNA isolated from various wild-type maize tissues: (lanes 1–3) shoot segments of 2–3-wk-old seedlings from which all fully expanded leaves had been removed: the basal-most 2 cm (lane 1), 3–5 cm from the shoot base (lane 2), and 6–8 cm from the shoot base (lane 3); (lane 4) mature leaf tissue; (lane 5) embryos at 21 d after pollination; (lane 6) ear primordia 0.5–2-cm long. Blots were stripped and reprobed with ubiquitin to confirm RNA integrity and equal loading.

vegetative shoot tips probed with an affinity-purified antibody raised against a TAN1 COOH-terminal peptide. In Fig. 3 B, an equivalent blot is probed with an mAb raised against the NH2-terminal portion of TAN1 encoded by exons 1 and 2 (TAN75). Both antibodies predominantly label a single protein band at ~43 kD, close to the predicted molecular mass of TAN1 (41 kD). Sequential centrifugation of crude cell extracts at gradually increasing speeds of 3,000–15,000 g shows that the majority of this protein sediments at 6,000–9,000 g, indicating that it is associated with a relatively large or dense structure in cell extracts. However, since this protein cannot be solubilized with detergents, it does not appear to be associated with a membrane-bound organelle (see supplemental material available at http://www.jcb.org/cgi/content/full/152/1/231/DC1). Parallel to results from the Northern blot analysis of Tan1 mRNA, this protein is not detected in 3,000–10,000 g pellets from extracts of shoot segments composed of expanding (EXP) or differentiating (DIF) leaf tissue (Fig. 3 A).

As shown in Fig. 3, C and D, the 43-kD protein identified by both antibodies is reduced, but not eliminated, in mutants homozygous for each of the three tan1 mutant alleles. Notably, the truncated form of Tan1 protein encoded by the tan1-gt1 allele (see Fig. 1 A) would not be recognized by the COOH-terminal peptide antibody and would not comigrate with full-length TAN1. Thus, it appears that the 43-kD protein recognized by anti-TAN1 antibodies in tan1-gt1 shoot tip extracts is the product of another gene, which is similar enough to TAN1 to be recognized by antibodies against nonoverlapping NH2- and COOH-terminal fragments of TAN1 as well as three other polyclonal antibodies raised against other fragments of TAN1 covering its entire length (data not shown).

An overlay assay was used to show that TAN1 protein can bind directly to MTs. Fig. 4 A shows Coomassie blue-stained Escherichia coli extracts separated by SDS-PAGE before or after induction of a His-tagged TAN1 protein (arrow). After transfer to membranes, E. coli extracts were incubated with or without MTs polymerized from bovine brain tubulin. Subsequent detection with an anti-β-tubulin antibody showed that MTs bound to the His-tagged TAN1 protein (Fig. 4 A). A similar experiment with maize vegetative shoot tip extracts is shown in Fig. 4 B. Anti-β-tubulin detects endogenous maize tubulin with or without prior incubation of the blots with polymerized MTs. In addition, this antibody detects MTs bound to a 43-kD protein that comigrates with TAN1 and is reduced in abundance in the tan1-gt1 mutant extract. The residual MT-binding protein of the same molecular mass as TAN1 seen in the tan1-gt1 mutant extract presumably corresponds to the 43-kD protein in this extract recognized by anti-TAN1 antibodies (see Fig. 3, C and D), suggested earlier to be the product of a Tan1-related gene.

Proteins Recognized by Anti-TAN1 Antibodies Are Associated with the Cytoskeleton in Dividing Cells

Leaf primordium cells labeled with mAb TAN75 are shown in Fig. 5, A–G. This antibody labels cells at all stages of the cell cycle in a punctate manner. In interphase cells, antibody labeling is distributed uniformly through-
out the cytoplasm but is excluded from the nucleus (Fig. 5 A). Examination of thin sections confirmed that TAN75 labeling is not restricted to the cortex as interphase MTs are but is evenly distributed throughout the cytoplasm. In mitotic cells, TAN75 labeling is also observed throughout the cytoplasm but is preferentially associated with the cytoskeleton. A prophase cell labeled with TAN75 (Fig. 5 B) and anti-β-tubulin (Fig. 5 C) illustrates the coincidence of TAN75 labeling with the PPB. Similarly, mitotic cells labeled with TAN75 (Fig. 5, D and F) and anti-β-tubulin (Fig. 5, E and G) show that proteins recognized by TAN75 are predominantly associated with the spindle and phragmoplast. Fig. 5, H–N, shows that labeling of leaf primordium cells with the affinity-purified COOH-terminal peptide antibody produces very similar results.

Consistent with the results of Western blot experiments suggesting that these antibodies recognize a TAN1-related protein in tan1 mutants, we found that cell labeling with TAN75 and the COOH-terminal peptide antibody is not eliminated or qualitatively altered in any of the tan1 mutants, including tan-gt1 mutants. However, peptide competition experiments confirm that the COOH-terminal peptide antibody specifically labels proteins sharing epitopes with TAN1: preincubation of the antibody with COOH-terminal TAN1 peptide–coupled beads reduced labeling to background levels (Fig. 5 P), but preincubation with beads coupled to an unrelated peptide had no effect (Fig. 5 O). From these results, in combination with the Western blot data and the observation that antibodies directed against nonoverlapping parts of TAN1 show equivalent cell labeling patterns, we conclude that these patterns reflect the intracellular distribution of a family of two or more related proteins that includes TAN1.

**Discussion**

Previous work showed that *Tan1* is required for spatial control of cytokinesis during maize leaf development. In
The *Tan1* gene is expressed in regions of active cell division and encodes a highly basic protein distantly related to the basic MT-binding domain of vertebrate APC proteins. Antibodies raised against various fragments of the *Tan1* protein specifically recognize a 43-kD protein band detectable only in extracts from regions of active cell division, which apparently includes at least one *Tan1*-related protein in addition to *Tan1* itself. A genomic DNA fragment shown to hybridize with the *Tan1* probe at low but not high stringency could correspond to a gene 80–90% identical to *Tan1* at the nucleotide and protein levels, sharing most if not all epitopes recognized by the anti-*Tan1* antibodies employed in this study. Similarly, antibodies raised against individual maize profilins cross-react with other family members that are 85% identical at the amino acid and nucleotide levels (Kovar et al., 2000).

Efforts are currently underway to identify *Tan1*-like proteins for direct comparison with *Tan1*.

*Tan1* protein produced in *E. coli* and extracted from maize shoot tips can bind directly to MTs in an MT overlay assay. Furthermore, proteins recognized by anti-*Tan1* antibodies are preferentially associated with the MT-containing structures in dividing cells that are misoriented in *tan1* mutants (PPBs, spindles, and phragmoplasts). The apparent lack of association of these proteins with MTs in interphase cells suggests that their interaction with the cytoskeleton is regulated in a cell cycle–dependent manner.

Together, our results suggest that *Tan1* protein participates in the orientation of cytoskeletal structures in dividing cells through an association with MTs. An interesting possibility is that *Tan1* mediates interactions between these structures and the cell cortex that are necessary for their proper orientation, such as those that guide phragmoplasts to cortical division sites previously occupied by PPBs.

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