A Divergent Cellular Role for the FUSED Kinase Family in the Plant-Specific Cytokinetic Phragmoplast

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Summary

The FUSED (FU) Ser/Thr protein kinase family has a key role in the hedgehog signaling pathway known to control cell proliferation and patterning in fruit flies and humans [1, 2]. The genomes of *Arabidopsis thaliana* and rice each encode a single *Fu* ortholog, but their role is unknown. Here, we show that cytokinesis-defective mutants, which we named *two-in-one* (*tio*), result from mutations in *Arabidopsis Fu*. Phenotypic analysis of *tio* mutants reveals an essential role for TIO in conventional modes of cytokinesis in plant meristems and during male gametogenesis. TIO also has a key role in nonconventional modes of cytokinesis (cellularization) during female gametogenesis. We demonstrate that TIO is tightly localized to the midline of the nascent phragmoplast and remains associated with the expanding phragmoplast ring. These data reveal the evolution of a divergent role for the Fu kinase family as an essential phragmoplast-associated protein that functions in different cell type-specific modes of cytokinesis in plants.

Results and Discussion

The hedgehog (Hh) signaling pathway has a central role in the development of vertebrates and invertebrates [1, 2]. Cells interpret the levels of the secreted morphogen Hh through a large intracellular complex, the Hh signaling complex (HSC). The HSC is well characterized in *Drosophila* and includes the kinesin-related protein Costal 2 (Cos2), the Ser/Thr protein kinase Fused (Fu), the transcription factor Cubitus interruptus (Ci), and the PEST domain protein Suppressor of Fused [Su(fu)] [1, 2]. The HSC regulates processing of various forms of Ci that lead to repression or activation of Ci-dependent target genes. Although plants possess a single *Fu*

ortholog, it is intriguing that no other components of the Hh signaling pathway exist in the complete *Arabidopsis* and rice genome sequences. This suggests that plant Fu proteins could act in distinct cytoplasmic signaling complexes that have evolved independently in plants.

In a genetic screen [3] for pollen cell division defects, we identified *two-in-one* mutants (*tio-1*, *tio-2*) that were shown to arise from mutations in *Arabidopsis Fu*. The ontogeny of haploid pollen grains within flower involves only two mitotic cell divisions and provides a tractable system to identify regulators of the division process [4]. Haploid microspores normally divide unequally at pollen mitosis I to form a larger vegetative cell and smaller generative cell. Only the generative cell divides further to form the two sperm cells in mature tricellular pollen. In contrast to the wild-type, *tio* mutants produce binucleate pollen grains that arise from failure of cytokinesis at pollen mitosis I. Heterozygous *tio-1* and *tio-2* mutants produce approximately 35% aberrant pollen grains including binucleate, uninucleate, and collapsed pollen (see Figures 1A–1D and Table S1 in the Supplemental Data available with this article online). A more severe T-DNA insertion allele, *tio-3*, showed approximately 50% mutant pollen in heterozygous plants demonstrating complete penetrance. In heterozygous *tio-3* mutants in the *quartet* [5] genetic background, in which the four products of meiosis remained attached, wild-type and mutant pollen segregated 2:2, suggesting that *tio* mutations act in the haploid gametophyte (Figure 1E). Ultrastructural analysis confirms that binucleate mutant *tio* pollen contain two free nuclei and remain uncellularized (Figures 1F and 1G). In reciprocal test crosses with wild-type plants, all three *tio* mutations completely block male transmission, preventing the isolation of homozygous mutants, and are transmitted through the female at less than 10% efficiency (Table S2). This demonstrates a requirement for *TIO* function during male and female gametogenesis.

To examine the origin of cytokinesis defects in *tio* mutants, we examined the ontogeny of microspores and pollen by staining isolated spores for nuclei, and for the presence of dividing callose walls. Polar nuclear migration and asymmetric nuclear division at pollen mitosis I occur normally in *tio* mutants (Figures 2A–2C). At early bicellular stage in wild-type, the generative cell remains attached to the parent wall, and a complete callose wall surrounds the lens-shaped generative nucleus (Figures 2C and 2F). In *tio-1* and *tio-2*, however, approximately 35% (n = 108 and 202) of dividing microspores have incomplete callose walls (Figures 2C and 2F) that increases to 50% (n = 330) in *tio-3* (see Table S3). Incomplete callose walls in *tio* mutants are correctly positioned at the generative cell pole but do not persist and are degraded before mid-bicellular pollen stage (data not shown). Subsequently, at mid-bicellular pollen stage, when the generative cell nucleus is highly condensed in wild-type, in approximately 35% of pollen in *tio-1* and *tio-2*, and 50% in *tio-3*, the smaller generative pole nucleus remains round and relatively uncondensed
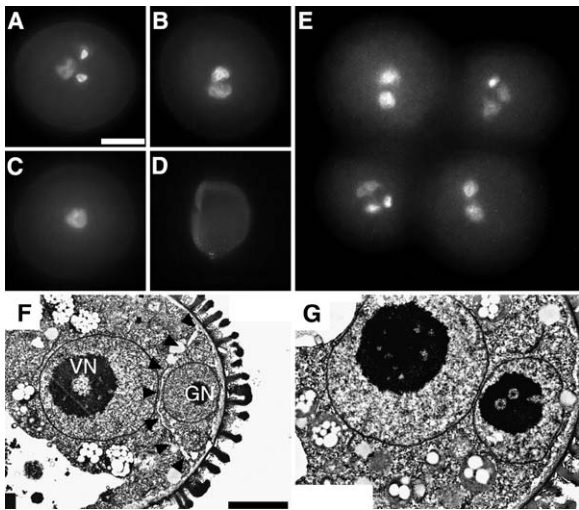
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Figure 1. Pollen Phenotypes in *tio* Mutants

(A–D) Four classes of pollen grains shed from mature anthers of heterozygous *tio* mutants viewed by fluorescence microscopy after DAPI staining. Tricellular (wild-type) (A), binucleate (B), uninucleate (C), and collapsed pollen (D).

(E) Mature pollen tetrad from a *tio-3* heterozygote in the *quartet* background showing segregation of wild-type and binucleate pollen.

(F and G) Ultrastructure of wild-type (F) and binucleate (G) pollen grains at early bicellular stage in *tio-1* heterozygotes. The vegetative nucleus (VN) is located centrally in the vegetative cytoplasm, and the new hemispherical cell wall marked by arrowheads encloses the generative cell and generative nucleus (GN).

Scale bars represent 10 μm (A–E) and 3 μm (F and G).

and does not divide further (Figures 2D and 2E). These data suggest that TIO is not required for positioning or establishment of the cell plate but has a specific role in cell plate expansion.

Given the strong effect on female transmission, we examined haploid embryo sacs of *tio* mutants for cytokinesis defects. Cytokinesis during female gametogenesis is uncoupled from mitosis, resulting in cellularization of the multinucleate embryo sac. Analysis using confocal laser scanning microscopy showed that until early FG5 stage [6], mitosis in *tio* embryo sacs proceeded normally to the

eight nucleate stage (data not shown). Mature embryo sacs, however, showed various numbers (2–5) of nuclei located toward the micropylar pole without visible cellular boundaries (Figures 3A and 3B). After fertilization, mutant embryo sacs did not develop further and remained uncellularized (Figures 3C and 3D). These data demonstrate that TIO also has an important role in the nonconventional mode of cytokinesis (cellularization) that occurs during female gametogenesis.

TIO was positionally cloned by mapping *tio-1* and *tio-2* to an interval of 39 kb within BAC clone F14I3 containing *Arabidopsis Fu* homology (see Figure 4A and Figure S1). Because of discrepancies in gene annotation in public databases, we verified and constructed the complete 4 kb *TIO* cDNA by RT-PCR and sequence analysis. Isolation of the T-DNA insertion allele *tio-3*, which shows a stronger *tio* pollen phenotype and more severely reduced female transmission, further confirmed the identity of *TIO*.

The lack of male transmission of *tio* mutations prevented the isolation of homozygous mutants and assessment of the potential role of *TIO* in somatic cytokinesis. Therefore, we adopted an inducible RNAi approach to suppress *TIO* expression in developing seedlings [7]. In the presence of the inducer β-estradiol, transgenic *TIO-RNAi* seedlings showed stunted development of young leaves and a striking swollen root tip phenotype (Figure 3E) resulting in arrested growth. Transverse sections through arrested shoot and root meristems revealed multinucleate cells with incomplete cell walls (Figures 3F and 3G). Irregularly expanded root tips contained highly enlarged cells often with greater than ten nuclei, demonstrating that mitosis was uncoupled from cytokinesis (Figures 3H and 3I). Moreover, Western blot analysis showed that TIO protein levels were decreased in roots of *TIO-RNAi* plants. (see Figure S3). Therefore, we conclude that *TIO* has an essential role in somatic cell cytokinesis and is required to maintain plant meristem structure.

The TIO kinase domain is closely related to those in dFu and hFU, with putative ATP binding and Ser/Thr kinase-active site residues conserved (Figure 4B). Moreover, there are no other *TIO*-related sequences in *Arabidopsis*, but the unique ortholog in rice (*OsTIO*) has
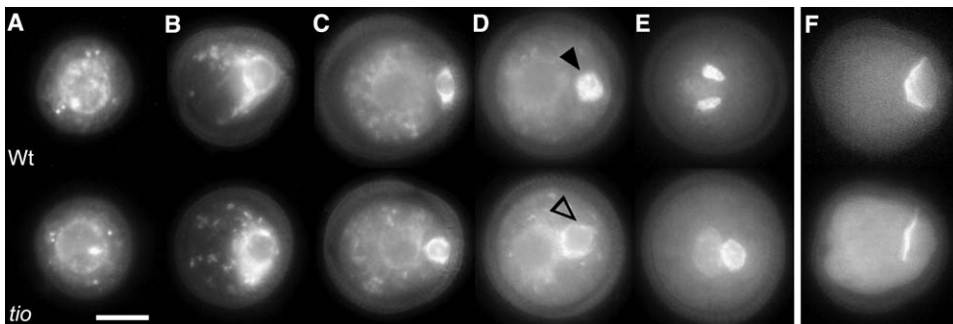

Figure 2. Pollen Development and Cytokinesis-Defective Phenotypes in *tio* Mutants

(A–E) DAPI-stained pollen from wild-type (top panel) and *tio-1* (bottom panel) at early microspore (A), late microspore (B), early bicellular (C), mid-bicellular (D), and late tricellular (E) stages. In wild-type, after detachment of the generative cell from the parent cell wall the highly condensed generative cell nucleus (closed arrowhead) divides to form two sperm cells in wild-type; however, in mutant *tio* pollen the generative pole nucleus (open arrowhead) does not divide, and pollen remains binucleate.

(F) Pollen at early bicellular stage stained for callose walls with aniline blue. Scale bar represents 10 μm.
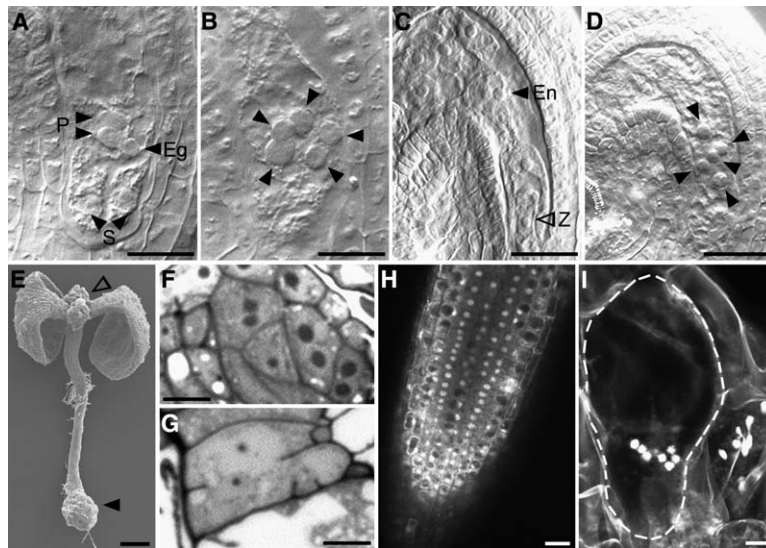
Figure 3. Embryo Sac Phenotypes in *tio-1* and Seedling Phenotypes in *TIO-RNAi* Lines

(A–D) Wild-type (A and C) and *tio-1* (B and D) ovules before (A and B) or after (C and D) fertilization. (A) In wild-type ovules, two polar nuclei (P), the egg nucleus (Eg), and two synergid cell nuclei (S) are separated by cellularization. (B) Misplaced nuclei (arrowheads) in *tio-1* embryo sac without cell boundaries. (C) Zygote (Z) and endosperm (En) nuclei are indicated. (D) Arrested *tio-1* embryo sac with five free nuclei.

(E) Scanning electron micrograph of *TIO-RNAi* seedling induced with 5 μM 17β-estradiol for 8 days showing abnormal development of shoot (open arrowhead) and root (closed arrowhead).

(F and G) Multinucleate cells with incomplete cell walls in sections of shoot and root meristems, respectively.

(H and I) Optical sections of uninduced (H) and induced (I) *TIO-RNAi* root tips stained with propidium iodide showing highly enlarged cells (outlined) with multiple nuclei. Scale bars represent 20 μm (A and B), 40 μm (C and D), 1 mm (E), 10 μm (F and G), and 25 μm (H and I).

a conserved length and domain structure (Figure 4B). Phylogenies constructed from either the kinase domains or the entire FU sequences revealed that plant and protozoan sequences are more closely related, with metazoan sequences being significantly more divergent (see Figure S2). A recent analysis of invertebrate and vertebrate FU sequences using ClustalW concluded that hFU and dFu share six colinear blocks of similarity in their C-terminal regions [8]. However, when we extended this analysis to include FU sequences from plants and the protozoan *Leishmania* (lFU), we did not detect the same colinear regions of similarity. Instead, we identified a conserved C-terminal domain (see Figure 4B and Figure S2). Within this domain, plant and *Leishmania* proteins contain four predicted Armadillo/β-catenin (ARM) repeats (Figure 4B and Figure S4). In addition, two to three overlapping HEAT repeats were also detected in all proteins except dFu. The C-terminal domain was less well conserved in hFu and dFu and did not contain predicted ARM repeats. The ARM repeat domain could therefore represent a structural or protein interaction domain unique to the functions of plant and protozoan FU proteins.

The T-DNA insertion at aa 276 in *tio-3* creates a premature stop codon immediately downstream of the kinase domain (Figure 4A). We were unable to amplify mutant transcripts from *tio-3* by RT-PCR (Figure 4C). Therefore, *tio-3* appears to be a null mutation consistent with the robust cytokinesis phenotypes in *tio-3* (Table S1). We sequenced *tio-1* and *tio-2* alleles and found that both contained point mutations creating a premature stop codon at amino acid position 943. *tio-1* contained an additional amino acid substitution (L737→Q) (Figure 4A). We detected mutant transcripts in *tio-1* and *tio-2* heterozygotes by sequencing RT-PCR products, suggesting that *tio-1* and *tio-2* are able to produce C-terminally truncated TIO protein. The incompletely penetrant pollen cytokinesis phenotypes in *tio-1* and *tio-2* further suggest that C-terminally truncated TIO retains partial function

because cytokinesis is completed normally in approximately 30% of pollen carrying *tio-1* or *tio-2* mutant alleles. We conclude that the C-terminal 379 amino acids of TIO including the ARM repeat domain are not essential for TIO function, but they may have a regulatory role associated with TIO localization or activity of the kinase domain.

RNA analysis revealed that *TIO* is broadly expressed in roots, stems, leaves, flowers, and developing pollen, consistent with the role of TIO in cytokinesis in somatic and reproductive cell types (Figure 4D). A single major protein at approximately 130 kDa was detected in protein extracts from *Arabidopsis* cultured cells and seedlings with an antiserum to the central region (aa 266–393) of TIO expressed in *E. coli* (Figure 4E).

To determine the subcellular localization of TIO, *Arabidopsis* cultured cells were subjected to triple staining with anti-TIO, anti-tubulin, and DAPI. Cytokinesis, in somatic plant cells, involves two plant cell-specific cytoskeletal arrays, the preprophase band (PPB) of microtubules (Mts) and the phragmoplast. The phragmoplast, an antiparallel array of two sets of Mts and actin filaments, directs cell plate formation through delivery of Golgi-derived vesicles to the phragmoplast midline. Lateral translocation of the phragmoplast as a ring involves depolymerization and repolymerization of Mts from the center toward the cell periphery [9]. We observed a relatively weak TIO signal in the nucleus of cells at early prophase with the PPB present (Figures 5A–5C). A diffuse TIO signal remained in the cytoplasm until late anaphase (Figures 5D–5F). At telophase, however, TIO was tightly localized to the midline of the phragmoplast, in the region where the phragmoplast Mts overlap (Figures 5G–5I). Subsequently, TIO remained associated with the expanding phragmoplast as a complete ring and disappeared when the phragmoplast Mts depolymerized on contact with the mother cell wall (Figures 5J–5L). TIO is not associated with phragmoplast Mts along their length and only appears tightly localized to the midline
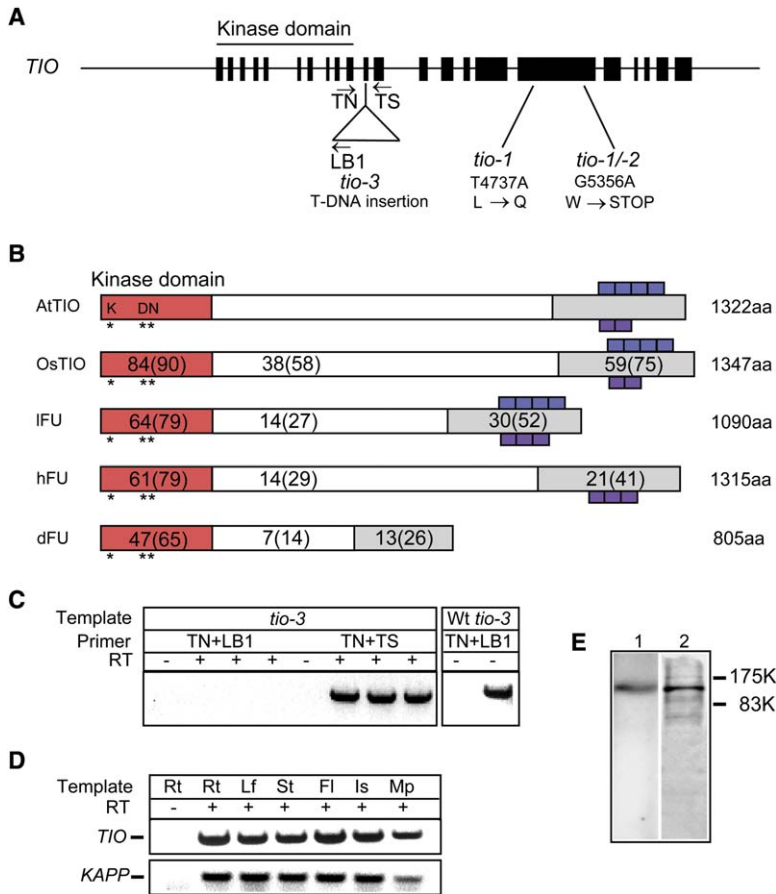
Figure 4. *TIO* Gene Structure and Expression

(A) Genomic structure and positions of *tio* mutations. Exons are shown as black bars, and the lesions in three *tio* alleles are shown.

(B) Domain structure of *Arabidopsis* TIO and FU proteins from rice (OsTIO, AK102130), *Leishmania* (IFu, CAB95259), human (hFU, AAF97028), and *Drosophila* (dFu, P23647). The percentage amino acid identity and similarity (in parentheses) between TIO and its homologs are shown in each domain. Single asterisk represents ATP binding site (Lys, K) at amino acid residue 35, and double asterisks represent kinase-active sites (Asp and Asn, DN) at 127 and 132 in the kinase domain. ARM and HEAT repeats are shown as upper blue and lower purple boxes.

(C) RT-PCR analysis of *tio-3* expression. Three leaf RNA samples were used to examine expression from either wild-type or the mutant *tio-3* allele. RT, reverse transcriptase.

(D) RT-PCR analysis of *TIO* expression in wild-type RNA samples from root (Rt), leaf (Lf), stem (St), flower (Fl), immature spores (Is), or mature pollen (Mp). + or −, RT-PCR reactions with or without RT. Intron-spanning primers were chosen to distinguish cDNA amplification from genomic DNA. KAPP, kinase-associated protein phosphatase (U09505) used as a positive control for integrity of RNA samples.

(E) Western blot analysis with anti-TIO antiserum. Fifty micrograms of proteins from *Arabidopsis* cultured cells (lane 1) and seedlings (lane 2) was separated by SDS-PAGE and immunostained with the TIO antiserum.

after phragmoplast assembly. The association of TIO with the expanding phragmoplast ring and the failure of cell plate expansion in *tio-3* null mutants (Figure 2F) demonstrate the requirement for TIO in the phragmoplast during cell plate expansion.

Cytokinesis is executed in distinct ways in plants and animals. In animals, daughter cells are formed by inward constriction by an actinomyosin contractile ring; however, plant cells generally divide centrifugally by building a cell plate. Moreover, plant cells have adopted cell type-specific modes of division [10]. Despite these differences, cell type-specific mechanisms of cytokinesis share overlapping structural features, and there is evidence that different modes of cytokinesis involve common molecular components [9]. For example, many embryo cytokinesis-defective mutants also display defects in endosperm cellularization [11]. Likewise, *gem1* and *gem2* mutants are cytokinesis defective during male and female gametogenesis [12]. However, GEM1 (also known as MOR1) is a MAP215 family Mt-associated protein that has a wider role in Mt stability not restricted to cytokinesis [13, 14]. Our data show that TIO is not required for positioning or establishment of the cell plate but has a more specific role in cell plate expansion.

In conclusion, we have shown that the *Arabidopsis* FU kinase TIO has an essential role in the phragmoplast during cell plate expansion in different modes of cytokinesis. Although FU functions have evolved independently in plants and animals, the indirect association of dFu with Mts through binding to the kinesin Cos2

suggests a possible mechanism of action for TIO. dFu uses both the kinase domain and the C-terminal domain to bind Cos2 [15]. Therefore, TIO might interact with a kinesin-related protein through the highly conserved kinase domain. Plants contain a large number of kinesin-related proteins [16], including several that are associated with the phragmoplast midline [17–20]. Importantly, the activation of the NACK-NPK1 MAPK signal transduction pathway regulating phragmoplast expansion in different cell types in tobacco and *Arabidopsis* is known to involve kinesin-like proteins [17, 21, 22]. These NPK1-activating kinesin-like proteins—NACK1 and NACK2 in tobacco [17] and their *Arabidopsis* orthologs, HINKEL [23] and TETRASPORE [24]—therefore represent candidate binding partners for TIO.
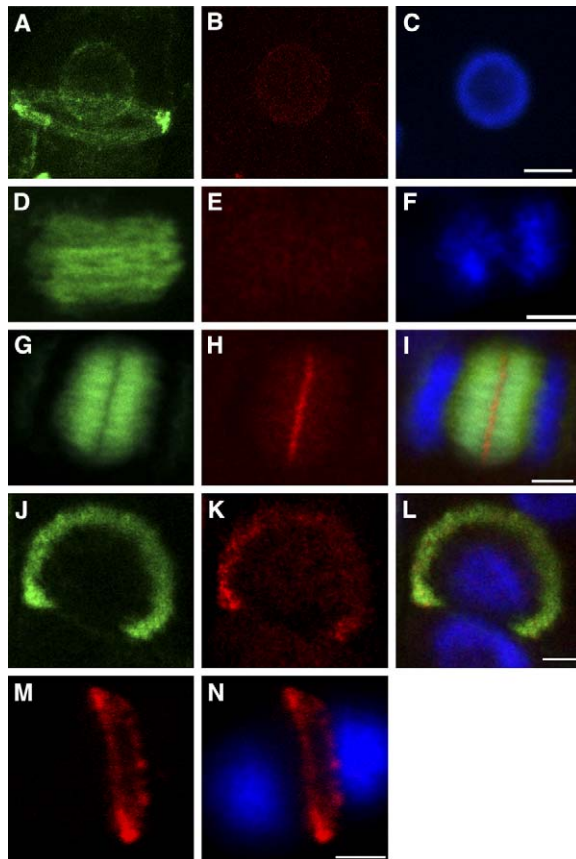
Figure 5. TIO Decorates the Phragmoplast Midline during Cytokinesis

Arabidopsis suspension culture cells were stained for tubulin, TIO, and DNA at various stages of mitosis. Optical sections show anti-tubulin (green), anti-TIO (red), and DAPI (blue) fluorescences that are triple merged in (I) and (L) and double merged in (N). Preprophase band (A–C), late anaphase spindle (D–F), early phragmoplast (G–I), late phragmoplast (J–N). Scale bar, 5 µm.

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Accession Numbers

The GenBank accession numbers of the full-length TIO cDNA sequence (Ler accession) and the correctly annotated TIO genomic sequence (Col-0 accession) are DQ149983 and DQ153170, respectively.