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Condensin and cohesin knockouts in *Arabidopsis* exhibit a *titan* seed phenotype

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Summary

The *titan* (*ttn*) mutants of *Arabidopsis* exhibit striking alterations in chromosome dynamics and cell division during seed development. Endosperm defects include aberrant mitoses and giant polyploid nuclei. Mutant embryos differ in cell size, morphology and viability, depending on the locus involved. Here we demonstrate that three *TTN* genes encode chromosome scaffold proteins of the condensin (SMC2) and cohesin (SMC1 and SMC3) classes. These proteins have been studied extensively in yeast and animal systems, where they modulate chromosome condensation, chromatid separation, and dosage compensation. *Arabidopsis* contains single copies of *SMC1* and *SMC3* cohesins. We used forward genetics to identify duplicate T-DNA insertions in each gene. These mutants (*ttn7* and *ttn8*) have similar *titan* phenotypes: giant endosperm nuclei and arrested embryos with a few small cells. A single *SMC2* knockout (*ttn3*) was identified and confirmed by molecular complementation. The weak embryo phenotype observed in this mutant may result from expression of a related gene (*AtSMC2*) with overlapping functions. Further analysis of *titan* mutants and the *SMC* gene family in *Arabidopsis* should provide clues to chromosome mechanics in plants and insights into the regulation of nuclear activity during endosperm development.

Keywords: *Arabidopsis*, condensin, cohesin, endosperm, SMC, *titan*.

Introduction

The *SMC* gene family encodes chromosome scaffold proteins (condensins and cohesins) that are required for *s*tructural *m*aintenance of *c*hromosomes in yeast (Strunnikov *et al.*, 1993; 1995) and play a central role in chromosome condensation (Koshland and Strunnikov, 1996), sister chromatid cohesion (Michaelis *et al.*, 1997), dosage compensation (Lieb *et al.*, 1998) and recombination repair (Jessberger *et al.*, 1996). The genetics and biochemistry of these proteins have been extensively characterized in organisms ranging from bacteria to humans (Heck, 1997; Hirano, 1999; Holmes and Cozzarelli, 2000; Strunnikov and Jessberger, 1999). SMC proteins are myosin-like chromosomal ATPases that have a common rod-hinge-rod structure with an N-terminal ATP binding domain and a C-terminal DNA binding domain. Rods are composed of 300–400 amino acids that differ in sequence but maintain a conserved coiled-coil secondary structure. SMC proteins form antiparallel dimers with ATP and DNA binding domains at each end (Strunnikov and Jessberger, 1999). Energy provided by ATP hydrolysis enables these protein complexes to play an active role in chromosome folding. Two different classes of heterodimers (SMC2/SMC4 and SMC1/SMC3) are found in eukaryotes. The SMC2/SMC4 complexes (condensins) generally function in chromosome condensation and dosage compensation whereas SMC1/SMC3 complexes (cohesins) are involved in sister chromatid cohesion and chromosome segregation. Little is known about the structure and function of SMC proteins in plants. One family member (MIM) required for efficient homologous recombination and

related in sequence to Rad18 in yeast (Fousteri and Lehmann, 2000) has been described in *Arabidopsis* (Mengiste *et al.*, 1999). Loss of function of this gene results in hypersensitivity to chromosome damage following mutagenesis.

Our interest in SMC proteins began with a screen for mitotic cell division mutants disrupted in seed development in *Arabidopsis*. Three recessive *titan* mutants were identified and the corresponding genes mapped to different chromosomes (Franzmann *et al.*, 1995; Liu and Meinke, 1998). The common feature among these mutants is the presence of giant endosperm nuclei. Mutant embryos contain a few giant cells (*ttn1*), several small cells (*ttn2*), or phenotypically normal cells (*ttn3*). Two contrasting models for *TTN* gene function were proposed: involvement with either cell cycle control or the structural mechanics of mitosis (Liu and Meinke, 1998). Several additional *titan* mutants have subsequently been recovered from a large collection of T-DNA insertion lines generated at Syngenta (McElver *et al.*, 2001). One of these mutants (*ttn5*) resembles *ttn1* in phenotype and encodes a small GTP protein (ARL2) related to ADP ribosylation factors (McElver *et al.*, 2000). *TTN1* encodes a large regulatory protein known as tubulin-folding cofactor D (Tzafrir *et al.*, 2002). This class of proteins interacts with ARL2 in fission yeast (Radcliffe *et al.*, 2000) and humans (Bhamidipati *et al.*, 2000) to regulate microtubule dynamics. One network of *TTN* gene products is therefore required for normal microtubule function during seed development (Tzafrir *et al.*, 2002).

The importance of chromosome scaffold proteins in endosperm development became apparent when *TTN3* was found to encode an SMC2 condensin. The phenotype of *ttn3* is unique among *titan* mutants identified to date. Mutant endosperm contains aberrant mitotic figures, giant nuclei and a large number of chromosomes (Liu and Meinke, 1998). Despite these abnormalities, embryo development proceeds in a normal manner and viable mutant plants are produced. We began our molecular characterization of *titan* mutants and the *SMC* gene family with *ttn3* because it was the first tagged mutant identified. Insertional mutants defective in two other *SMC* genes were subsequently identified through forward genetic screens of tagged embryo-defective mutants (McElver *et al.*, 2001). We present in this report a description of five *titan* mutants disrupted in three different *SMC* genes of *Arabidopsis*. Our results indicate that SMC proteins perform essential functions during embryo and endosperm development. The availability of *Arabidopsis* knockouts in known condensin and cohesin genes should provide further insights into SMC functional diversity in eukaryotes.

Results

Cloning and sequencing of TTN3

Based on hybridization patterns and band intensities with T-DNA probes, we concluded that at least four T-DNA repeats totaling approximately 65 kb were present at the *ttn3* locus. TAIL-PCR (Liu *et al.*, 1995) was then used to clone the flanking sequence. Two bands (650 bp and 400 bp) were produced after tertiary reactions using a T-DNA (TL-1) primer in combination with degenerate (AD-2) and random (OPA-2) primers. These PCR products were cloned, sequenced, and shown to be identical in the overlapping region. Six clones were identified when the 650 bp fragment was used to screen a genomic library. Two overlapping clones totaling 9413 bp were sequenced (GenBank No. AF271730) before completion of the genome project.

An inflorescence cDNA library was screened at high stringency using a 5.5-kb PCR product amplified from one of the genomic clones. Three overlapping cDNAs were recovered. Additional sequence was obtained by RT-PCR. Sequence analysis revealed a 3.7-kb ORF. The genomic fragment contained a 6.2-kb coding region with 19 introns, 1.5 kb putative promoter region and 1.7 kb 3′ non-coding sequence. The T-DNA inserted into the 14th intron and the 650 bp TAIL-PCR product contained the 15th exon and parts of the adjacent introns. The deduced gene structure is shown in Figure 1. This region was later sequenced from adjacent P1 clones (MMI9 and K19B1) as part of the genome initiative. The peptide sequence predicted from that effort is one amino acid shorter than the one derived from our cDNA sequence.

Confirmation of TTN3 isolation

Restriction fragment length polymorphisms between wild-type and mutant plants were identified with several enzymes when the cloned genomic DNA was used as a probe. PCR amplification using primers from the right border and 12th intron yielded a product of expected size

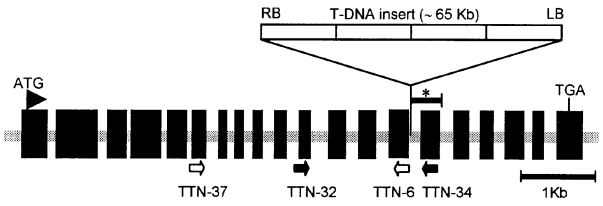

Figure 1. Structure of *TTN3* gene composed of 20 exons (boxes) and 19 introns (lines).
Locations of the T-DNA insert in the mutant allele, right border (RB) and left border (LB) sequences, and the 650 bp TAIL-PCR product (asterisk) are noted. Arrows mark locations and directions of primers used to study gene expression. The T-DNA insert is not drawn to scale.

(500 bp) from mutant but not wild-type samples. This is consistent with our conclusion that flanking plant DNA was not deleted. Molecular complementation with a 7.5-kb genomic fragment confirmed that the cloned region corresponded to *TTN3*. Of 87 independent transformants identified among 6000 *ttn3/ttn3* T_1 plants selected on Basta, 37 were analysed for endosperm phenotype and all but one had the *titan* defect corrected. The exception may have received an inactivated insert. Transformants contained one insert (11 lines), two inserts (10 lines) or multiple inserts (15 lines) based on segregation of Basta-resistant progeny in the T_2 generation. Plants with multiple copies of the transgene were larger and flowered later than expected. Lines with one or two inserts flowered after 25 ± 2 days, whereas those with multiple inserts flowered after 33 ± 3 days. The cellular basis for this difference remains to be determined.

TTN3 encodes an SMC2 condensin

The deduced TTN3 protein has 1177 amino acids and shows high homology to conserved regions of SMC2 condensins. Overall, TTN3 exhibits 44% identity to XCAP-E from *Xenopus* and HCAP-E from human, 42% to Scll from chicken, 35% to Cut14 from *S. pombe*, 34% to Smc2 from *S. cerevisiae* and 28% to MIX1 from *C. elegans*. Homology to other classes of SMC proteins is much lower: 16% to XCAP-C (SMC4-like) and 17% to xSMC1 and xSMC3 from *Xenopus*. The relationship between TTN3 and other SMC proteins is shown in Figures 2 and 3. TTN3 contains all of the conserved SMC motifs: (a) the N-terminal NGSGKSN domain; (b) the central hinge region with four glycines within the GD(5X)G(3X)GG box; (c) the C-terminal DNA-binding domain; and (d) the QG box (AA 147–148) of unknown function. When compared only with SMC2 proteins, TTN3 shows a much higher level of sequence homology. For example, the N-terminal ATP-binding domain in TTN3 has an extended motif [FNAITG(L/Y)*NGSGKSN*ILD(A/S)ICF] that is conserved only in SMC2 proteins. Similarly, the C-terminal DNA binding domain is expanded to: ELSGGQRSL(X)AL(S/A)LI(7X)PAP (X)YILDE(V/I)DAALDLSHT. Secondary structure analysis of TTN3 revealed the presence of another conserved feature of SMC proteins: two long arms (AA 160–500 and AA 660–1030) with a high tendency to form coiled-coil structures (Figure 2b).

Arabidopsis contains two SMC2 genes

Southern blots were used to search for *Arabidopsis* sequences related to *TTN3* before completion of the genome project. Results obtained were consistent with the presence of a *TTN3* homolog. This gene (*AtSMC2*; At3g47460) is located on chromosome 3 (F1P2.10) and encodes a predicted protein of 1171 amino acids that shares 74% sequence identity and 81% similarity with TTN3. The relationship between AtSMC2 and selected SMC proteins from other species is shown in Figure 3. Expression of *AtSMC2* during reproductive development has been confirmed through identification of an EST (GenBank AV566225) obtained from green siliques (Asamizu *et al.*, 2000). Mutants disrupted in this putative condensin gene remain to be identified.

TTN3 expression analysis

TTN3 is expressed at low levels and is the only member of the *SMC* family without an EST sequence in public databases. After Northern blots failed to detect *TTN3* transcripts, quantitative RT-PCR was performed using RNA samples from leaves, stems, flowers and siliques (Figure 4). Two pairs of primers were used: one (TTN-32, TTN-34) located at opposite sides of the T-DNA insert and another (TTN-37, TTN-6) located upstream of the insert. The results after a minimum number of PCR cycles (22) followed by hybridization with a *TTN3* probe showed that in wild-type plants, *TTN3* is expressed at low levels in leaves, intermediate levels in stems and maturing siliques, and higher levels in flowers and young siliques. There was no difference between the amount of product formed with TTN-32/TTN34 and TTN-37/TTN-6 primer pairs. PCR products were sequenced to confirm that *TTN3* transcripts were being detected. *TTN3* is therefore not an endosperm-specific gene even though the mutant phenotype is restricted to the developing endosperm. When primers upstream of the insertion (TTN-37/TTN-6) were used with tissue from homozygous mutant plants, results similar to those obtained with wild-type plants were observed and a strong signal was detected in flowers and young siliques. This signal disappeared when primers (TTN-32/TTN-34) flanking the insertion were used. However, a very weak signal similar to that seen in wild-type leaves was detected in all RNA samples from mutant plants after 30 PCR cycles (data not shown). This result was reproducible but unexpected because the large T-DNA insert should have prevented recovery of such a product. We propose that a peculiar insert structure in *ttn3* facilitates T-DNA removal from at least some of the processed transcripts.

Cytoskeleton of mutant endosperm

Immunocytochemistry and fluorescence microscopy were used to study cytoskeletal organization in mutant and wild-type seeds (Figure 5). Immunolabeling with anti-α-tubulin and counter-staining with DAPI demonstrated that the radial arrays of microtubules around interphase nuclei and the formation of spindles and phragmoplasts during mitosis are not visibly affected in mutant endosperm.

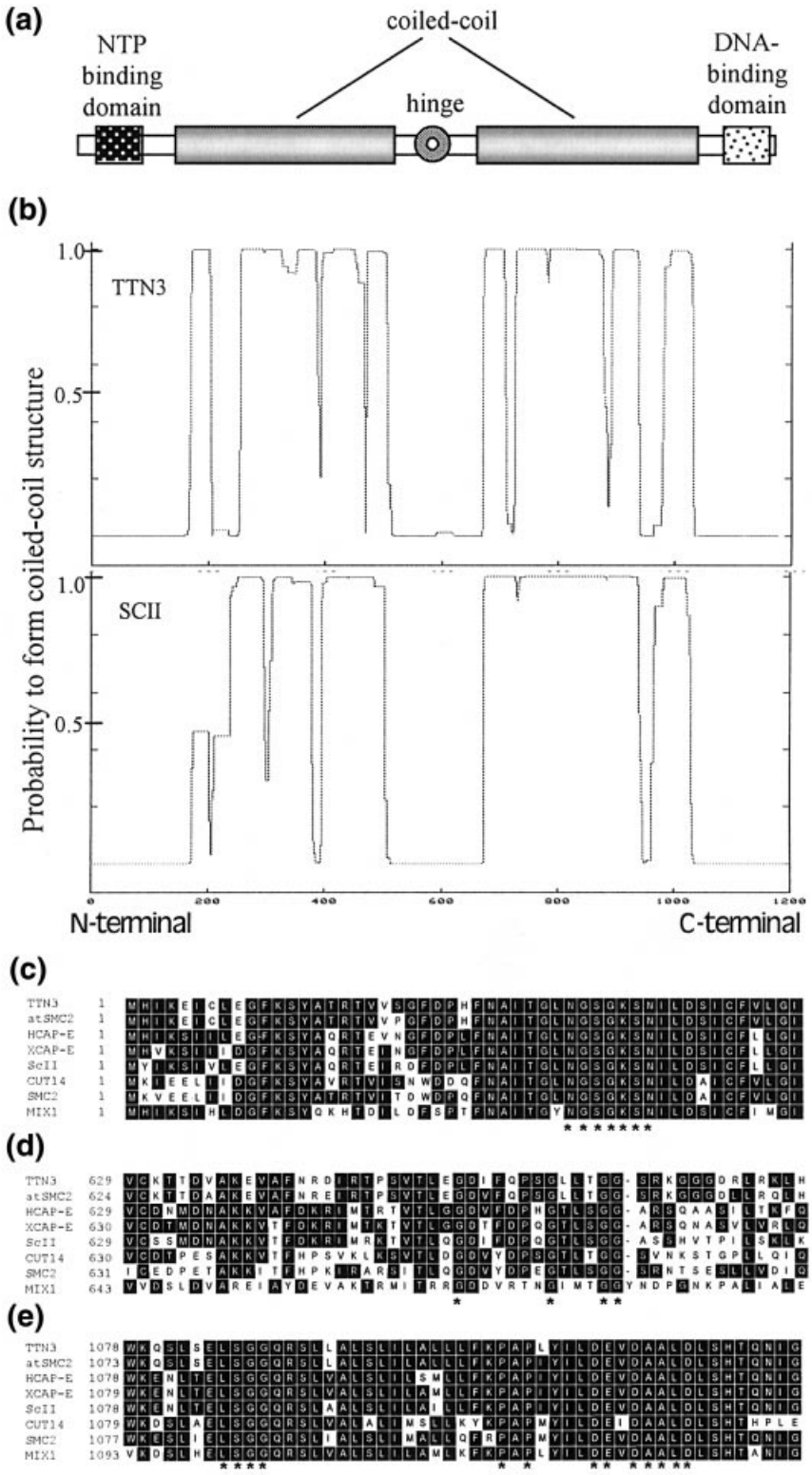

Figure 2. Conserved structure of SMC2 proteins.
(a) Common domains found in all SMC proteins.
(b) Likelihood of forming coiled-coil structures in TTN3 and chick SCII proteins. The horizontal axis is co-linear with (a) above.
(c–e) Amino acid sequence comparison of TTN3 with other SMC2 condensins at the N-terminal (c), central hinge (d), and C-terminal (e) regions. Asterisks mark the locations of amino acids conserved in all SMC proteins. Accession numbers are listed in Figure 3.

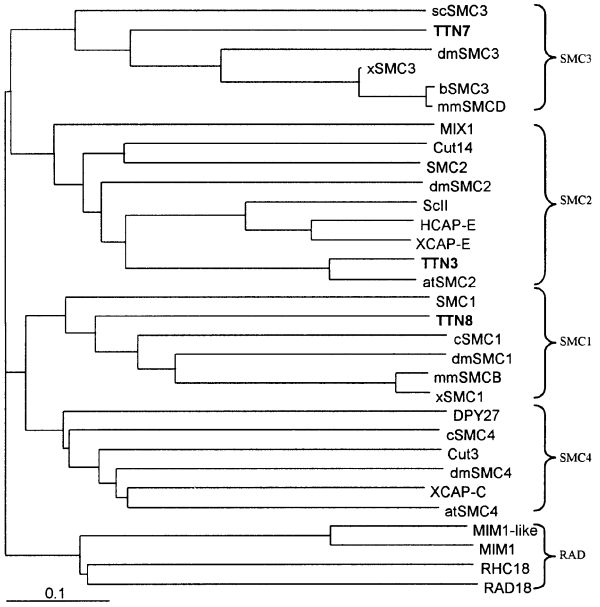

Figure 3. Predicted evolutionary relationships among a representative sample of SMC proteins.
Tree constructed using the ClustalX program (http://www-igbmc.u-strasb.fr/BioInfo/) with complete protein sequences. Accession numbers and source organisms: scSMC3 (*S. cereviseae*): Y14278; TTN7 (*A. thaliana*): AC007290; dmSMC3 (*D. melanogaster*): U30492; xSMC3 (*X. laevis*): AF051785; bSMC3 (*B. taurus*): AF072713; mmSMCD (*M. musculus*): AF047601; MIX1 (*C. elegans*): U96387; Cut14 (*S. pombe*): D30787; SMC2 (*S. cerevisiae*): U05280; dmSMC2: AF179287; ScII (chicken): X80792; HCAP-E (human): AF092563; XCAP-E (*X. laevis*): U13674; TTN3: AF271731; atSMC2: AL132955; SMC1 (*S. cerevisiae*): L00602; TTN8: AL138650; cSMC1: AF039716; dmSMC1: AF225909; mmSMCB: AF047600; xSMC1: AF051784; DPY27 (*C. elegans*): P48996; cSMC4: Q20060; Cut3 (*S. pombe*): D30788; dmSMC4: AF185287; XCAP-C (*X. laevis*): U13673; atSMC4: AB015468; MIM1-like (*A. thaliana*): AB010070; MIM1: AF120933; RHC18 (*S. cerevisiae*): Q12749; RAD18 (*S. cerevisiae*): P53692. Scale bar = 0.1 amino acid substitutions per residue.

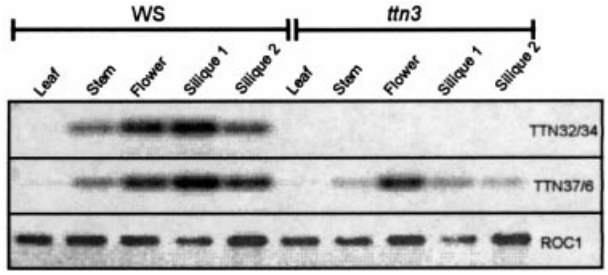

Figure 4. Expression of *TTN3* shown by quantitative RT—PCR and hybridization with *TTN3* cDNA.
Total RNA isolated from wild-type (WS) and homozygous mutant (*ttn3*) plants was reverse transcribed and amplified using the primer pairs shown in Figure 1. One pair (TTN-37/TTN-6) is located upstream of the insertion site; the other (TTN-32/TTN-34) spans the insertion site. ROC1 was used as a positive control without hybridization. Immature siliques were harvested: (1) up to the torpedo stage of seed development, when endosperm tissue is abundant; and (2) at the cotyledon stage of seed development, when little endosperm remains.

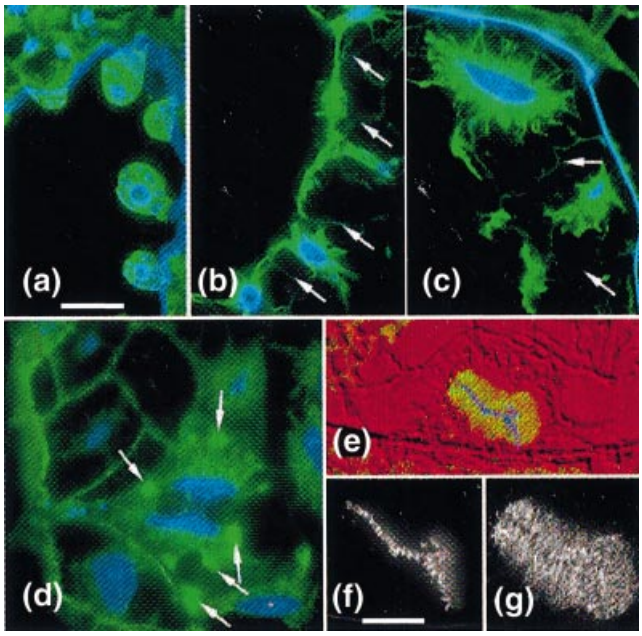

Figure 5. Wild-type (a,b) and mutant (c–g) endosperm stained with DAPI (blue) and immunolabeled with anti-α-tubulin (green).
(a) Nucleate phase of endosperm development with microtubules radiating from nuclei around periphery of embryo sac.
(b) Alveolar phase with characteristic microtubule configurations. Arrows mark early stages of cellularization.
(c) Microtubules radiating from mutant endosperm nuclei at the onset of cellularization (arrows).
(d) Endosperm cell at anaphase with remnants of multiple phragmoplasts (arrows).
(e) Large mitotic spindle in the developing endosperm.
(f–g) Image from (e) restricted to DAPI (f) and anti-α-tubulin (g) fluorescence. Scale bars = 20 μm (a–e) and 15 μm (f,g).

However, there is a change in overall configuration of the microtubule cytoskeleton because of the disturbance of nuclear division and the altered shapes and positions of endosperm nuclei. These irregular divisions prevent the formation of a well-organized coenocyte characteristic of wild-type seeds (Brown *et al.*, 1999). Cellularization of mutant endosperm occurs with an aberrant alveolar phase, resulting in large cells with irregular shapes. Some multinucleate cells have remnants of multiple phragmoplasts or folded mitotic plates (Figure 5d). We conclude that *ttn3* does not disrupt endosperm mitosis through changes in microtubule organization and that altered nuclear features are responsible for abnormalities observed throughout endosperm development.

SMC1 and SMC3 knockouts

The sequenced *Arabidopsis* genome contains a single *SMC1* gene (At3g54670; T5N23.30) located on chromosome 3 and a single *SMC3* gene (At2g27170; F20F1.4) on chromosome 2. Tagged mutants disrupted in these cohesin genes were identified through forward genetics as part of a large-scale screen of embryo-defective

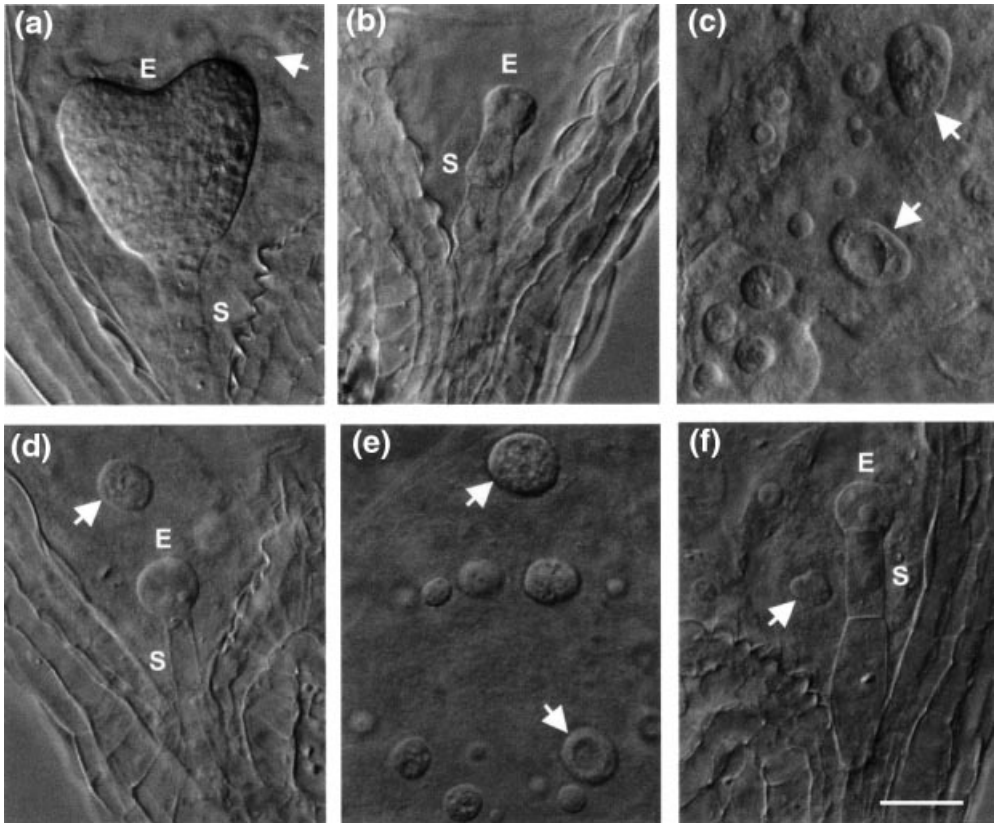

Figure 6. Seed phenotypes of cohesin knockouts.
(a) Wild-type seed at the heart stage of development.
(b–f) Mutant seeds from *ttn7–1* (b), *ttn7–2* (c), *ttn8–1* (d,e), and *ttn8–2* (f) heterozygotes. The apical embryo cell (E), suspensor (S), and endosperm nucleoli (arrowheads) are visible. Scale bar = 30 μm.

mutants (McElver *et al.*, 2001). The *SMC1* knockouts (*ttn8–1, ttn8–2*) and *SMC3* knockouts (*ttn7–1, ttn7–2*) all exhibit a strong *titan* phenotype similar to that of *ttn2* (Liu and Meinke, 1998) and *ttn9* (Tzafrir *et al.*, 2002) with significant defects in both embryo and endosperm development. Examples of seed phenotypes are shown in Figure 6. The mutant embryo becomes arrested very early in development, without cell or nuclear enlargement, and is usually composed of fewer than five cells. The consistency of this embryo phenotype in cleared seeds examined with Nomarski optics is shown in Table 1. Cellularization of the endosperm is blocked but differentiation between the early embryo proper and suspensor can be detected. Enlargement of endosperm nuclei and nucleoli resembles that found in other *titan* mutants. The presence of some seeds with a weak endosperm phenotype (Table 1) is a characteristic of *titan* mutants that remains to be explained (Tzafrir *et al.*, 2002).

The tagging status of each insertion line was resolved by screening Basta-resistant transplants for the presence of the seed mutation (McElver *et al.*, 2001). No wild-type plants were found among 178 *ttn7–1,* 142 *ttn7–2,* 170 *ttn8–1, and* 127 *ttn8–2* resistant transplants, consistent with the absence of recombination between the T-DNA insert and mutant gene. TAIL-PCR was then used to isolate plant DNA sequences flanking the insertion sites (McElver *et al.*, 2001). Sequence information was obtained from both sides of each insert. No evidence was found of chromosomal aberrations associated with the insertion sites. Insert locations and predicted cohesin gene structures are shown in Figure 7. The isolation of duplicate mutant alleles with similar phenotypes and independent insertions at different points in the same locus indicates that the *TTN7* and *TTN8* genes have been identified. An EST for *TTN7* (AV567365) has been recovered from green siliques (Asamizu *et al.*, 2000) and several ESTs for *TTN8* (AV548094, AV554219, AA650868, T41869, AA720322) have been identified from a variety of plant tissues (Asamizu *et al.*, 2000; Newman *et al.*, 1994). These results are consistent with widespread expression of these essential genes throughout the plant life cycle.

Knockouts in three different *SMC* genes (*SMC1*, *SMC2*, and *SMC3*) have therefore been found to give a *titan* phenotype. Mutations in the single *Arabidopsis SMC4*

Table 1. Phenotypic variation observed in mutant seeds[a]

	Number of seeds observed with specified mutant phenotype					
	Endosperm *titan* phenotype[b]			Arrested embryo phenotype		
Mutant	Strong	Moderate	Weak	Preglobular	Globular	N.D.[c]
ttn7–1	84	1	15	61	0	39
ttn7–2	70	21	9	61	0	39
ttn8–1	82	7	11	37	0	63
ttn8–2	80	9	5	55	0	45

[a]100 cleared seeds from each mutant were examined with Nomarski optics.
[b]Seeds with giant endosperm nucleoli were classified as strong, those with nucleoli of intermediate sizes were called moderate, and those with smaller nucleoli were considered weak.
[c]Not detected because the embryo was too small.

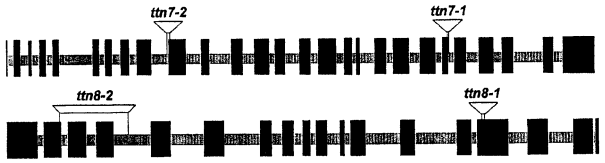

Figure 7. T-DNA insertion sites for knockouts of *TTN7* and *TTN8*.
Locations of exons (boxes) and introns (lines) are based upon current genome annotation (*Arabidopsis* Genome Initiative, 2000). *TTN7* is about 7.8 kb in length with 27 predicted exons; *TTN8* is about 7.9 kb in length with 18 predicted exons. Each insertion disrupts at least one exon. Distances between vertical lines connecting each insertion to a gene location reflect deletions associated with T-DNA integration. Deletion sizes are 9 bp (*ttn7–1*), 22 bp (*ttn7–2*), 24 bp (*ttn8–1*) *and* 923 bp (*ttn8–2*).

gene (At5g48600; K15N18.7) remain to be recovered although an EST (AV532932) from floral tissues (Asamizu *et al.*, 2000) has been identified. Disruption of another *SMC*-like gene (*MIM*) related to yeast *RAD18* alters the efficiency of homologous recombination (Mengiste *et al.*, 1999). All major classes of *SMC* genes have therefore been identified in *Arabidopsis*. Four of these have interesting knockout phenotypes consistent with important functions in growth and development.

Discussion

Seed development has been subjected to extensive genetic analysis in *Arabidopsis* (Goldberg *et al.*, 1994; Laux and Jurgens, 1997; Meinke, 1995). Large numbers of genes must be expressed as the zygote divides in a regulated manner to form a multicellular embryo. Disruption of any non-redundant gene with an essential function results in an embryo-defective phenotype (Meinke, 1994). The frequency of *emb* mutants recovered following mutagenesis is consistent with the presence of 500–750 *EMB* genes in *Arabidopsis* (Franzmann *et al.*, 1995; McElver *et al.*, 2001). Protein functions range from general metabolism (Patton *et al.*, 1998) and cell biology (Lukowitz *et al.*, 1996; Rojo *et al.*, 2001) to chloroplast maintenance (Apuya *et al.*, 2001; Uwer *et al.*, 1998) and the regulation of transcription (Hardtke and Berleth, 1998; Lotan *et al.*, 1998; Luerssen *et al.*, 1998). The triploid endosperm tissue in *Arabidopsis* has also been subjected to genetic analysis (Berger, 1999). Particular attention has been given to mutants with inappropriate endosperm development in the absence of fertilization (Grossniklaus *et al.*, 1998; Luo *et al.*, 1999; Ohad *et al.*, 1999; Sorensen *et al.*, 2001; Yadegari *et al.*, 2000). These studies have revealed the importance of polycomb proteins in regulating early endosperm formation in *Arabidopsis*. Detailed cytological studies (Brown *et al.*, 1999; Olsen, 2001) have provided additional insights into patterns of nuclear division (Boisnard-Lorig *et al.*, 2001) and cellularization (Otegui and Staehelin, 2000) during endosperm development.

The *titan* mutants exhibit some of the most striking defects in embryo and endosperm development observed to date. The common feature that defines these mutants is dramatic enlargement of endosperm nuclei, some of which may exceed the size of a normal globular embryo (Liu and Meinke, 1998). Mutant embryos differ in cell size, morphology, and viability, depending on the locus involved and strength of the mutant allele. Related mutants with similar phenotypes have also been described by Mayer *et al.* (1999). At least nine different genes have been identified that give a *titan* phenotype when disrupted (Tzafrir *et al.*, 2002). Novel features of *ttn3* include embryo viability, chromosome condensation, endosperm cellularization, normal microtubule arrays, and nucleolar fragmentation late in development. Based on mutant phenotypes and double mutant analysis, we originally proposed that *TTN* genes played a direct role in either cell cycle control or the structural mechanics of mitosis (Liu and Meinke, 1998). The cloning of three *TTN* genes reported here provides evidence in support of the second

model. We have identified all four classes of *SMC* genes in *Arabidopsis* through database searches and found that mutations in *SMC1* (*ttn8*), *SMC2* (*ttn3*), and *SMC3* (*ttn7*) result in a *titan* phenotype. These knockouts should facilitate a comprehensive genetic dissection of condensin and cohesin function in plant growth and development.

The importance of cohesins in seed development is underscored by the embryonic lethality and endosperm defects observed in *SMC1* and *SMC3* knockouts. The similarity of *ttn7* (*SMC3*) and *ttn8* (*SMC1*) seed phenotypes is consistent with known physical interactions between cohesins in other systems (Hirano, 2000; Jones and Sgouros, 2001). The distinctive cellular phenotypes of knockouts in yeast and *Arabidopsis* may result from differences in cell-cycle regulation. The apparent absence of cohesin gene redundancy in *Arabidopsis* allows the null phenotype to be observed. The presence of functional gametophytes, small embryos, and polyploid endosperm nuclei in mutant seeds may reflect the diffusion of trace amounts of functional protein from surrounding heterozygous tissues. Different cell-cycle checkpoints in the *Arabidopsis* embryo and endosperm may explain why disruption of cohesin function results in cell abortion in the mutant embryo while DNA replication and nuclear enlargement continue in the endosperm.

Two unrelated cohesins identified in *Arabidopsis* (Bai *et al.*, 1999; Bhatt *et al.*, 1999; Kleinow *et al.*, 2000) are not functional homologs of the proteins described here. The *SYN1* gene (Bai *et al.*, 1999), also known as *DIF1* (Bhatt *et al.*, 1999) and named for meiotic defects observed in knockout mutants, encodes a non-SMC cohesin related to Rad21 of *S. pombe* and Scc1 of *S. cerevisiae* (Jones and Sgouros, 2001). The other gene (*MSS2*), which is not associated with a known mutant phenotype, was identified in a screen for heterologous suppressors of *snf4* deficiency in yeast (Kleinow *et al.*, 2000) and encodes a protein with limited sequence homology to SMC1. The cohesins described in this report appear to be central components of a conserved protein complex required for normal chromosome function.

We also demonstrate here that condensins are required for normal seed development in *Arabidopsis*. The mutant phenotype in this case is more difficult to interpret because of the presence of a related gene (*AtSMC2*) with potentially overlapping functions and the unexpected discovery of trace amounts of processed *TTN3* transcript in mutant plants. Removal of the large T-DNA insert from some *ttn3* transcripts during RNA processing could nevertheless provide an interesting system for studying splicing mechanisms in plants. Identifying additional *ttn3* alleles through forward genetic screens may present a challenge if they also exhibit a subtle embryo phenotype. Reverse genetics may therefore be required to confirm the null phenotype of *AtSMC2* knockouts. Double mutants between *TTN3* and *AtSMC2* knockouts should result in embryo lethality if condensins indeed perform an essential function.

The appearance of condensed chromosomes and mitotic figures in *ttn3* endosperm is atypical for *titan* mutants and surprising given that condensins are required for chromosome condensation in other organisms (Hirano, 1999; Koshland and Strunnikov, 1996). We propose that TTN3 is part of an SMC complex that plays a more specialized function in *Arabidopsis*. This model is consistent with the specialized functions of MIX1 (SMC2) and DPY-27 (SMC4) in dosage compensation in nematodes (Chuang *et al.*, 1994; Lieb *et al.*, 1998). According to this model, AtSMC2 serves a more direct role in chromosome condensation. Whether AtSMC4 interacts with TTN3, AtSMC2, or both remains to be determined. The discovery that condensins localize to rDNA regions at the G2/M phase of the cell cycle in yeast and that segregation of rDNA is disrupted in the corresponding *smc2* mutants (Freeman *et al.*, 2000) suggests that nucleolar enlargement characteristic of *titans* may result from defects in condensin function.

We propose that several different perturbations can disrupt chromosome dynamics and result in polyploidy and nuclear enlargement during endosperm development in *titan* mutants (Tzafrir *et al.*, 2002). SMC proteins may be the final targets for many of these perturbations by mediating the relationship between chromosome dynamics, nuclear morphology, and cell cycle progression. This raises the interesting possibility that SMC proteins also modulate some of the novel features of free nuclear division characteristic of coenocytic endosperm development in wild-type seeds. The molecular isolation of *TTN2* and *TTN9*, which exhibit mutant phenotypes similar to those of cohesin knockouts, may provide further insights into factors that interact with SMC proteins in plants and function in part to regulate endosperm development.

Experimental procedures

Molecular cloning of TTN3

The *ttn3* mutant was isolated from a T-DNA population (Feldmann, 1991) in the Wassilewskija (WS) ecotype and maintained as described (Liu and Meinke, 1998). Genetic analysis indicated that the mutation was caused by T-DNA insertion at a single locus (Liu and Meinke, 1998). To analyze T-DNA structure at the insertion site, genomic DNA from *ttn3/ttn3* plants was digested with *Eco*RI, *Hin*dIII and *Sal*I, separated by gel electrophoresis, and probed with T-DNA left border (LB), right border (RB) and internal pBR322 DNA (Castle *et al.*, 1993). TAIL-PCR was used to clone genomic DNA flanking the insert (Liu *et al.*, 1995). Three primers (TL-1: 5′ CAGCCAATTTTAGACAAGTATCA 3′; TL-2: 5′ AACTGTAATGACTCCGCGCAATA 3′; TL-3: 5′ TCTGGGAATGGCGTAACAAAGGC 3′) were designed based on the LB sequence. TL-1 is closest to the border and TL-3 is most distant. A

degenerate primer [AD-2: 5′ NGTCGA(G/C)(A/T)GANA(A/T)GAA 3′] and 10-mer random primer (OPA-2: 5′ TGCCGAGCTG; Operon Technology, Alameda, CA, USA) were selected for TAIL-PCR. A WS λZAPII genomic library (Syngenta, Research Triangle Park, NC, USA) was screened using the cloned flanking sequence as a probe. Two of the five clones isolated were subjected to fine mapping (FLASH, Stratagene, La Jolla, CA, USA) and sequenced. RFLP analysis was performed using a 5.5-kb PCR-amplified genomic DNA as a probe and hybridizing with genomic DNA from wild-type and mutant plants to determine whether the cloned DNA was linked to the insert. Three λZAP cDNA clones (3.0 kb, 2.2 kb, and 0.7 kb) isolated from a L*er* inflorescence library (CD4–6, ABRC) were sequenced and compiled to produce a partial cDNA sequence. RT-PCR was then used to clone the missing 5′ end using a primer 46 bp upstream of the ATG. Several independent PCR products were cloned and sequenced to determine the full-length cDNA (3790 bp) and coding region (3531 bp). *TTN3* copy number was examined by digesting genomic DNA with *Eco*R1 and *Bam*H1, hybridizing at 50°C with a *TTN3* fragment lacking internal restriction sites for these enzymes, and then washing at medium stringency (2xSSC/0.1%SDS, 50°C). After autoradiography, the blot was washed again at high stringency (0.1×SSC/0.1%SDS, 65°C) before a second exposure to film. The same blot was then stripped, hybridized with a PCR-amplified *AtSMC2* genomic fragment, and washed at high stringency.

Cytology of mutant seeds

Seeds prepared for visualization of microtubules were cryo-fixed, freeze-substituted, and embedded in butyl methyl methacrylate (Wittich *et al.*, 1999). Sections 2–3 μm in thickness were washed in acetone, blocked, and labeled with monoclonal anti-α-tubulin (1 : 100, Sigma) and goat antimouse antibody (GaM-IgG) conjugated to Alexa 488 (1 : 200, Molecular Probes, Leiden, The Netherlands). Sections were stained with 5 μg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI) to visualize DNA and analyzed with a Nikon Microphot epifluorescence microscope with excitation filters at 365 nm (DAPI) and 488 nm (Alexa). Images were recorded with a cooled HCCD camera (Quantix, Photometrics, Tucson, AZ, USA).

Expression analysis

Total RNA was isolated (Wan and Wilkins, 1994) from wild-type and homozygous mutant leaves, stems, flowers, and siliques. PCR primers located upstream (TTN-37: 5′ CCTCTGAACA-AAATCCAATCTT 3′ and TTN-6: 5′ GCATGTATCAACTTGAGC-TCGGC 3′) and flanking the insertion (TTN-32: 5′ TGTTTATGCACAGTTGGAGCTGA 3′ and TTN-34: 5′ CCTGATT-GATCTGATTGGAGC 3′) were designed for quantitative RT-PCR detection of *TTN3* expression. PCR products with minimal thermal cycles were separated by gel electrophoresis, transferred to nylon membranes, and hybridized with a 2.9-kb *TTN3* partial cDNA probe. Rotamase cyclophilin (ROC1; primers: 5′ CGGGAAGGATCGTGATTGA 3′ and 5′ CCAACCTTCTCG-ATGGCCT 3′) was used as a control for ubiquitous gene expression (Lippuner *et al.*, 1994).

Molecular complementation

A 7.5-kb (*Sal*I-*Bam*H1) genomic fragment containing the entire ORF and a 1.4-kb promoter sequence was cloned into a binary vector (pCSA104) obtained from Syngenta (McElver *et al.*, 2001). The vacuum infiltration method (Bechtold and Pelletier, 1998) was used to deliver the *TTN3* gene to *ttn3/ttn3* plants. Transformants were identified by plating progeny seeds on 50 μM Basta (glufosinate ammonium; Crescent Chemical Co., Hauppauge, NY, USA). Seeds from transgenic plants were analyzed with Nomarski optics (Liu and Meinke, 1998).

Identification of cohesin knockouts

More than 120 000 T-DNA insertion lines in the Columbia ecotype were generated at Syngenta and screened for seed mutations (McElver *et al.* 2001). Linkage between the T-DNA insert and mutant phenotype was demonstrated by transplanting resistant seedlings from selection plates to soil and scoring resulting plants for the seed mutation. Plant sequences flanking insertion sites in tagged mutants were obtained through TAIL-PCR and subjected to detailed analysis (McElver *et al.*, 2001). Mutant phenotypes were determined by examining cleared seeds with Nomarski optics (Liu and Meinke, 1998; Meinke, 1994). Images were captured with a Nikon DXM1200 digital imaging system and E600 compound light microscope.

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