Arabidopsis CHROMOSOME TRANSMISSION FIDELITY 7 (AtCTF7/ECO1) is required for DNA repair, mitosis and meiosis.

指導教授: 趙光裕 (Dr. Guang-Yuh Jauh)
研究生: 何伯樂 (Pablo Alberto Bolaños Villegas)
博士位生論文

題目: 阿拉伯芥基因CTF7/ECO1修復DNA有絲分裂和減數分裂的作用

姓名: 何伯樂 (Pablo Alberto Bolaños-Villegas) 學號: 8096041115

經口試通過特此證明

論文指導教授 (Advisor):

趙光裕博士 (Dr. Guang-Yuh Jauh)

論文考委員 (Members):

羅誌升博士 (Dr. Wan-Sheng Lo)

王中茹博士 (Dr. Chung-Ju Wang)

易玲輝博士 (Dr. Ling-Huei Yih)

張松彬博士 (Dr. Song-Bin Chang)

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中 文 摘 要

細胞分裂時如何將遺傳訊息—DNA妥切及完整地傳遞給後代子細胞，對於真核生物的存活及繁衍十分重要。當細胞分裂時，被複製的染色分體（姊妹染色體）需要緊緊相連在一起，之後此染色分體會被忠實地分離，直到細胞分裂後期。已知在酵母菌中，姊妹染色體的凝聚發生在S期且需要Establishment of cohesion 1 (Eco1) 蛋白質的參與。Eco1失去正常的功能，會導致正在分裂中細胞內的同源染色體分離失敗，與使得以同源互換為主的DNA 修復失去功能，進而導致細胞凋亡。 之前研究室中利用生物資訊學的方式從阿拉伯芥T-DNA插入突變株資料庫中獲得許多無法產生具同源後代（non-homozygous progeny）的突變株，本篇研究中，我們針對其中一個類似Eco1 的基因－CHROMOSOME TRANSMISSION FIDELITY 7/ESTABLISHMENT OF COHESION (CTF7/ECO1) 上的兩株阿拉伯芥T-DNA插入突變株，我們將之命名為ctf7-1和ctf7-2，並分別利
用細胞學、遺傳學及分子生物學針對該基因的分子功能及作用機制進行一系列研究。外表型觀察顯示ctf7-1和ctf7-2突變株呈現植株極度矮小、 花藥發育不良及不孕的現象。利用顯微鏡觀察發現突變株花藥內的花粉發育缺失是導致無法產生同源後代的主因。藉由流式細胞儀分析突變株的體細胞後發現營養細胞內多倍套數DNA含量增加；掃描式電子顯微鏡觀察亦發現葉片表皮細胞的表面積顯著增加。進一步利用QPCR分析與細胞分裂及DNA修復相關的基因表現，發現一些與DNA修復相關的基因（例如BRCA1和PARP2）在突變株的營養細胞內表現量顯著增加，但一些與進入endocycle相關基因的表現量則無明顯變化。進一步用QPCR偵測突變株營養組織中的基因表現，發現跳躍基因COPIA28和soloLTR的表現量增加，推測CTF7/ECO1可能參與基因靜默的調控。綜合以上現象推斷，ctf7-1和ctf7-2突變株的 養細胞失去DNA修復及細胞分裂的能力。 對於突變株中花粉母細胞的減數分裂 分析發現 ，整個染色體的結構發生改變且染色體分離 在細胞分裂過程中發生嚴重缺陷；除此之外，染色體中心粒凝聚的現象和凝聚於中心粒的染色體接合次單元數皆有減少之趨勢。利用QPCR分析突變株花粉內參與減數分裂相關基因，發現DMC1、RAD51C以及S-phase licensing factor-CDC45基因的表現量皆顯著增加。 總而言之，本實驗結果證明了阿拉伯芥 CTF7/ECO1對於維持染色體的完整性及在剪敵分裂上扮演很重要的角色。
Abstract

Proper transmission of DNA in dividing cells is crucial for the survival of eukaryotic organisms. During cell division, faithful segregation of replicated chromosomes requires their tight attachment, known as sister chromatid cohesion, until anaphase. Sister chromatid cohesion is established during S-phase in a process that requires an acetyltransferase that in yeast is known as Establishment of cohesion 1 (Eco1). Inactivation of Eco1 typically disrupts chromosome segregation and homologous recombination dependent DNA repair in dividing cells ultimately resulting in lethality. We report here the isolation and detailed characterization of two homozygous T-DNA insertion mutants for the Arabidopsis thaliana Eco1 homologue, CHROMOSOME TRANSMISSION FIDELITY 7/ESTABLISHMENT OF COHESION 1 (CTF7/ECO1), called ctf7-1 and ctf7-2. Mutants exhibited dwarfism, poor anther development and sterility. Analysis of somatic tissues by flow cytometry, scanning electron microscopy and QPCR identified defects in DNA repair and cell division, including an increase in the area of leaf epidermal cells, an increase in DNA content, and the upregulation of genes involved in DNA repair including BRCA1 and PARP2. No significant change was observed in the expression of genes that influence entry into the endocycle. Analysis of meiocytes identified changes in chromosome morphology and defective segregation; reduced cohesion at centromeres and a reduction in the abundance of chromosomal-bound cohesion subunits. Transcript levels for several meiotic genes, including the recombinase genes, DMC1 and RAD51C, and the S-phase licensing factor, CDC45 were elevated in mutant anthers. Moreover QPCR in vegetative tissues indicated upregulation of transposable elements COPIA28 and soloLTR, which suggest an additional role for CTF7/ECO1 in gene silencing. Taken together our results demonstrate that Arabidopsis CTF7/ECO1 plays important roles in preservation of genome integrity and meiosis.
Abstract (in Spanish)

La sobrevivencia en organismos eucarióticos depende en gran manera de una adecuada repartición de ADN a la hora en que una célula se divide. Asimismo durante la división celular cada par de cromátidas debe estar adherida a la otra para segregar correctamente durante anafase. A este tipo de adhesión le se conoce como cohesión cromosomal o de cromátidas. La cohesión de cromátidas se establece durante la síntesis de ADN gracias a la acción de una acetil transferasa conocida en el hongo *Saccharomyces cerevisiae* (levadura) como Establecimiento de cohesión (Eco1). La inactivación de esta enzima en células que se dividen causa defectos durante la segregación de cromosomas y durante la reparación de ADN, lo cual conlleva a la muerte celular.

En este estudio se reporta la caracterización de dos mutantes creados a partir de transferencia de ADN extracromosómico (ADN-T) en la planta modelo *Arabidopsis thaliana*. Los alelos corresponden a insertos de ADN-T en el gen homólogo de Eco1 en *Arabidopsis*, mejor conocido por su nombre en inglés como *CHROMOSOME TRANSMISSION FIDELITY 7/ESTABLISHMENT OF COHESION 1 (CTF7/ECO1)*. A estos alelos se les llamó *ctf7-1* y *ctf7-2*. La presencia de ambos alelos en condición homocigota causa enanismo, pobre desarrollo de anteras, esterilidad, y defectos en la segregación de cromosomas en la raíz. Análisis de hojas jóvenes por medio de citometría de flujos, microscopía electrónica de barrido y PCR cuantitativo permitió identificar defectos en la reparación de ADN y en la división celular evidenciados por un aumento en el área de células epidermales, un mayor contenido de ADN en las mismas, y un incremento significativo en la expresión de genes claves en la reparación de ADN como lo son *BRCA1* y *PARP2*. Empero, no se observó cambios en la expresión de genes que regulan la entrada al endociclo.

Por otra parte análisis de células meióticas de polen permitió identificar defectos en la morfología y segregación de cromosomas, pérdida de cohesión en los centrómeros, y una reducción en la cantidad de proteínas cromosomales que regulan la cohesión. En anteras también se detectó un aumento en la expresión de varios genes meióticos como lo son las recombinasas *DMC1* y *RAD51C*, y el factor de síntesis de ADN *CDC45*. Por último en plántulas se detectó un incremento en la expresión de los elementos genéticos transponibles *COPIA28* y *soloLTR*, lo cual sugiere que *CTF7/ECO1* juega un papel en el silenciamiento de estos elementos. En conjunto los resultados de esta investigación llevan a concluir...
que el gen \textit{CTF7/ECO1} es importante para resguardar la integridad del genoma, para la realización de la mitosis y de la meiosis, y puede haber desarrollado nuevas funciones relacionadas con el control de elementos genéticos transponibles.
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Introduction: establishment of chromosome cohesion in *Arabidopsis thaliana*

Precise cell division with transmission of genetic information is a key process controlling growth and development in all eukaryotic organisms (Peters and Bhaskara, 2009). Chromosomes need to be properly replicated and condensed then attached to the spindle fibers in order to be distributed evenly among daughter cells (Díaz-Martínez and Clarke, 2009). The cohesin complex is critically important for these processes. Compliance to this program ensures timely growth and development of unicellular organisms such as yeast, and proper formation of tissues and organs in multicellular organisms such as animals and plants (Skibbens, 2010; Wu et al., 2010).

Proteins from the STRUCTURAL MAINTENANCE OF CHROMOSOMES (SMC) family and associated non-SMC factors, are essential for the regulation of higher order chromosomal structure in eukaryotes (Schubert, 2009). SMC complexes are mostly composed of canonical SMC proteins, about 1000 amino acids in length, and are loaded onto chromosomes during telophase and G1 before DNA replication (Wu and Yu, 2012). Each complex involves 1) a heterodimer in which both proteins interact through a hinge domain, which opens to allow loading, 2) a 50 nm long coiled-coil domain, and 3) a globular ATPase head composed of lobe I, containing Walker A and B motifs to bind ATP, and lobe II, which contains an ABC motif that binds Yphosphates of ATP (Nasmyth, 2011; Chan et al., 2012). Transient interaction of both lobes across both proteins allows binding and hydrolysis of ATP, which is essential for the interaction of the complex with DNA (Nasmyth, 2011). A fourth component are the kleisin subunits that connect the two heads to form a ring that topologically embraces nascent chromatid fibers (Peters et al., 2008; Watanabe, 2012). This topological entrapment allows each chromatid to be used as a template for homology-dependent DNA repair during DNA synthesis at the S-phase (Murakami et al., 2010), and binds sister chromatids to each other for proper spindle orientation and segregation during the G2/M phase (Beckouet et al., 2010, Appendix 1). Chromosome cohesion involves cohesin complexes that include SMC3, SMC1, SCC3 and one of several different kleisins (Schubert, 2009). Cohesins are also important for the repair of double strand breaks in mitotic DNA caused by exposure to radiation or chemical agents post-replication, a task performed by cohesin complexes that include SMC5, SMC6A/B and δ-kleisins NSE4A/B (Watanabe et al., 2009; Callegari et al., 2010; Kim et al., 2010a)(Nasmyth, 2011) Cohesins are also required for assembly for replication complexes during the S-phase, for assembly of the axes of synaptonemal complexes during the prophase stage of meiosis, for
orientation of kinetochores during meiosis I (Nasmyth, 2011), for suppression of recombination within rDNA sequences (Wu and Yu, 2012), for repair of stalled replication forks (Tittel-Elmer et al., 2012), and for regulation of transcriptional activity in vertebrates (Rudra and Skibbens, 2013).

The SMC family of proteins also includes several complexes collectively referred to as condensins. Condensins are believed to regulate chromosome organization and condensation during mitosis and meiosis in eukaryotic cells (Wu and Yu, 2012). In vertebrates two types of complexes are known to exist, condensin I and condensin II, which feature common subunits SMC2 and SMC4, but differ in subunits CAP-D2/D3, CAP-H/2, and CAP-G/2 (Wu and Yu, 2012). Condensins are believed to control chromosome condensation either by destabilizing cohesin rings, or by promoting decatenation of sister chromatids through interaction with topoisomerase II-α (Cuylen and Haering, 2011). It has also been shown that in *Xenopus laevis* and *Drosophila melanogaster* both condensin complexes induce positive supercoiling on DNA in the presence of topoisomerase IIα, and that depletion of condensins lead to loss of chromosome condensation (Baxter and Aragón, 2012). In worm (*Caenorhabditis elegans*) changes in chromosome condensation have been shown to affect meiotic DNA break distribution and crossover frequency, perhaps by disturbing the distribution of DNA loops in which double strand breaks are thought to occur (Mets and Meyer, 2009).

Assembly of SMC complexes around chromosomes has been extensively studied in budding yeast (*Saccharomyces cerevisiae*), and humans (Dorsett and Ström, 2012). In eukaryotes the key regulator of cohesion establishment is believed to be a lysine acetyltransferase known as Establishment of cohesion 1 (Eco1) in yeast, Esco1/2 in humans and mice, Deco and San in *Drosophila*, Eso1 in *S. pombe* (Nasmyth, 2011; Whelan et al., 2012b), and XEco1/2 in *Xenopus* (Higashi et al., 2012). Lysine acetyltransferases transfer an acetyl radical (COCH3) from acetyl-CoA to the ε-amino group of a K residue (Kim and Yang, 2011). This modification is also known as Nε-acetylation and is different from Nα-acetylation, which is associated with co-translational modification and affects protein stability. Moreover Nε-acetylation is reversible and dynamic and its level depends on the antagonistic activity of acetyltransferases and deacetylases (Kim and Yang, 2011). Acetylation of lysine residues in the ATPase domain of cohesins is thought to reduce the rate of ATP hydrolysis, which controls the closure and opening of the cohesin ring (Kim and Yang, 2011). Alternatively, acetylation might inhibit the interaction with the negative regulator of cohesion RAD61 (WAPL in vertebrates) (Rudra and Skibbens, 2013).
In yeast acetylation of key lysine residues K112, K113 and K84, K210 of SMC1 and SMC3, respectively, by Eco1 is believed to stabilize the ring and facilitate binding to the α-kleisin, Sister chromatid cohesion 1 (Scc1), until anaphase (Beckouet et al., 2010). Then two events occur in sequence, first the enzyme separase cleaves Scc1 to open the ring, followed by deacetylation of SMC1 and SMC3 by the Histone lysine deacetylase 1 (Hos1) to facilitate recycling of SMC1 and SMC3 (Rivera and Losada, 2010). Acetylation of the other members of the SMC family including condensins (SMC2-SMC4), SMC5 and their corresponding kleisins has also been shown to occur in human myeloid leukemia cells (Choudhary et al., 2009), suggesting that acetylation is a common regulatory mechanism shared by all SMC complexes.

There is ample biochemical and genetic evidence for the link between Eco1 activity, acetylation of SMC proteins for cell viability In budding yeast, replacement of key lysine residues by arginine in SMC3 is lethal, while replacement by asparagine or threonine enables cells to grow in the absence of functional Eco1 (Nasmyth, 2011). Replacement of several lysines for glutamine in subunit Scc1 also allows for repair of double strand breaks during the M-phase in cells which have lost the acetyltransferase activity of Eco1 (Wu and Yu, 2012), while overexpression of Eco1 induces generation of cohesion during the M-phase without the requirement for double strand breaks on DNA (Wu and Yu, 2012). In *Xenopus* egg extracts immunodepletion of XEco1/2 leads to loss of chromatic cohesion and abolishes acetylation of Smc3 (Higashi et al., 2012), while in mice deletion of *Esco2* leads to loss of sister chromatid cohesion, loss of Smc3 acetylation, loss of DNA repair activity (during the S phase) and eventually, to cell death (Whelan et al., 2012a).

In humans, point mutations in the Eco1 homolog, ESCO2, leads to congenital abnormalities exemplified by Roberts Syndrome (RBS) (Vega et al., 2005). In RBS patients only 10-20% of cells show abnormal mitosis; however all cells are hypersensitive to DNA damaging agents and show premature centromere separation (van der Lelij et al., 2009; Whelan et al., 2012b; Vega et al., 2005). Recent studies on CTF7 in yeast and mice Eco1 and Esco2 mutants suggest that mutations in the C-terminal acetyltransferase domain have little effect on S-phase cohesion and chromosome segregation, but increase the sensitivity to DNA-damaging agents, thereby phenocopying RBS cells (Lu et al., 2010; Whelan et al., 2012a). Mutations in the N-terminus mostly lead to defects in cohesion, and often to loss of chromosomes during mitosis (Lu et al., 2010; Whelan et al., 2012a). Moreover, in yeast it has been observed that haploid-strains defective in Eco1 are not able to sporulate, while diploid heterozygous strains are normal (Rudra and Skibbens, 2012). In mice heterozygous conditional-*Esco2* mutants show no phenotype, while homozygous embryos die at the eight-cell stage (Whelan et al., 2012b). These findings have led to the
suggestion that Eco1 activity is dosage-dependent (Rudra and Skibbens, 2012; Whelan et al., 2012b), a claim made earlier by Skibbens (2010) who suggested that a decrease in yeast Eco1 activity may compromise DNA repair first, and chromatid pairing second.

Interestingly until recently nothing was known about the biological function of the Arabidopsis thaliana only Eco1 homolog. Then Jiang et al. (2010) showed that Arabidopsis CTF7/ECO1 encodes an acetyltransferase with the ability to rescue yeast ecol deletion mutants and capable to self-acetylate. Arabidopsis CTF7/ECO1 encodes a 345 amino acid protein, which contains a conserved N-terminal PIP box required to interact with the replication fork subunit PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) and necessary to enforce cohesion (Jiang et al., 2010; Higashi et al., 2012), and a Zinc finger domain, important for chromatin binding. At the C-terminus of the protein is the acetyltransferase domain, required to acetylate cohesin factors (Jiang et al., 2010; Higashi et al., 2012; Rudra and Skibbens, 2012, Appendix 2). Heterozygous ctf7-1 mutants showed asynchronous female development, while homozygous embryos were found to arrest before or at the globular stage. In this document we report the identification and characterization of homozygous ctf7-1 and ctf7-2 T-DNA insertion mutants and show that CTF7/ECO1 is required to establish sister chromatid cohesion during male meiosis, and to allow proper cell division in vegetative tissues. We also show that CTF7/ECO1 is required for DNA repair and discuss these results in the context of a complex regulatory network.
Materials and Methods

Plant material and growth

The wild type (ecotype Columbia) and T-DNA insertional lines, SALK_059500 (ctf7-1), and SAIL_1214G06 (ctf7-2) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA; http://abrc.osu.edu). Seeds were surface sterilized in 30% sodium hypochlorite and germinated on ½ X Murashige and Skoog (MS) medium without sucrose, followed by stratification at 4°C for 96 hrs in the dark. Seedlings grown at 21°C, on a 16 hr photoperiod and 60% relative humidity for approximately 5 days after the emergence of the radicle. Seedlings were then genotyped following emergence of either cotyledons or first true leaves and used for the experiments. Remaining seeds were germinated on soil to further characterize the phenotype of the mutants.

Molecular analysis of CTF7/ECO1

The Arabidopsis At4g31400 locus was first selected for study during a screen for potential gametophytic mutants. The T-DNA lines SALK_059500 (ctf7-1) and SAIL_1214G06 (ctf7-2) were selected in this study since previous work showed that both independent lines display a similar phenotype, and line ctf7-1 could be successfully complemented by transformation with the Gateway binary vector pFGC5941 carrying the full genomic sequence of At4g31400 (Jiang et al., 2010). Genomic DNA was extracted from segregant ctf7-1 and ctf7-2 T3 seedlings. Plants were genotyped with specific primer pairs for their corresponding T-DNA inserts and wild type locus. At least five independent complementation lines were analyzed using specific primers for the ctf7-1 insertion, for the pFGC5941 vector and for exons 3, 4 and 5 of CTF7/ECO1. Resistance to BASTA, and plant fertility were also analyzed. The complete list of primers used can be found in Table 1.
Morphological characterization of ctf7-1 and ctf7-2

Images of seed set were recorded after dissection of at least 30 siliques from 7-week old plants under a Carl Zeiss stereo Lumar V12 fluorescence stereomicroscope (Carl Zeiss, http://microscopy.zeiss.com/ microscopy) connected to a Carl Zeiss AxioCam MRC5 CCD unit. Whole anther morphology was analyzed by staining with Alexander staining for 24 hrs at 50°C (Alexander, 1969), or by sectioning followed by staining with toluidine blue. Pollen viability was analyzed by releasing mature pollen grains into fluorescein diacetate (FDA) (Sigma-Aldrich, http://www.sigmaaldrich.com) solution for 15 min in the dark followed by observation under an Olympus BX51 epifluorescence microscope coupled to an Olympus DP70 CCD unit (Olympus, http://www.olympus-global.com/en/corc/company/lifescience). Analysis of in vivo pollen tube growth was performed as described by Szumlanski and Nielsen (2009) and emasculated pistils from the wild type and homozygous ctf7-1 (ctf7-1) were cross-pollinated and collected after 24 hrs. Tissue was obtained from five week-old plants, with three biological replicates and at least 100 pollen grains per replicate were used to estimate pollen viability.

Analysis of subcellular localization of CTF7/ECO1

Protoplasts prepared from the leaves of four-week old Arabidopsis plants were co-transformed with 35S:CTF7/ECO1:GFP and nuclear marker construct 35S:ERF4:mRFP. Transformed protoplasts were observed by two-photon laser confocal microscopy and analyzed with the Zeiss LSM Image Browser version 3.5.

Analysis of ultrastructure

Ultrastructure of mature microspores was analyzed by transmission electron microscopy on a Philips CM 100 unit (Philips, http://www.research.philips.com). Samples were prepared by fixation on gluteraldehyde 2.5% and formaldehyde 4%, in 0.1 M sodium phosphate buffer, pH 7.0 at room
temperature for 4 hrs. After three 20 min buffer rinses, samples were postfixed on osmium oxide 1%. Samples were dehydrated in an acetone series, embedded on Spurr’s resin, and sectioned on a Leica Ultracut E microtome (Leica Microsystems GmbH, http://www.leica-microsystems.com). Ultra-thin sections (70-90 nm) were stained with 6% uranyl acetate and lead citrate 0.4%. Sections were observed at 80 kVolts. Preparation of anther sections followed the same procedure, however semi-thin samples (1 µm) were stained instead with 10% toluidine blue in 1% Na-Borex for 1-2 min, and observed directly under a light microscope. Development stages were assigned according to Sanders et al. (1999). For the analysis of leaf cell structure, the first true leaves of 1 week-old seedlings were excised, frozen on liquid nitrogen and then transferred to a sample preparation chamber set at -160°C. After 5 min the temperature was raised to -85°C and the sample was sublimed for 15 min. After coating with platinum at -130°C the sample was transferred to a PP2000T Cryo-SEM System chamber (Quorum Technologies, http://www.quorumtechnologies.com) and observed at -160°C on a FEI Quanta 200 scanning electron microscope (FEI, http://www.fei.com) set at 20 kVolts. Results were obtained from three different biological samples containing at least three technical repeats.

Chromosome spreads

In order to analyze male meiosis anthers were isolated and digested as described (Yang et al., 2011). Following digestion, cells were transferred onto poly-L-lysine slides (Sigma-Aldrich), and covered with a cover slip. The slides were frozen on dry ice and the cover slips quickly removed. The dried slides were stained with 1.5 mg/mL 4’, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, http://www.vectorlabs.com). In order to analyze mitosis, root tips from seedlings grown on agar were excised and placed within 200 µL PCR tubes. Fixation and digestion was performed within the tubes, and digestion was extended to 2 hrs.

Fluorescence in situ hybridization

Fluorescence in situ hybridization was conducted on chromosome spreads using a probe that was prepared from the 180 bp centromeric repeat sequence (CEN), which was previously amplified by PCR
from the pAL1 clone and then labeled using the Fluorescein-High Prime DNA labeling Kit (Roche, https://www.roche-applied-science.com), alternatively CEN probes were also prepared by amplifying the 180 bp repeat directly from genomic DNA. The CEN probe was then used in hybridization solution at 5 mg/mL. Chromosomes were counterstained with DAPI and observed under an epifluorescence microscope.

**Antibodies and immunolocalization**

SMC3 and SYN1 were localized in buds prepared as described previously (Yang et al., 2011). Rabbit polyclonal antibodies against SYN1 and SMC3 were then detected with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:500) (Molecular Probes, http://zt.invitrogen.com) with or without Alexa Fluor 594 goat anti-mouse secondary antibody (1:500) and observed under an epifluorescence microscope.

**Flow cytometry**

Cells from fresh leaves were isolated and stained with the CyStain PI Absolute P Kit (Partec, http://www.partec.com). Finally the nuclei suspension was run through a MoFlo XDP Laser Cell Sorter (Beckman Coulter, https://www.beckmancoulter.com) and results were analyzed with Summit V5.0 software (Beckman Coulter) from at least three different biological samples.

**Quantitative-real-time PCR**

Total RNA was extracted with the RNeasy Plant Minikit (Qiagen, http://www.qiagen.com/) from WT and *ctf7-1* seedlings (1-week-old), and from meiotic anthers collected from mature WT and *ctf7-1* plants. Total mRNA from meiotic anthers was further amplified with the MessageAmp II aRNA Amplification Kit (Ambion, http://zt.invitrogen.com). First-stranded cDNA was prepared from total RNA
with the M-MLV reverse transcriptase system (Promega, http://www.promega.com) according to manufacturer’s instructions. For quantitative PCR, a Power SYBR Green I Master Mix (Applied Biosystems, http://www.appliedbiosystems.com) was used with 150-200 nM primers, 20 ng/µl cDNA and 50 µl of RT reaction product. Reactions were run and analyzed on the AB 7500 Real Time PCR System (Applied Biosystems). Melting curves analyses and negative controls were used to exclude primer-dimer artifacts and low-specificity in the amplification. Quantitative reactions were done in triplicate and averaged. Primers specific for the 3’ end of transcripts were either designed on Primer Express version 3.0 (Applied Biosystems) or adapted from relevant references (Preuss and Britt, 2003; Takahashi et al., 2010; Czechowski et al., 2005). The complete list of primers used can be consulted at Table 1.

**DNA comet assay**

Mutant and wild-type plants were treated with DNA-damaging agent bleomycin sulfate (Sigma-Aldrich), dissolved in liquid½ X MS medium at a concentration of 50 µg/mL, for 1 hr, then washed and places on liquid ½X MS medium without bleomycin. Cell suspension samples were prepared according to Kozak et al. (2009), and then processed with the Trevigen CometAssay Kit (Trevigen, www.trevigen.com) according to the instructions of the manufacturer. Slides containing the suspension were stained with SYBR Green I (Molecular Probes) and observed under an Olympus BX51epifluorescence microscope with the FITC filter. The percent of double strand breaks per nucleus was calculated with TriTek Comet Score software version 1.5 (TriTek Corporation, tritekcorp.com), from 80 different nuclei on three different slides. The percent of damage remaining after a given repair time ($t_x$) was estimated according to Kozak et al. (2009). Results were obtained from at least three different biological samples, with three technical repeats and a negative control.

**Bisulfite sequencing**

The Qiagen DNeasy Plant Mini Kit (Qiagen, www.qiagen.com) was used to isolate high purity genomic DNA from ctf7-1 homozygous plants and the wild type. After isolation approximately 1 µg per line was digested with PstI (New England Biolabs, www.neb.com) for 5 hrs, and then purified with the
Gene Clean Kit (MP Biomedicals, www.mpbio.com). Approximately 500 ng of purified DNA was treated with the Qiagen Epitect Bisulfite Kit (Qiagen, www.qiagen.com) to deaminate cytosines. After treatment four specific loci plus a negative control were amplified by PCR, purified and ligated to the T-Easy vector. Selection of loci was performed with the assistance of the Arabidopsis Epigenome Maps of the Salk Institute (http://neomorph.salk.edu/ epigenome/epigenome.html), and only loci that showed substantial cytosine methylation were chosen including: IGN23, IGN25, COPIA28 and soloLTR. Gene PHAVOLUTA (PHV) was used as a negative control. Approximately 4-20 clones per loci were sequenced. Sequencing results were aligned and analyzed with Sequencher 5.1 (Gene Codes Corporation, http://genecodes.com) and online tool CyMATE (Cytosine Methylation Analysis Tool for Everyone, http://www.cymate.org/). The complete list of primers used can be consulted at Table 1.

**Accession Numbers**

Sequence data from this report can be found in the Arabidopsis Genome Initiative or GenBank/EMBL under the following accession numbers:

At2g27040 (AGO4), At3g48190 (ATM), At5g40820 (ATR), At4g21070 (BRCA1), At5g01630 (BRCA2B), At3g19590 (BUB3.1), At3g25100 (CDC45), At3g48750 (CDK1A), At5g18620 (CH17), At4g31400 (CTF7/ECO1), At4g37490 (CYCB1;1), At3g22880 (DMC1), At1g19100 (DMS11/MORC6), At2g16390 (DRD1), At5g14620 (DRM2), At5g63110 (HDA6), At3g25980 (MAD2), At5g11510 (MYB3R4), At5g56580 (NQK1), At2g40030 (NRPE1), At4g02390 (PARP2), At5g20850 (RAD51), At2g45280 (RAD51C), At3g54670 (SMC1), At2g27170 (SMC3), At5g15920 (SMC5), At5g61460 (SMC6B), At4g25120 (SRS2), At5g55300 (TOPOI-α), At5g55310 (TOPOI-β), At3g23890 (TOPOII-α), At5g63920 (TOPOIII-α).
Results

Homozygous ctf7-1 and ctf7-2 plants are viable but exhibit defects in vegetative and reproductive development

It was previously shown that approximately 25% of the seed in siliques of heterozygous ctf7-1 plants (ctf7-1/+), exhibit defects in zygote and embryo development including arrest by the early globular stage (Jiang et al., 2010), suggesting that inactivation of Arabidopsis CTF7/ECO1 results in embryo lethality. During the analysis of segregating populations of progeny of the ctf7-1/+ (SALK_059500) and ctf7-2/+ (SAIL_1214G06) T-DNA lines (Jiang et al., 2010; Figure 1a), we identified several slow growing, dwarf plants (Figure 1b). At about the same time analysis of the subcellular localization of AtCTF7 in Arabidopsis protoplasts indicated that AtCTF7/ECO1 localizes to the nucleus (Figure 1c), a result that is in agreement with the hypothesis that Arabidopsis CTF7/ECO1 is an essential nuclear protein required for growth (Jiang et al., 2010). Indeed, genotyping indicated that dwarf plants were homozygous for the T-DNA insert and segregated at a very low frequency (below 4%), a rate that deviated significantly from a 1:3 Mendelian ratio (Figure 1d). The phenotype of ctf7-1 and ctf7-2 homozygous mutants is undistinguishable, therefore, it was decided to focus efforts on the characterization of ctf7-1, which had been successfully complemented using the full genomic sequence of CTF7/ECO1, plus its native promoter (Jiang et al., 2010). This line is referred to as the ctf7-1 complementation line (Com) in this report. Quantitative real-time PCR (QPCR) showed that plants homozygous for either ctf7-1 (ctf7-1) or ctf7-2 (ctf7-2) contain less than 20% of wild type CTF7 mRNA levels corresponding to exon five, located downstream of the corresponding T-DNA inserts (Figure 1e). Amplification of CTF7 cDNA with primers spanning the T-DNA insert was not possible in ctf7-1 plants, suggesting that the ctf7-1 mutation gives rise to truncated versions of the transcript, a situation previously observed in mutations for human ESCO2 (Vega et al., 2005). Sequencing experiments using primers specific to the T-DNA suggest that both mutant lines contain only one T-DNA insert.

Developmental defects are widespread in ctf7-1 and ctf7-2 seedlings, including defects in the distribution of leaves on the stem (e.g. phyllotaxy). In WT all leaves are arranged in a spiral while in ctf7-1 and ctf7-2 this arrangement shows modifications, including additional basal leaves and clusters of
modified, small rosette leaves (Figure 2a). Defects in root development were also observed including reduction in the length of the elongation zone and root swelling (Figure 2b).

The morphology of ctf7-1 flowers was also abnormal, with enlarged papillae, defective anthers, and a very limited amount of pollen (Figure 1f). Crossing experiments showed that mutant stigmas allowed the germination of WT pollen, but no mature siliques were recovered after pollination, suggesting that both male and female gametophytes are defective in ctf7-1 (Figure 1g). No defects were observed in the germination of pollen from the ctf7-1 complementation line or ctf7-1/+ plants (Figure 1g), suggesting that ctf7-1 heterozygous plants are not defective in the development of mature microspores.

Unlike heterozygous ctf7-1, and ctf7-2 plants, mature siliques of ctf7-1 and ctf7-2 plants were unable to develop viable seeds (Figures 1h and 1i). Approximately 22% of the ctf7-1/+ seed reaches full size but the embryos arrest by at the globular stage (Jiang et al., 2010); however in ctf7-1 and ctf7-2 all seeds arrest development before cellularization of the endosperm (Figure 1h). Analysis of emasculated flowers suggested that unlike the case in wild type siliques, unfertilized ctf7-1 and ctf7-2 ovules degrade within 2 days after emasculation of the anthers (Figure 2c).

**CTF7/ECO1 is essential for microsporogenesis**

Reciprocal pollination experiments showed that ctf7-1 pollen is defective. Alexander staining revealed a reduction in the size and number of viable pollen grains in ctf7-1 and ctf7-2 anthers, which was not observed in heterozygous anthers. Pollen from ctf7-1 and ctf7-2 is poorly stained due to the lack of cytoplasm (Figure 3a). Staining with fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970) revealed that less than 6% of ctf7-1 and ctf7-2 pollen was viable, as opposed to over 85% in WT, the complementation and heterozygous lines (ctf7-1/+ , ctf7-2/+ ) (Figure 3b). Also staining with the DNA dye 4',6-diamidino-2-phenylindole (DAPI) indicated that less than 4% of ctf7-1 and ctf7-2 pollen fully develops into mature tricellular pollen(Figure 3c). This prompted us to examine anther and pollen development in ctf7-1 and ctf7-2 plants.
Anther development in Arabidopsis starts with the formation of bilateral primordia that features locules, and vascular tissue (stages 1-4). Once the primordia are established, archesporial cells within the anther give rise to the endothecium, middle layer, tapetum and pollen mother cells (stage 5). At this stage *ctf7*-1 anthers already appear smaller than in the WT (Figure 4a). Alterations in microsporogenesis were first observed during stages 6 and 7 when normally meiosis is completed and tetrads are formed. Irregular division of pollen mother cells (stage 6) and the formation of irregular tetrads (stage 7) were observed in *ctf7*-1 anthers. Anthers in *ctf7*-1 remain smaller than WT as development continues, and development of the connective tissue appeared compromised as well. After the release of microspores at stage 8, the anthers of WT and *ctf7*-1 continued to develop in a similar fashion; the microspores became vacuolated and the tapetum degenerated (stages 9 and 10). However at stage 11, when microspores normally enter into mitosis and the stomium and septum degrade, *ctf7*-1 microspores appeared shrunken and there was no noticeable degradation of the stomium and septum. By stage 12, WT anthers contained fully developed tricellular pollen and the anthers were undergoing anthesis. In contrast, most *ctf7*-1 pollen appeared dead and no opening of the stomium and septum was observed.

Finally, severe defects in the ultrastructure of *ctf7*-1 and *ctf7*-2 pollen was observed, including the relative absence of an electron-dense cytoplasm, vegetative nuclei and sperm cells (Figure 4b). These results demonstrate that microsporogenesis and anthesis is defective in *ctf7* homozygous mutants.

*ctf7*-1 male meiocytes display defects in chromosome condensation, sister chromatid cohesion and the distribution of cohesin proteins

Based on observations in other systems (Baudrimont *et al.*, 2011; Rudra and Skibbens, 2012), we expected that inactivation of CTF7/ECO1 activity should block the establishment of sister chromatid cohesion and result in meiotic defects. In order to investigate this possibility we analyzed meiotic chromosome spreads in *ctf7*-1 plants. Alterations were observed from the earliest stages examined, with the first noticeable difference between in *ctf7*-1 and WT plants being the presence of fewer meiocytes overall throughout meiosis. It is not clear if this is due to the fact that the plants are smaller and less healthy, or if some *ctf7*-1 microsporocytes arrest and abort prior to meiosis. Some variability was also observed in the phenotypes at different stages of meiosis, with some meiocytes appearing relatively
normal; however most meiocytes shared common phenotypes, which are described below. During preleptotene WT chromosomes showed faint labeling of chromosomes with chromocenters that stain deeply (Ross et al., 1997; Figure 5a), while in ctf7-1 plants no chromosome axes were recognizable and the chromocenters stained very faintly (Figure 5a). Similar to WT, chromosome condensation was observed during leptotene in ctf7-1; although at somewhat reduced levels (Figure 5b). During zygotene chromosome alignment was reduced in ctf7-1 (Figure 5c) and ultimately a mixture of unpaired and unevenly paired chromosomes were observed at pachytene (Figure 5d). During diplotene, a decondensed mass of chromatin was typically observed in ctf7-1; no individual separated chromosomes were visible (Figure 5e). In contrast to the five bivalents observed in WT at diakinesis (Mercier et al., 2005; Figure 5f) a mixture of uncondensed chromatin, unpaired chromosomes, and possibly some bivalents were observed in ctf7-1 (Figure 5f). Beginning at diplotene and diakinesis and continuing through meiosis II ctf7-1 meiocytes typically appeared less condensed than their WT counterparts. A relatively small number of cells (10%) also appeared to contain extra chromosomes (Figures 5e-5g), although it is not clear whether this was caused by defects in chromosome segregation or DNA replication.

During metaphase I a mass of DNA, possibly chromosomes, congregated at the equatorial plane in ctf7-1, however individual chromosomes and/or bivalents were difficult to identify (Figure 5g). In contrast to WT (Figure 5h), chromosomes of ctf7-1 did not segregate evenly at anaphase I, resulting in chromosome bridges, lagging chromosomes and a random distribution of chromosomes (Figure 5h). At telophase I in ctf7-1 individual chromosomes could be identified while the organelle band in the equatorial region of the cells was diffuse and difficult to visualize (Figure 5i). At metaphase II and anaphase II the chromosomes were irregularly scattered around the cell in ctf7-1 (Figures 5j-5k). Finally at telophase II nuclear membranes formed around random groups of DNA resulting in polyads in ctf7-1 (Figure 5i).

To further investigate meiosis and in particular sister chromatid cohesion and chromosome pairing, in situ hybridization was conducted with the 180 bps centromere (CEN) repeat as a probe (Armstrong et al., 2001). In leptotene, approximately ten unpaired and well-dispersed CEN signals were observed in WT (11 ± 2, n = 10), while in ctf7-1 irregular CEN signals were typically observed (Figure 6a). By zygotene, the number of CEN signals was reduced to approximately 5 ± 1 (n = 10) in WT. In ctf7-1 roughly double the number of CEN foci (12 ± 1, n = 7) were observed, consistent with a defect in synapsis (Figure 6b).
Increased CEN foci (8 ± 1, n = 4), were also observed during pachytene in ctf7-1; in addition the signals were more dispersed and less well defined than in WT, often appearing not as discrete foci but rather as long extended segments. Five easily identifiable CEN foci were observed in WT cells at diakinesis, however, 20 or more CEN signals were typically found in ctf7-1 (Figure 6d).

During late metaphase I/early anaphase I, five pairs of CEN signals (9.1 ± 1, n = 10) were observed in the WT, while over 20 CEN signals appeared randomly dispersed around the nucleus in ctf7-1 (Figure 6e). By late anaphase I masses of DNA, some without CEN signals, some with two signals, and clusters of CEN signals were observed in ctf7-1 (Figure 6f). Later in development ctf7-1 microspores containing varying numbers of CEN signals; some with 20 or more signals could be observed (Figure 6g). “Extra” CEN signals were also observed in interphase nuclei of some anther somatic cells of ctf7-1 plants (Figure 6h). In contrast WT microspores and interphase anther cells always contained five and ten CEN foci, respectively.

We next investigated the loading and distribution of the SYN1 and SMC3 cohesin proteins on chromosomes of ctf7-1 meiocytes. As has been demonstrated previously, SYN1 and SMC3 display similar distribution patterns on WT meiotic chromosomes (Yang et al., 2011; Figure 7). Diffuse nuclear labeling is observed at interphase. Beginning at early leptotene and extending into zygotene both proteins decorated the developing WT chromosomal axes. During late zygotene and pachytene the proteins lined the synapsed chromosomes. As meiosis progressed from diplotene to diakinesis the chromosome-associated cohesin signals became progressively weaker and more diffuse.

Similar to our observations in the chromosome spreading and CEN FISH experiments, approximately half of the ctf7-1 meiocytes displayed relatively normal SYN1 and SMC3 labeling patterns. However, in most cells the labeling for both proteins was very weak and irregular (Figure 7). Very little SMC3 and SYN1 signal was present in the nucleoplasm at interphase (Figure 7a). A diffuse labeling of the chromatins was first observed during leptotene (Figure 7b), with some labeling of thread-like structures in some cells during early zygotene (Figure 7c). However, in most cases the SYN1 and SMC3 signals were diffuse and became progressively weaker as meiosis progressed into pachytene, diplotene and diakinesis stages (Figures 7d-7g).
Several key genes for DNA repair and cell cycle progression are upregulated in *ctf7-1* plants

In addition to its critical role in the establishment of sister chromatid cohesion during DNA replication, CTF7/ECO1 may be involved in DNA repair and cell cycle progression (Lu et al., 2010; Lyons and Morgan, 2011). We therefore investigated the effect of the *ctf7-1* mutation on the expression of a number of genes required for DNA repair and cell cycle progression. A pathway analysis using AraNet (Lee et al., 2010) suggested a strong functional linkage for these genes \( \text{p} = 1.054 \times 10^{-82} \). The relative expression levels of the selected genes were measured in triplicate through QPCR. As shown in Figure 8, large and statistically significant increases (>four fold) in transcript levels were observed for several genes in *ctf7-1* plants, including: *ATM* (a kinase), *BRCA1* (an ubiquitin ligase), *PARP2* (a polymerase), *RAD51* (a gene involved in homology-dependent DNA repair), *CYCB1;1* (a G2/M checkpoint gene) and *TOPOII-a* (a topoisomerase) (Xie and Lam, 1994; Garcia et al., 2003; Preuss and Britt, 2003; Takahashi et al., 2010; Thomson and Guerra-Rebollo, 2010). Smaller increases (approximately two-fold) were observed for *SMC5*, *SMC6B*, and *SRS2* (a helicase) (Ira et al., 2003; Watanabe et al., 2009), while no significant change was observed for *ATR* (a kinase involved in single strand break repair) (Yoshiyama et al., 2009), the M-phase checkpoint genes *MAD2* and *NQK1* (Takahashi et al., 2010), and gene *CDKA1*, which regulates the transition from mitosis to endocycle (Dissmeyer et al., 2007). Expression of other topoisomerases was not detected in either WT or *ctf7-1* samples, including *TOPOI-a*, and *TOPOI-b* (Takahashi et al., 2002).

Given the developmental defects observed in *ctf7-1* meiocytes, the transcript levels of highly expressed meiotic genes (Yang et al., 2011 b) was measured by QPCR as well. As shown in Figure 9, statistically significant increases in transcript levels were observed for *ATM* and *ATR*, *BRCA2B*, *RAD51C*, *DMC1*, *SMC1* and *SMC3*, and *CDC45*, a gene that codes for an S phase licensing factor and is required for meiosis (Stevens et al., 2004). No significant increase was observed for the mitotic checkpoint gene *BUB1.3*. Expression of several other genes was not detected; including SRS2, mitotic topoisomerases *TOPOII-a*, *TOPOI-a*, *TOPOI-b* (Takahashi et al., 2002), meiotic *TOPO III-a* (Hartung et al., 2008) or endoreplication factor *MYB3R4* (Takahashi et al., 2010).
The ability of ctf7-1 plants to repair DNA double breaks was tested using the comet assay (Kozak et al., 2009), which has been employed in Arabidopsis mutants with defects in either chromosome cohesion or DNA repair proteins (Takahashi et al., 2010). Seven-day-old WT and ctf7-1 seedlings were exposed to a bleomycin solution (50 µg/mL) for 1 hour and the percentage of DNA present in nuclei tails after recovery times of 0, 30 and 60 minutes was used to estimate the level of double strand breaks remaining in each sample (Figure 10). In WT most double strand breaks were repaired after 30 minutes and only approximately 29% remained after one hour. In contrast approximately 79% of all double-strand breaks remained unrepaired after one hour in ctf7-1 plants, indicating that CTF7/ECO1 activity is required for DNA repair in Arabidopsis.

ctf7-1 and ctf7-2 plants are defective in mitotic cell cycle progression

The extreme dwarf phenotype of ctf7-1 and ctf7-2 plants suggested that similar to the situation in yeast (Moldovan et al., 2006), Arabidopsis CTF7 plays an important role in cell cycle progression. To determine if ctf7-1 and ctf7-2 cells show cell cycle arrest, we analyzed the morphology and density of pavement and stomata cells in the first true leaves of 7-day-old seedlings by cryo-Scanning Electron Microscopy (cryo-SEM). In WT, pavement and stomata cells were small (Figure 11a), developed at a density of 1070 and 590 cells/mm² (Figure 11b), and covered an approximate area of 892 µm² (for pavement cells) and 88.0 µm² (for stomata) (Figure 11c). Similar values were observed for the ctf7-1 complementation line (Com) (Figures 11a-11b). In cells of ctf7-1 and ctf7-2 there was a significant reduction in the density of both pavement cells (580 cells/mm²), and stomata (250 cells/mm²) (Figure 11b), accompanied by a significant increase in the average area of pavement cells (1300 µm²/cell) (Figure 11c); however no change was observed in the area of stomata cells under cryo-SEM.

Mitotic cell cycle progression was further examined by analyzing the DNA content of leaf cells. Intact nuclei were isolated from the first leaves of 7-day-old plants, followed by flow cytometry. In WT and complementation line samples, approximately 71-73% of all nuclei had a haploid DNA content of 2, ‘2C’, which reflects normal entry into mitosis and cell division (Figure 11d). Approximately 16 -17 % of WT and complementation line nuclei showed a 4C value, which represents those cells that have completed DNA replication but have not entered the G2/M phase. In contrast, 55-56% of ctf7-1 and ctf7-2 nuclei
showed a 4C value (Figure 11d), suggesting a defect in the ability of cells to advance into M-phase after DNA replication.

**ctf7-1 and ctf7-2 plants are defective in mitotic chromosome segregation**

In order to obtain direct evidence of the role of Arabidopsis CTF7/ECO1 in mitotic chromosome segregation, root tips were excised from 2-week old seedlings corresponding to WT, Com, ctf7-1, and ctf7-2. Seedlings developed in ½ MS agar plates at density of 120 plants per plate, 10-12 plates per line. Mutant seedlings were selected visually based on short height, short root length, and dark leaf color. Approximately 40 seedlings per line were recovered. After excision of the root tip, tissues were prepared following the protocol used for meiocytes, except that tissues were handled inside 200 µL PCR tubes, and digestion with enzymes was extended to two hrs. As observed in Figure 12, it was possible to observe cell division from interphase to telophase. In both WT and Com, 10 chromocenters were observed during interphase (Figures 12a and 12e), which corresponds to a diploid number of chromosomes, while in ctf7-1 (Figure 12i) and ctf7-2 (Figure 12m) the chromocenters often appeared decondensed and in some cases an excess of 10 chromocenters was observed. During metaphase WT (Figure 12b) and Com (Figure 12f) cells typically displayed 10 condensed chromosomes; however, chromosomes in cells of ctf7-1 (Figure 12j) and ctf7-2 (Figure 12n) appeared less condensed and irregularly shaped. At anaphase WT (Figure 12c) and Com (Figure 12g) cells showed even segregation of chromosomes, and individual chromosomes could be recognized, while chromosomes in cells of ctf7-1 (Figure 12k) and ctf7-2 (Figure 12o) looked intertwined and stretched; and condensation was defective. In telophase, WT (Figure 12d) and Com (Figure 9h) cells showed segregation of chromosomes into two well-condensed masses of equal size, but in ctf7-1 (Figure 12l) and ctf7-2 (Figure 12p) chromosome bridges persisted and the chromosomes often recondensed into a single unevenly shaped mass of DNA (Figures 12l and 12p).

In fact, in the WT and Com lines 8 to 15% of the cells are unable to segregate chromosomes evenly, but up to 77 and 84% of cells in ctf7-1 and ctf7-2 are unable to do so (Figure 13). Moreover these results are statistically significant (p<0.001, Student’s t test). Taken together these results suggest that CTF7/ECO1 has a critical role in the regulation of chromosome segregation during mitotic cell division.
**ctf7-1 and ctf7-2 plants show upregulation of transposons and DNA methylase DRM2**

Co-expression analysis with the bioinformatics tool Gene Angler (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) (Toufighi et al. 2005) was performed to test whether Arabidopsis CTF7/ECO1 could influence the transcription of other genes. The program calculates Pearson correlation coefficients for genes based on a cut-off threshold ‘r’, and returns candidates with a coefficient higher than the cut-off (Toufighi et al., 2005). A query at the ‘r’ level of 0.65 returned several genes including DEFECTIVE IN MERISTEM SILENCING (DMS11), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), all key genes involved in RNA-directed DNA methylation (RdDM). This process is unique to plants and is responsible for enforcing gene silencing (Eun et al., 2012).

The expression of these targets was then evaluated by QPCR in ctf7-1. In ctf7-1 there was significant upregulation of DMS11, and DRD1 and very significant upregulation of DRM2 (Figure 14). Other targets upregulated were RdDM-factor ARGONAUTE 4 (AGO4), and CHROMATIN REMODELING FACTOR 17 (CH17). In contrast HISTONE DEACETYLASE 6 (HDA6) showed significant downregulation (Appendix 3). Subsequent PCR in both ctf7-1 and ctf7-2 showed strong and significant upregulation of transposons COPIA28 (15-fold), soloLTR (10-fold) and DRM2 (6000-fold, or alternatively 12-fold in a logarithmic scale) (Figure 14).

*The ctf7-1 mutant shows variable changes in the methylation of cytosine residues across intergenic regions and retrotransposons*

It is believed that in *Arabidopsis* the RNA-dependent DNA Methylation (RdDM) process is enforced by the DNA methylases DRM2, MET1 and CMT3 (Kankel et al., 2003; Preuss et al., 2008). This process is also involved in large scale chromatin remodeling at centromeres and ribosomal sequences (Kankel et al., 2003). It has been reported that mutants corresponding to the SMC-like gene DMS11/MORC6 show changes in the methylation status of cytosines across intergenic regions and retrotransposons (Lorkovic et al., 2012), therefore it was inferred that a mutant for the SMC regulator *CTF7/ECO1* might show a
similar phenotype. Analysis of the sequence of intergenic loci IGN23, IGN25 and retrotransposons COPIA28 and soloLTR indicated that in ctf7-1 and WT the bulk of methylation (50-80%) corresponds to the CG type, which is enforced by METHYLTRANSFERASE 1 (MET1) (Kankel et al., 2003) and that a minority (about 40%) corresponds to CHG and CHH methylation (Figure 15%). CHROMOMETHYLASE 3 (CMT3) and DRM2 enforce these other types of methylation (Preuss et al., 2008). In ctf7-1 there appears to be an important reduction in the absolute amount of CG methylation across intergenic regions (20% reduction), coupled to a modest increase in CHG and CHH methylation (Figure 15). Retrotransposon COPIA28 shows a slight reduction in the amount of methylation across all contexts (1-5%), while soloLTR shows the opposite (Figure 15). No obvious pattern can be deduced, however variable changes in methylation have been observed too at the same loci in the dms11 mutant (Lorkovic et al., 2012).
**Discussion**

Accumulating evidence suggests that assembly of cohesion rings around nascent chromatids allows efficient DNA repair during mitosis by guaranteeing the availability of an intact template (Schubert et al., 2009). In yeast and mammals, a direct link exists between acetylation of cohesin rings and cohesion (Peters and Bhaskara, 2009). Acetylation of cohesin rings occurs at conserved lysine residues in SMC proteins during S-phase and to a lesser extent during the G2/M-phase (Onn et al., 2009). Further, establishment of cohesion requires functional interactions with subunits of the replication fork (Sherwood et al., 2010). In yeast this process is regulated by the acetyltransferase Establishment of cohesion 1 (Eco1), which targets both SMC subunits and the PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) subunit of the replication fork (Sherwood et al., 2010).

In contrast to budding yeast, very little is known about the role of Arabidopsis CTF7/ECO1. In this study we characterized the roles of Arabidopsis CTF7/ECO1 in both mitosis and meiosis by characterizing plants homozygous for a T-DNA insertion in AtCTF7/ECO1 (ctf7-1 and ctf7-2). Given that CTF7 is a single copy gene in Arabidopsis and complete inactivation of CTF7 is typically lethal, our ability to obtain plants homozygous for the T-DNA insertion was unexpected. The presence in ctf7-1 and ctf7-2 plants of relatively normal levels of transcript having the potential to encode the N-terminus of the protein, and reduced but detectable levels of RNA encoding the C-terminus, (which comprises the acetyltransferase domain) raises the possibility that truncated forms of the protein may be produced in some cells, which allows some nuclear division. While further experiments are required to determine if partially functional forms of the protein are formed, the possibility that low levels of CTF7 activity are present in at least some cells is consistent with the fact that we observe severe defects in development and DNA repair first, and somewhat more mild defects in chromosome cohesion during nuclear division. A dosage effect has been observed for cohesins in other organisms (Rudra and Skibbens, 2012) and this also appears to be the case for Arabidopsis. Plants heterozygous for ctf7-1 and ctf7-2 showed defects during female gametophyte development, which requires three rounds of mitosis (Figure 2c), but no developmental defects were found in microspores, which require only two rounds of mitosis (Figure 2b) (Chang et al., 2011). Also no obvious defects in vegetative growth were detectable in heterozygous plants, which is consistent with the hypothesis that a small reduction in acetyltransferase activity does not significantly impact either cohesion or cell division (Whelan et al., 2012a). However, ctf7-1 homozygous plants had severe defects in male gametophytic development, including defects in anther development.
(Figure 4), defects in SMC3 and SYN1 binding to meiotic chromosomes (Figure 7), and dramatically reduced cohesion at centromeres (Figure 6). Alterations in the cohesion distribution can affect formation of the synaptonemal complex, impair RAD51-mediated formation of chiasmata (Longhese et al., 2009), and affect orientation of the kinetochores (Chelysheva et al., 2005), and result in defective pollen formation and sterility, as we observed in ctf7-1 (Figure 4, and Figures 1h-1i).

Homozygous ctf7-1 and ctf7-2 plants are dwarf (Figure 1c), suffer from cell cycle arrest (Figure 7d) and are unable to segregate chromosomes properly during mitotic cell division (Figure 12). Moreover, leaf cells from ctf7-1 plants are unable to efficiently repair DNA double strand breaks (DSBs) (Figure 10) and contain elevated levels of transcripts of genes required for DSB repair (Figure 8), such as the effector kinase gene Ataxia Telangietasia Mutated (ATM), known to be required for recruitment of checkpoint and repair factors (Branzei and Foiani, 2008), polymerase PARP2, recombination mediators RAD51 and BRCA1, SMC genes SMC5 and SMC6B (MIM), and checkpoint regulator CYCB1;1, which triggers arrest at the G2/M phase (Garcia et al., 2003; Schubert et al., 2009; Yoshiyama et al., 2009). Interestingly in Schizosaccharomyces pombe genes rad51 and brc1 are required for homologous recombination and are epistatic to the Smc5/6 complex, suggesting a functional role for Smc5/6 in homologous recombination (Wu and Yu, 2012). Indeed, biochemical experiments suggest that in yeast both SMC proteins are required to promote homologous recombination between sister chromatids (De Piccoli et al., 2006). In budding yeast the Smc5/6 complex is also required for the repair of double strand breaks caused by radiation and chemicals such as Mitomycin C (Santa Maria et al., 2007), and repair of stalled replication forks (Ampatzidou et al., 2006), while in S. pombe Smc6 is required for activation of the G2/M checkpoint (Wu and Yu, 2012). In Arabidopsis the G2/M checkpoint supervises the successful completion of the S-phase and allows an increase in cyclins that trigger the entry into mitosis (Gutiérrez, 2009). In Arabidopsis this checkpoint is also characterized by upregulation of BRCA1, PARP, RAD51 and B-type cyclins (Gutiérrez, 2009).

Taken together our results suggest that loss of CTF7/ECO1 activity in Arabidopsis induces defects in mitotic DNA repair similar to what has been observed in yeast, including activation of the G2/M checkpoint response. Presumably this might be a result of the accumulation of stalled or collapsed replication forks. Nonetheless we also observed upregulation of antihelicase gene SRS2, which is believed to repress ectopic recombination in yeast and Arabidopsis (Ira et al., 2003; Blanck et al., 2009). This
The anthers of ctf7-1 plants contained elevated transcript levels for a number of genes (Figure 9). In humans and worm it has been shown that both cohesins and condensins have a role during meiosis. Cohesins are required for formation of the lateral element of the synaptonemal complex (Garcia-Cruz et al., 2010), while condensins control the distribution of DNA loops in which recombination takes place (Mets and Meyer, 2009). In ctf7-1 anthers we observed upregulation of the meiotic recombinase gene DMC1 and its associated factor RAD51C, the ATR kinase gene which is required for the loading of meiotic recombinases (Kurzbauer et al. 2012), RAD51 co-factor BRCA2B, cohesin subunits SMC1 and SMC3, and the Minichromosome Complex Maintenance (MCM) subunit gene CDC45, which is required for proper meiotic entry into the S-phase (Stevens et al., 2004). In humans proteins of the MCM group are known to show reversible acetylation of lysines (Choudhary et al., 2009), a trademark of N-acetyltransferase activity, while in Xenopus they are known to interact with the cohesins during the S-phase (Sherwood et al., 2010). In yeast the recombinases Rad51, Dmc1 and the cohesins are known to interact physically and genetically during meiosis (Hong et al., 2013), and it is proposed that cohesins alter the structure of double strand breaks to a) favor single end invasion by Rad51 and b) allow Dmc1 to regulate Rad51-mediated strand exchange with the homolog, not the sister chromatid (Hong et al., 2013). An outcome of this cooperation is the formation of double Holliday Junctions, which are then cleaved by endonucleases and resolved into meiotic crossovers (Lilienthal et al., 2013).
All these genes are highly expressed during meiosis in WT Arabidopsis plants (Yang et al., 2011b), therefore a further increase could be interpreted as evidence that ctf7-1 plants may experience DNA recombination stress, as concluded for Arabidopsis mcm mutants (Takahashi et al., 2010). Interestingly, changes in the activity of Arabidopsis CDC45, RAD51C, and BRCA2 have all been linked to meiotic chromosomal fragmentation due to pre-replicative stress and the failure to perform homologous recombination (Stevens et al., 2004; Abe et al., 2005; Kurzbauer et al. 2012), a phenotype somewhat similar to what we observe in ctf7-1 meiocytes (Figures 5 and 6). Nonetheless the presence of extra chromosomes in those ctf7-1 meiocytes makes it necessary to formulate an alternative hypothesis. In human cells failure of the separase to cleave a mutant SCC1 α-kleisin leads to the formation of diplochromosomes, which are chromosomes in which four sister chromatids are aligned in parallel (Hauf et al., 2001). In these mutant ssc1 cells it is believed that anaphase cells re-enter interphase with the sister chromatids still attached, and then re-replicate their DNA, resulting in the formation of diplochromosomes (Hauf et al., 2001). However we ignore if ctf7-1 shows defects in the localization or processing of the meiotic Arabidopsis SSC1 homolog SYN1 (Cai et al., 2003), although that is a likely scenario since immunolocalization experiments already showed defects in loading of α-kleisin SYN3 (Figure 7). A third, more complex explanation would involve disruption in the activity of the MCM complex. The MCM and ORC complexes cooperate to limit origin firing to only once per cell cycle (Archambault et al., 2005). However defects in loading, binding, phosphorylation, or expression of the corresponding proteins or genes lead to rereplication and a very strong DNA damage response specific to double strand break formation (Archambault et al., 2005). Other than an increase in expression of CDC45, there is no evidence for this hypothesis; however one could speculate that CTF7/ECO1-mediated acetylation might be required for proper loading of the MCM complex, and that in ctf7-1 loss of acetylation might lead to poor MCM complex activity, derepression of rereplication and formation of diplochromosomes.

Taken together, these results suggest that CTF7/ECO1 activity is extremely important for mitotic cell cycle progression, meiosis, mitosis, and DNA repair in Arabidopsis. The dramatic developmental defects observed in ctf7-1 plants are not observed in humans containing mutations in ESCO2, which induce defects in cohesion and DNA damage repair, but not in chromosome segregation (van der Lelij et al., 2009). A role for ESCO2 in human meiosis has remained mostly hypothetical (Hogarth et al., 2011). Likewise, in Drosophila melanogaster, Eco1 homologs are required for checkpoint activation, and
chromatid cohesion (Williams et al., 2003), but a direct role in meiosis has not been demonstrated (Pimenta-Marques et al., 2008).

Finally, some of the observed developmental defects may be the result of impaired acetylation of proteins other than SMC1/SMC3 and SMC5/6. For instance condensin subunit SMC2A/CAP-E1 has been shown to play an important role during meiosis (Siddiqui et al., 2003), while condensin subunits HEB1 and 2 directly influence root and root hair development under Boron stress (Sakamoto et al., 2011). Moreover the Arabidopsis genome harbors several SMC-like genes that have been show to impact organ development, gene expression and chromatin compaction (Schatlowski et al., 2010; Moissiard et al., 2012) and whose relationship with CTF7/ECO1 has not yet been determined. One of such genes is DMS11/MORC6, whose expression is slightly increased in ctf7-1 mitotic cells (Appendix 1). DMS11 corresponds to an SMC-like protein that when bound to partner DMS3 forms a heterodimer that features an ABC-like ATPase domain, an SMC coiled-coil domain and a hinge domain, all features found in canonical SMC proteins. Moreover the DMS11/DMS3 complex is able to bind to DNA and hydrolyze ATP (Lorkovic et al., 2012). DMS11 mutants exhibit changes in DNA methylation at intergenic regions, a decrease in histone repressive mark H3K9me2, and upregulation of several transposable elements including COPIA28 and soloLTR (Lorkovic et al., 2012; Moissiard et al., 2012). Interestingly, in both ctf7-1 and ctf7-2 there is a significant increase in the expression of COPIA28, soloLTR, and a very strong increase in the expression of de novo DNA methylase DRM2, however changes in methylation are variable (Figure 15), a significant reduction is observed for CG methylation in intergenic loci IGN23 and 25, while a small reduction in all contexts (CG, CHG, and CHH) is observed in COPIA28. In soloLTR the opposite is observed, a slight increase in methylation occurs in all methylation contexts. Therefore more experiments are required to test whether CTF7/ECO1 is able to interact physically with DMS11 or other proteins.

A recent survey of the human acetylome found 3600 acetylation sites on 1750 proteins that perform functions related (but not exclusive) to RNA splicing, DNA damage repair, cell cycle, chromatin remodeling, nuclear transport, actin cytoskeleton remodeling and ribosomal biogenesis (Choudhary et al., 2009). It is not known what proteins ESCO1 and 2 directly target, nonetheless one could expect that the number is large and that many different processes are involved.
In conclusion we have shown that *Arabidopsis CTF7/ECO1* plays critical roles in meiosis, mitosis, and DNA repair and is essential for microsporogenesis and anther development. It also appears that Arabidopsis *CTF7/ECO1* may have evolved novel functions related to gene silencing. While further work is required to dissect the regulatory mechanism of *Arabidopsis CTF7/ECO1* the availability of plants homozygous for CTF7 mutations provides a valuable system to study this important enzyme.
Conclusions and Prospects

The results presented in this dissertation suggest that in *Arabidopsis* the gene *CTF7/ECO1* controls cell division during both meiosis and mitosis, and that organs such as roots, leaves, and sexual organs require proper expression of *CTF7/ECO1* to achieve full development. The severity of the phenotype observed in homozygous *ctf7-1* and *ctf7-2* plants can also be interpreted as suggesting that highly mutagenic but faster forms of DNA repair such as non-homologous end joining, or translesion synthesis may not be able to compensate for the loss of cohesion-dependent homologous repair. Our results may also suggest that establishment of chromosome cohesion and cell cycle progression are uniformly interlocked across all tissues, and that it may not be possible to dissociate both processes. For example in yeast it is possible to recover growth in *eco1* deletion colonies by introducing a second mutation in the SUMO E3 ligase gene *siz1* (Moldovan et al., 2006). In yeast this mutation reduces SUMOylation of lysine residues in the Proliferating Cell Nuclear Antigen (PCNA) (a subunit of the replication fork) which leads to increased activity of recombinase *rad51*, and enhances the affinity of the replication fork for DNA (Nagai et al., 2011). An important consequence is that lysine residues in the PCNA become ubiquitylated and this leads in turn to increased recruitment of low-fidelity, no proofreading, polymerases that are able to advance through serious DNA lesions (Nagai et al., 2011).

It is unclear whether the same results can be obtained in *Arabidopsis* mutants for *CTF7* and *SIZ1*. Preliminary experiments with *ctf7-1/siz1-2* double homozygous mutants have produced variable results (data not shown), and attempts to either resynthesize or propagate the original stocks have been unsatisfactory. Another tentative strategy to recover growth would be to increase ectopic DNA recombination as a way to: 1) improve repair of double strand breaks in DNA b) weaken cell cycle checkpoint responses (Ira et al. 2003). In fact *Arabidopsis* has at least one gene, the antihelicase *FANCM1* that represses ectopic recombination during meiosis and mitosis (Crismani et al., 2012; Crismani and Mercier, 2012). Mutants for this gene show an increase in recombination (Crismani et al., 2012; Crismani and Mercier, 2012), however it is unknown if this gene is able to cross talk with cell cycle genes. Quite surprisingly eukaryotic homologs of *CTF7/ECO1* might have evolved functions that go beyond cohesion, DNA recombination and chromosome segregation (Rudra and Skibbens, 2013; Appendix 4). In *ctf7-1* and *ctf7-2* it is possible to observe an increase in the expression of transposons and genes that take part in RNA-dependent DNA methylation, a finding that could be interpreted as suggesting a role for
Arabidopsis CTF7/ECO1 in such a process. If that were the case subsequent generations of ctf7 mutants might exhibit increased defects in growth and development.

In Arabidopsis dissociation of cohesion-dependent homologous recombination from the cell cycle circuitry might be necessary to study the impact of CTF7-directed lysine acetylation in functions such as gene expression by Chip-on-Chip or microarray experiments. In humans this function is known to be regulated by the interaction of cohesins with the CTCF insulator, and by binding of cohesins to transcription start sites (Bose and Gerton, 2010). One possibility would be to employ conditional RNAi lines as done recently by a competing team from India (Singh et al., 2013). Such CTF7-RNAi lines showed mild vegetative phenotypes, partial loss of fertility and could be propagated easily (Singh et al., 2013), unlike the T-DNA lines that we employed.

In conclusion our present knowledge on the biology of CTF7/ECO1 is still very limited because: a) the network of proteins and genes that interact with CTF7/ECO1 is not characterized yet, and b) it is not known what are the factors that modulate expression of the gene. More effort will be required to better understand CTF7/ECO1 function in plant organisms, and to better understand its evolutionary history, so that future agricultural and biotechnological applications can be developed in the near future.
Figure 1. Homozygous ctf7-1 and ctf7-2 plants are dwarf and exhibit male sterility.

a) Diagram shows genomic organization and T-DNA insertion sites in the Arabidopsis CTF7 locus. Dark boxes represent exons. The primer sets used for genotyping of both T-DNA lines (59LP, 59RP and LBP1.3 for ctf7-1; 12LP, 12RP and LB1 for ctf7-2) and Quantitative RT PCR (1F, 1R, 2F 2R, 3F and 3R) are indicated. (b) The Arabidopsis CTF7/ECO1 protein colocalized with the ERF4 nuclear marker in leaf protoplasts. (c) Homozygous ctf7-1 and ctf7-2 plants are dwarf and fail to develop mature siliques, however transformation of ctf7-1 heterozygous plants with the full genomic sequence of CTF7/ECO1 allowed normal development in complementation homozygotes (Com). (d) Less than 4% of the progeny of self-pollinated heterozygous ctf7-1 and ctf7-2 (ctf7-1/+, ctf7-2/+) plants were homozygous (ctf7-1, ctf7-2). Segregation of progeny for both T-DNA alleles was non-Mendelian (not 1:3), and the respective P values for the Chi square test (with two degrees of freedom) were highly significant, suggesting serious developmental defects. (e) QPCR experiments with primers complementary to exons 3, 4 and 5, which flank the T-DNA inserts in ctf7-1 and ctf7-2 indicated a significant reduction in CTF7/ECO1 expression downstream of the respective T-DNA insert, while the ctf7-1 complementation line (Com) showed values similar to WT. Results are shown as means ± SD (n = 3) from three biological samples. Asterisks represent significant differences (*P<0.5, **P < 0.01; Student’s t test) relative to WT. (f) After anthesis, free pollen grains were easily identifiable on the surface of wild-type stigma, but not on ctf7-1. Distribution of the petals, sepals and anthers was also affected in ctf7-1 flowers. (g) Aniline blue staining of self- and reciprocal-pollinated pistils showed normal elongation of pollen tubes from WT, ctf7-1 heterozygous (ctf7-1/+), and the ctf7-1 complementation line (Com) inside wild-type and ctf7-1 pistils, but no seed was recovered in the latter. No elongating pollen tubes were found inside either wild-type or ctf7-1 pistils after pollination with ctf7-1 pollen grains. (h) Compared to the WT, siliques of heterozygous ctf7-1 and ctf7-2 plants contained a higher percentage of defective (pale) or aborted seeds. Nevertheless, ctf7-1 and ctf7-2 plants only produced immature siliques without normal seeds. (i) Counts of seeds per silique indicate recovery of seed development in the ctf7-1 complementation line (Com), while ctf7-1 and ctf7-2 homozygotes show complete sterility. Scale bars = 1 cm for b, 10 µm for b, 0.5 mm for f and h, and 0.25 mm for g.
Figure 2. Phenotypes associated to the *ctf7-1* and *ctf7-2* homozygous mutants.

(a) WT plants before anthesis (approximately four-week-old) showed a normal phyllotaxy (arrangement of leaves on the stem), however in *ctf7-1* and *ctf7-2* phyllotaxy appears altered, including (but not limited) to additional basal leaves, development of small, multiple rosette leaves and loss of cauline leaves (See accompanying diagram for details). (b) Seven day-old seedlings stained with propidium iodide (PI). The WT, complementation line (Com), and heterozygous *ctf7-1* and *ctf7-2* (*ctf7-1/+*, *ctf7-2/+*) displayed roots.
with a clearly defined elongation zone, however roots in \textit{ctf7-1} and \textit{ctf7-2} showed a short elongation zone, swelling and overstaining of cells. (c) Two days after emasculation, wild-type (WT) ovules decolorized on chloral hydrate solution remained intact, but the ovules present in siliques of the \textit{ctf7-1} and \textit{ctf7-2} mutants collapsed. The results suggest that the ovules in \textit{ctf7-1} and \textit{ctf7-2} siliques were probably unfertilized. Abbreviations: B, basal leaf; Ca, cauline leaf; Ct, cotyledon; R, rosette leaf; r, modified rosette leaf. Scale bars = 1 cm in a, 100 µm in b, and 0.25 mm in c.
Figure 3. Homozygous *ctf7-1* and *ctf7-2* pollen is not viable.

(a) Staining of pollen with Alexander’s stain indicated presence of cytoplasm and viability in the wild-type (purple), complementation line (Com) and heterozygous *ctf7-1* and *ctf7-2* (*ctf7-1/+*, *ctf7-2/+*); however anthers of *ctf7-1* and *ctf7-2* were small and contained aborted pollen (bluish color). (b) Staining with fluorescein diacetate (FDA) indicated that less than 6% of pollen in *ctf7-1* and *ctf7-2* were viable compared to over 85% of pollen in the wild-type, complementation line, and heterozygous *ctf7-1* and *ctf7-2*. (c) Staining with DNA dye DAPI indicated that less than 5% of mature pollen from *ctf7-1* and *ctf7-2* has recognizable sperm cells and vegetative nuclei (tricellular and bicellular pollen). In the WT, complementation line and heterozygous *ctf7-1* and *ctf7-2* the values are over 95%. Data are shown as means ± SD (*n* = 100) from three biological samples. Asterisks represent significant differences (**P < 0.01, ***P < 0.001; Student’s *t* test) relative to WT. Scale bars = 50 µm in a, 10 µm in b and c.
Figure 4. Anther dehiscence and microsporogenesis are defective in homozygous *ctf7-1* plants.

(a) Sections of developing anthers revealed that *ctf7-1* had multiple anther developmental defects, including: a reduction in anther size (all stages), unsynchronized release of free tetrads (stage 7), release of irregular microspores (stage 8), lack of pollen mitosis II (stage 11), and failure of the septum (Sn) and stomium (St) to degenerate during anthesis (stage 12). (b) Left, transmission electron microscopy (TEM) showed that homozygous *ctf7-1* and *ctf7-2* pollen grains were smaller, lacked cytoplasm, a vegetative nucleus and sperm cells, right, staining with DNA dye 4',6-diamidino-2-phenylindole (DAPI, bottom) confirmed that *ctf7-1* and *ctf7-2* pollen lack identifiable vegetative nuclei and sperm cells, unlike wild-type pollen were vegetative nuclei and sperm cells were clearly observed. Abbreviations: T, tapetum; PMC, pollen mother cell; StR, stomium regium, E, epidermis; En, endothecium; ML, middle layer; V,
vascular tissue; C, connective tissue; MC, meiotic cell; Td, tetrad; Msp, microspore; Sm, septum; PG, pollen grain; Fb, fiber bands, St, stomium. Scale bars = in a, 25 µm for stages 5-8 and 50 µm for stages 9-12; in b left, 50 µm, and right: 2 µm.
Figure 5. Homozygous ctf7-1 male meiocytes are defective in chromosome pairing and segregation.

DAPI stained male meiocytes from WT and ctf7-1 plants are shown. A number of alterations are observed. (a) Preleptotene. Chromosomes of ctf7-1 failed to distribute in the nuclear periphery. (b)
Leptotene. Chromosomes failed to form condensed threads and remained scattered pattern. (c) Zygotene. Chromosomes of did not pair and align along the chromosome axis and the recombination foci were poorly observed. (d) Pachytene. Defects in synopsis with unpaired regions observed. (e) Diplotene. Alterations in chromosome condensation, with a mixture of unpaired chromosomes. (f) Diakinesis. A mixture of unpaired chromosomes, univalents and potential chromosome fragments were observed. (g) Metaphase I. Individual bivalents were not observed. Many meiocytes appeared to contain “extra” chromosomes. (h) Anaphase I. Chromosomes failed to segregate properly. Lagging chromosomes and chromosome bridges were observed. (i) Telophase I. Chromosomes failed to condense properly at the poles, and lagging chromosomes were observed throughout the cell. (j) Metaphase II. Chromosomes did not align properly at the equatorial planes and remained scattered in the meiocyte. (k) Anaphase II. Chromosomes failed to segregate properly. (l) Telophase II. Polyads were observed. Scale bars = 10 μm.
Figure 6. Chromosomes in homozygous *ctf7-1* male meiocytes exhibit defects in cohesion, chromosome fragmentation and extra chromosomes.

Merged images of DAPI stained chromosomes (red) and centromere FISH signals (green) are shown for male meiocytes (a to f), microspores (g), and anther somatic cells (h). (a) Leptotene. Approximately ten discrete CEN signals are observed in WT, while clusters of CEN signals are observed in *ctf7-1*. (b) Zygotene. CEN signals reduced to five to six in WT, while >10, were typically found in *ctf7-1*. (c) Pachytene. Five compact CEN foci are observed in synapsed WT chromosomes. Greater than five extended foci are observed in *ctf7-1*. (d) Diakinesis. Five CEN foci are present in desynapsing WT chromosomes. Greater than 20 small CEN signals are observed in *ctf7-1*. (e) Metaphase I. Four to five
pairs of CEN signals are present in WT as bivalents attach to the spindle. Greater than 20 small CEN signals are observed in ctf7-1. (f) Anaphase I. Two groups of five CEN signals are present in WT. Variable numbers of CEN signals are observed in ctf7-1 with some “chromosomes” containing ten signals and others no CEN signals. (g) Microspore. Five CEN signals are present in WT. Variable numbers (10-20) of CEN signals are present in ctf7-1. (h) Interphase. WT Ten CEN signals are present in WT. Greater than ten CEN signals are present in ctf7-1. Scale bars = 10 µm.
Figure 7. Meiotic cohesin subunits SMC3 and SYN1 exhibit an altered distribution pattern in homozygous ctf7-1 male meiocytes.
Merged images of DAPI stained chromosomes (red) and SMC3/SYN1 (green) are shown. (a) Interphase. SMC3 and SYN1 are distributed throughout the nuclei of WT male meiocytes, in contrast little or no SMC3 or SYN1 was observed within the nuclei of *ctf7-1*. (b) Leptotene. SMC3 and SYN1 decorated WT chromosome filaments as they started to condense, while in *ctf7-1* only labeling is weak and diffuse. (c and d) Early and Late Zygotene. SMC3 and SYN1 decorate WT chromosome axes as chromosomes start to synapse. (e) Pachytene. SMC3 and SYN1 lined the synapsed WT bivalents, but in *ctf7-1* the labeling remained weak and diffuse. (f) Diplotene. SMC3 and SYN1 continue to label WT bivalents, while in *ctf7-1* both proteins appear as scattered, punctuate foci. (g) Diakinesis. Chromosome-associated SMC3 and SYN1 begin to weaken in WT and are absent in *ctf7-1* nuclei. Scale bars = 10 µm.
Figure 8. DNA-repair genes are upregulated in leaf tissue of ctf7-1.

cDNAs from one-week-old WT and ctf7-1 seedlings were generated and used in QPCR. Transcript levels of ATM, PARP2, BRCA1, RAD51, SMC5, TOPOII-a, and CYCB1;1 are elevated in ctf7-1. Data are shown as means ± SD (n = 3) from three biological samples. Asterisks represent significant differences (*P<0.5, **P < 0.01, ***P < 0.001; Student’s t test) relative to WT.
Figure 9. DNA recombination and canonical cohesin subunit genes are upregulated in meiocytes of ctf7-1.

cDNAs were isolated and amplified from ctf7-1 seedlings and used in QPCR. Transcript levels of ATM, ATR, RAD51C, BRCA2B, DMC1, SMC1, SMC3 and CDC45 are elevated in ctf7-1. Data are shown as means ± SD (n = 3) from three biological samples. Asterisks represent significant differences (**P < 0.01, ***P < 0.001; Student’s t test) relative to WT.
Figure 10. Homozygous ctf7-1 seedlings exhibit reduced DNA repair efficiency.

(Top) Electrophoresis of leaf nuclei isolated from one-week-old plants after exposure to the radiomimetic agent, bleomycin sulfate (50 µg/µL). The images were processed with software to estimate the length of comet tails, which in the neutral comet assay correspond to DNA with double strand breaks. Compared to the wild-type (WT), the efficiency of DNA repair was dramatically reduced in ctf7-1 (bottom). Efficiency is compared by comparing the amount of tail DNA between t(x) and t(0). The length of comet tails was used to estimate the percent of double strand breaks remaining on nuclei after 0, 30 and 60 minutes exposure to bleomycin. After one hour, wild-type nuclei efficiently repaired most of the damage and left about one third ($29.6 \pm 1.51 \%$) of the initial double strand-breaks unrepaired. In contrast ctf7-1 nuclei still exhibited substantial damage leaving approximately three quarters ($76.5 \pm 9.73 \%$) of all breaks unrepaired. Data are shown as means $\pm$ SD ($n = 80$) from three biological samples. Asterisks represent significant differences (**$P < 0.01$; Student's t test) relative to WT.
Figure 11. Leaves of ctf7-1 and ctf7-2 mutant seedlings are defective in mitotic cell division. (a) Cryo-Scanning Electron Microscopy (Cryo-SEM) revealed a dramatic increase in cell size of ctf7-1 and ctf7-2 pavement cells (adaxial). (b) Total cell number per 1 mm² leaf area. (c) Statistic quantification of average pavement cell area. (d) Flow cytometry analyses of leaf cells DNA content (‘C’) showed a significant increase (‘4C’) in ctf7-1 and ctf7-2. Data are shown as means ± SD (n = 100 for B and C, and 10 000 for D) from three biological samples. Asterisks represent significant differences (**P < 0.01, ***P < 0.001; Student’s t test) relative to WT.
Figure 12. Homozygous *ctf7-1* and *ctf7-2* mitotic cells are defective in chromosome segregation.

DAPI staining reveals defective chromosome segregation in homozygous *ctf7-1* and *ctf7-2* mitotic root tip cells. Interphase cells in both WT (a) and the *ctf7-1* complementation line (Com, e) show approximately 10 chromocenters corresponding to a diploid number of chromosomes. In cells of *ctf7-1* (i) and *ctf7-2* (m) the chromocenters often appeared decondensed and in some instances an excess of 10 chromocenters are observed. In metaphase, WT (b) and Com (f) cells typically show 10 condensed chromosomes; however, chromosomes in cells of *ctf7-1* (j) and *ctf7-2* (n) appear less condensed and irregularly shaped. In anaphase, WT (c) and Com (g) cells show even segregation of chromosomes, and individual chromosomes can be recognized, while chromosomes in cells of *ctf7-1* (k) and *ctf7-2* (o) appear intertwined and stretched; condensation...
is also defective. In telophase, WT (d) and Com (h) cells chromosomes segregate into two well-condensed masses of equal size, but in ctf7-1 (l) and ctf7-2 (p) chromosome bridges persist and the chromosomes often recondense into a single unevenly-shaped mass of DNA (l and p). Scale bars = 10 µm.
Figure 13. Chromosome segregation in homozygous *ctf7-1* and *ctf7-2* mitotic cells is statistically different from the WT.

Results suggest an increase in defective chromosome segregation. Data are shown as means ± SD (n = 100) from forty biological samples. Asterisks represent significant differences (***P < 0.001; Student’s t test) relative to WT.
Figure 14. Retrotransposons *COPIA28*, *soloLTR*, and the *de novo* DNA methyltransferase *DRM2* are upregulated in homozygous *ctf7-1* and *ctf7-2* mutants.

Data are shown as means ± SD (*n* = 3) from three biological samples. Asterisks represent significant differences (*P* < 0.5, **P* < 0.01, ***P* < 0.001; Student’s *t* test) relative to WT.
Figure 15. Intergenic regions and retrotransposons *COPIA28*, and *soloLTR* show variable changes in the percent of methylated cytosines in mutant *ctf7-1*. Genomic DNA was collected from at least three biological samples. Number of clones analyzed per locus: 4-20. Three different methylation contexts are shown: CG (symmetric), CHG (asymmetric) and CHH (asymmetric). H corresponds to nucleotides that are either A, C or T. The results correspond to absolute changes in the % of methylated cytosines, not the mean.
## Table 1. Primers used in this report.

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**QPCR**

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To quantify expression of transposable elements.

To quantify expression of genes involved in gene silencing and histone modifications.
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References


Appendix 1. Eco1 regulates the acetylation cycle of yeast Smc (cohesin) proteins. During the S phase, yeast Eco1 acetylates Smc3. This reaction is required for the establishment of sister chromatid cohesion. In the G2/M phases, cohesion has been established, and Smc3 acetylation may act to stabilize it. At the metaphase-to-anaphase transition, separase cleavage of the cohesin Scc1 allows Hos1 to deacetylate Smc3. Adapted from Beckouet et al. (2010).
Appendix 2. Schematic representations of ECO1/CTF7 proteins from different organisms. The ECO1/CTF7 domain (gray box), C2H2 zinc finger domain (black box), and PCNA Interacting Box (PIP Box) (thin gray line) are shown. Accession numbers are as follows: Arabidopsis CTF7 (EU077499), O. sativa CTF7 (Q7XY81), S. pombe Esol (O42917), S. cerevisiae Eco1 (P43605), Homo sapiens Esco1 (Q5FWF5), Drosophila Eco (Q9VS50), H. sapiens Esco2 (Q56N19). aa: Amino acids. Adapted from Jiang et al. (2012).
Appendix 3. The ctf7-1 mutant shows changes in the expression of genes involved in RNA-directed DNA Methylation (RdDM) and histone modifications. Data are shown as means ± SD (n = 3) from three biological samples. Asterisks represent significant differences (**P < 0.01, ***P < 0.001; Student’s t test) relative to WT. Gene information: ARGONAUTE 4 (AGO4, At2g27040), CHROMATIN REMODELING FACTOR 17 (CH17, At5g18620), DEFECTIVE IN MERISTEM SILENCING 11/MICRORCHIDIA 6 (DMS11/MORC6, At1g19100), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1, At2g16390), DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2, At5g14620), HISTONE DEACETYLASE 6 (HDA6, At5g63110) and NUCLEAR RNA POLYMERASE D1B (NRPE1, At2g40030).
Appendix 3. Proposed functions performed by Eco1 proteins across different eukaryotic organisms. During the S phase, each chromosome (gray line) is replicated to produce two sister chromatids. Each sister also becomes chromatinized (histone assembly and/or modification) and competent to condense. Cohesins (green) are deposited onto each chromatid by Scc2 and Scc4 (SYN1-4 in Arabidopsis) and during most of the cell cycle, but deposition (blue arc) is essential specifically during S phase (thick blue arc). The Smc3 subunit of chromatid-bound cohesin is acetylated by Eco1 and this modification is essential during S phase to convert cohesins to a tethering-competent state termed cohesion establishment (red arc). Eco1-dependent acetylation can occur prior to origin firing during S phase but does not participate in sister chromatid cohesion establishment. Cohesins remain chromatin associated through G2 phase and into mitosis (spindle microtubules are shown in red, condensed chromosomes are shown as gray ovals) to maintain sister chromatid identity over time. In response to DNA damage during G2 and M phase, Eco1 is reactivated (broken red arc). During this time, the Smc3 component of the cohesin complex is deacetylated (green arc) by Hos1. Although Hos1 activity appears to be crucial in late mitosis and into G1, some deacetylation can precede anaphase (not shown). During G1, a soluble pool of cohesins continues to interact with DNA, consistent with the known roles of cohesin in both promoting and repressing transcription (blue arrows). Adapted from Rudra and Skibbens (2013).
CURRICULUM VITAE

Name: 何伯樂 (Pablo Alberto Bolaños Villegas)

Study program: Taiwan International Graduate Program-Molecular and Biological Agricultural Sciences (TIGP-MBAS), National Chung Hsing University and Academia Sinica.

Date of birth: March 15, 1979.

Country: Costa Rica, Central America.

E-mail addresses: pablobln@gate.sinica.edu.tw, pabloalbv@hotmail.com.

Current lab address: Inst. Plant Microbial Biol. Lab #126, Academia Sinica, Academia Road #128, Sec. 2, Nankang District, Taipei City 11529, Taiwan.

Future address: Fabio Baudrit Agricultural Research Station, University of Costa Rica, P.O. Box 183-4050, Alajuela province, Costa Rica.

Interests: meiosis, cell biology, pollen development, plant breeding.

Education

MSc in Horticulture. 2007. National Pingtung University of Science and Technology. Taiwan.


Papers


**Reviews and book chapters**


**Conference papers**


Awards

1st Prize. Performance award. Arabidopsis CHROMOSOME TRANSMISSION FIDELITY 7 (AtCTF7/ECO1) is required for meiosis. 2013. Annual Retreat of the Molecular and Biological Agricultural Sciences Program, Taiwan International Graduate Program, National Chung Hsing University and Academia Sinica. September 13-14, Hui-Shan Forest Station, Taichung county, Taiwan.

1st Prize. Academic award. Arabidopsis CHROMOSOME TRANSMISSION FIDELITY 7 (AtCTF7/ECO1) is required for meiosis of sporocytes, mitosis and DNA repair. 2012. Annual Retreat of the Molecular and Biological Agricultural Sciences Program, Taiwan International Graduate Program, National Chung Hsing University and Academia Sinica. September 12 and 13, Taipei, Taiwan.

Young scientist travel grant award. 2012. Institute of Plant and Microbial Biology, Academia Sinica, Taiwan.