Running title: Role for CDC48/p97 during development

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Research Area: Cell Biology
In planta analysis of the cell cycle-dependent localization of AtCDC48A and its critical roles in cell division, expansion, and differentiation

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ABSTRACT

CDC48/p97 is a conserved homohexameric AAA-ATPase chaperone required for a variety of cellular processes but whose role in the development of a multicellular model system has not been examined. Here we have used reverse genetics, visualization of a functional Arabidopsis CDC48 fluorescent fusion protein, and morphological analysis to examine the subcellular distribution and requirements for AtCDC48A in planta. Homozygous Atcdc48A T-DNA insertion mutants arrest during seedling development exhibiting decreased cell expansion and display pleiotropic defects in pollen and embryo development. Atcdc48A insertion alleles show significantly reduced male transmission efficiency due to defects in pollen tube growth. YFP-AtCDC48A, a fusion protein that functionally complements the insertion mutant defects, localizes in the nucleus, cytoplasm, and is recruited to the division midzone during cytokinesis. The pattern of nuclear localization differs according to the stage of the cell cycle and differentiation state. Inducible expression of an Atcdc48A Walker A ATPase mutant in planta results in cytokinesis abnormalities, aberrant cell divisions, and root trichoblast differentiation defects apparent in excessive root hair emergence. At the biochemical level, our data suggests that the endogenous steady-state protein level of AtCDC48A is dependent upon the presence of ATPase-active AtCDC48A. These results demonstrate that CDC48A/p97 is critical for cytokinesis, cell expansion and differentiation in plants.
INTRODUCTION

Members of the AAA (ATPase Associated with different cellular Activities) ATPase protein family are characterized by either one (type I) or two (type II) 220-250 amino acid ATPase domains containing both conserved Walker A and B motifs per protomer (Beyer, 1997; Neuwald et al., 1999). The ATPase domains elicit protein conformational changes upon the nucleotide binding, hydrolysis, and product release that is believed to be required for the function of the mechano-chemical enzyme (Rouiller et al., 2000; Zhang et al., 2000; Rouiller et al., 2002; Beuron et al., 2003; DeLaBarre and Brunger, 2003; Huyton et al., 2003; Wang et al., 2003; Davies et al., 2005; DeLaBarre and Brunger, 2005; Beuron et al., 2006). The conservation and widespread use of the AAA domain suggests that AAA ATPase proteins may use common mechanisms that utilize their ATPase activity to carry out a wide range of cellular functions (Lupas and Martin, 2002). CDC48/p97 is a highly abundant type II AAA-ATPase (Peters et al., 1990) involved in cell cycle control (Moir et al., 1982) and cell proliferation (Egerton and Samelson, 1994). Gene disruption of CDC48/p97 in budding and fission yeasts (Fröhlich et al., 1991; Ikai and Yanagida, 2006), trypanosomes (TbVCP) (Lamb et al., 2001), mouse (Muller et al., 2007), and Drosophila (ter94) (Leon and McKearin, 1999) demonstrate that CDC48/p97 is essential in unicellular and multicellular organisms. At the molecular level, CDC48/p97 is involved in many distinct cellular processes (Woodman, 2003; Dreveny et al., 2004; Meyer, 2005; Jentsch and Rumpf, 2007). Current models indicate that the targeting of CDC48/p97 family members to cellular pathways is accomplished via recruitment by adapter proteins or cofactors. For example in mammalian cells, CDC48/p97 requires the cofactor p47 to mediate ER and Golgi membrane assembly (Kondo et al., 1997; Roy et al., 2000; Yuan et al., 2001) and nuclear envelope reformation (Hetzer et al., 2001). An additional cofactor complex, Ufd1-Npl4, is required for p97-mediated formation of the chromatin-associated nuclear envelope network, consolidation of the nuclear envelope (Hetzer et al., 2001), mitotic spindle dynamics (Cao et al., 2003; Cao and Zheng, 2004; Cheeseman and Desai, 2004), and ERAD, (ER-associated protein degradation) (Alzayady et al., 2005; Schuberth and Buchberger, 2005; Römisch, 2006).
Mutations in CDC48/p97 interacting proteins have provided insight into the physiological and developmental functions of CDC48/p97-dependent biochemical pathways. eyes closed (eyc), a loss-of-function allele of the Drosophila p47 adapter protein ortholog (Sang and Ready, 2002) shows nuclear envelope assembly defects in early zygotic divisions in Drosophila. In Arabidopsis, loss of PUX1, a negative regulator of CDC48/p97 function (Rancour et al., 2004; Park et al., 2007), results in accelerated plant growth due to increased cell division and expansion (Rancour et al., 2004).

Plant morphological development requires the coordination of cell division, expansion, and differentiation (Meijer and Murray, 2001; Beemster et al., 2003; Fleming, 2006). Our previous work has suggested that AtCDC48 may be directly involved in cell division and expansion (Rancour et al., 2004). The Arabidopsis genome encodes three CDC48 isoforms: AtCDC48A (At3g09840), AtCDC48B (At3g53230) and AtCDC48C (At5g03340). These isoforms are predicted to share 91% (AtCDC48B) and 95% (AtCDC48C) amino acid identity to AtCDC48A (Rancour et al., 2002). Expression of AtCDC48A mRNA is highest in proliferating cells of the vegetative shoot, root, and flowers in rapidly growing plants (Feiler, et al., 1995; Zimmerman, et al., 2004; Zimmerman, et al., 2005).

At the subcellular level AtCDC48A has been shown by immuno-fluorescence microscopy to be localized to the cytoplasm, nucleus, and to the phragmoplast midzone during cytokinesis (Feiler et al., 1995; Rancour et al., 2002). In addition, overexpression studies in plant protoplasts of fluorescent fusion protein (FFP)-tagged AtCDC48A have suggested that the chaperone is associated with the ER and plasma membrane (Aker et al., 2006; Aker et al., 2007).

The role of the CDC48/p97 protein family during growth and development has not been examined to date in planta. Here we show, through live-cell imaging, the expression and analysis of loss-of-function and inducible dominant-negative ATPase defective mutants, that AtCDC48A is essential for plant growth and development at various stages. These results provide the first direct evidence for CDC48/p97 function in plant cytokinesis, cell expansion and differentiation. In addition, our data support a role for AtCDC48A ATPase function in maintenance of steady-state AtCDC48A protein levels, thus suggesting a mode of protein turnover auto-regulation.
RESULTS

Molecular characterization and phenotypic analysis of Atcdc48A T-DNA insertion mutants

The gene encoding AtCDC48A is 3.3 kb in length and composed of 8 exons (Figure 1A). To characterize the biological function of AtCDC48A, we identified 3 independent Atcdc48A T-DNA insertion lines (Atcdc48A\textsuperscript{T-DNA} -1, -2, and -3). All three Atcdc48A alleles exhibited identical phenotypes. The T-DNA insertion site in each of the alleles was verified by PCR amplification and DNA sequence analysis. The T-DNAs in Atcdc48A\textsuperscript{T-DNA}-1, Atcdc48A\textsuperscript{T-DNA}-2, and Atcdc48A\textsuperscript{T-DNA}-3 were inserted in the 1\textsuperscript{st} intron, 3\textsuperscript{rd} exon, and 3\textsuperscript{rd} intron, respectively, of AtCDC48A (Figure 1A). All three T-DNA insertion sites are upstream of the sequences encoding the two ATPase domains. No viable soil-grown homozygous plants for any of the Atcdc48A\textsuperscript{T-DNA} insertion alleles were identified from progeny of self-fertilized heterozygous parent plants. To verify that the mutant alleles are recessive, progeny from self-fertilized heterozygous Atcdc48A\textsuperscript{T-DNA} mutants were grown on solid Murashige and Skoog (MS) (Murashige and Skoog, 1962) media and were monitored for growth. Approximately 4% of the germinated seedlings (14 seedlings from 346 seeds plated) on solid MS media were homozygous for the T-DNA insertion. Homozygous Atcdc48A\textsuperscript{T-DNA} seedlings arrested 1 day after germination and the mutants exhibited severely disorganized root morphology (Figure 1B). As shown in figure 1B, roots of homozygous Atcdc48A\textsuperscript{T-DNA} mutant were less than 0.5 mm in length with a root tip that was narrower than wild-type (Figure 1C). Morphologically, Atcdc48A\textsuperscript{T-DNA} mutant roots displayed abnormal cell files, with no apparent elongation or meristematic regions, and a disorganized root cap (Figure 1C). These results indicated that AtCDC48A is critical for seedling development and growth.

The allelic ratio of F\textsubscript{1} progeny from a self-fertilized heterozygous Atcdc48A\textsuperscript{T-DNA}/+ plant did not follow the predicted frequency of segregation for a typical recessive mutation, suggesting earlier defects in development. Therefore, 10 days after flowering, immature siliques from 2 generations of self-fertilized heterozygous plants (~40 siliques
of each generation) were examined. In a visual survey of seeds from heterozygous Atcdc48A<sup>T-DNA</sup> siliques, pale green seeds, suggesting embryo development defects (Meinke, 1994) (http://www.seedgenes.org/Tutorial.html), were observed at a frequency of 5 pale seeds out of 60 total (~8%) (Figure 1D). PCR-based genotypic analysis of embryos from the pale green seeds indicated they were homozygous for the Atcdc48A<sup>T-DNA</sup> insertions (Figure S1). To determine at which stage in embryo development homozygous Atcdc48A<sup>T-DNA</sup> mutants arrested, 5 days post-fertilization siliques from wild-type and heterozygous Atcdc48A<sup>T-DNA/+</sup> mutant plants were cleared with Hoyers solution (Liu and Meinke, 1998) and examined by light microscopy using differential interference contrast (DIC) optics. Atcdc48A<sup>T-DNA</sup> mutant embryo growth was arrested at the early heart stage of development (Figure 1E) supporting a role for AtCDC48A in plant embryogenesis.

PCR-based genotype analysis of 10 day-old soil grown surviving progeny from self-fertilized heterozygous Atcdc48A<sup>T-DNA/</sup> plants showed an altered segregation ratio (78 wild-type:114 heterozygote [41%:59%, respectively]; n=192) in contrast to the predicted frequency for a recessive embryo/seedling lethal mutation (1 wild-type:2 heterozygote [33%:67%, respectively]). The altered segregation ratio suggests impairment of mutant allele transmission through the gametes. To confirm this, male and female transmission efficiencies of the Atcdc48A<sup>T-DNA</sup> alleles were determined by performing reciprocal crosses between heterozygous mutants and wild-type plants. Pollen grains from the wild-type plants were able to fertilize Atcdc48A<sup>T-DNA/</sup> plants to yield progeny that segregated in the ratio of 1:0.73 (124 wild-type:91 heterozygote [58%:42%, respectively]; n=215). Using Atcdc48A<sup>T-DNA/</sup> pollen to fertilize wild-type stigmas resulted in a highly skewed segregation of progeny genotype (235 wild-type:13 heterozygote [95%:5%, respectively]; n=248). Atcdc48A<sup>T-DNA</sup> alleles show significantly reduced male transmission efficiency and modest female transmission efficiency defects. These results suggest a critical role for AtCDC48A in pollen development and/or function.
Analysis of pollen development in *Atcdc48A* mutants

To determine at which developmental stage *Atcdc48A* pollen was defective, the development, maturation and germination of pollen grains from wild-type and heterozygous *Atcdc48A*/*+ plants was analyzed by DIC and epifluorescence microscopy. Staining of nuclear DNA with 4,6-diamidino-2-phenylindole (DAPI) was used to analyze pollen developmental progression. No apparent defects in late microsporogenesis, including tetrad formation and microspore release were observed compared to wild type. After microspore release and pollen mitosis I and II, all the pollen grains from wild-type and *Atcdc48A*/*+ plants contained two generative and one vegetative nucleus (Figure 2A) and thus appeared normal. Two models are consistent with these data: either *Atcdc48A* mutant pollen arrest at an early developmental stage prior to late microsporogenesis (meiosis II) or, alternatively, during stages after pollen mitosis II that include the processes of pollen germination, pollen tube guidance and/or egg sac fertilization. To investigate these possibilities, the *Atcdc48A*-1 and *quartet* (*qrt*) mutant alleles were introgressed by cross-pollination between heterozygous *Atcdc48A*-1/*+ and *quartet* mutant plants. The *qrt* mutation results in pollen grains that remain physically associated in a tetrad and undergo synchronous development due to a defect in the pollen mother cell wall that results in microspore dissociation failure after meiosis II (Preuss et al., 1994; Rhee and Somerville, 1998). Pollen from wild-type *qrt* (*AtCDC48A;qrt*) and heterozygous *Atcdc48A*-1/*+;*qrt* lines was monitored by bright field microscopy (Figure 2B and C). As shown, four pollen grains of *Atcdc48A*-1/*+;*qrt* remained associated indicating that the *Atcdc48A*-1/*+;*qrt* mutation does not disrupt meiotic processes in pollen development.

To test whether pollen germination was affected by the *Atcdc48A*-1 insertion mutations, *in vitro* pollen germination assays were performed. Pollen tubes were observed protruding from all four germinating wild-type *AtCDC48A;qrt* pollen grains (Figure 2B). In contrast, only two pollen grains germinated from *Atcdc48A*-1/*+;*qrt* pollen (Figure 2C) with approximately 5% showing delayed growth of a third pollen tube (Figure 2C, arrows). These data indicate a critical role for AtCDC48A in pollen
germination and tube elongation that manifests in significant transmission inhibition of the \( Atcd48A^{T-DNA} \) insertion alleles.

**Characterization and localization of native promoter::YFP- AtCDC48A in homozygous \( Atcd48A^{T-DNA} \) seedlings**

To examine the tissue and subcellular localization of AtCDC48A, transgenic Arabidopsis plants expressing an N-terminal tagged yellow fluorescent (YFP) (Heim and Tsien, 1996) AtCDC48A fusion protein (YFP-AtCDC48A) under control of the native \( AtCDC48A \) promoter were generated. This construct was introduced into heterozygous \( Atcd48AT-DNA/+ \) plants and the T2 population was analyzed by both YFP screening and PCR genotyping (Figure S2) to assess complementation. The YFP-AtCDC48A construct rescued all the phenotypic defects associated with the \( Atcdc48AT-DNA \) mutants described above. Immunoblot analysis of protein extracts prepared from wild type and \( Atcd48A;YFP-AtCDC48A \) seedlings using anti-AtCDC48A and -GFP antibodies confirmed that the 118 kDa YFP-AtCDC48A fusion protein was intact (Figure S3).

Expression of YFP-AtCDC48A was detected throughout developing \( Atcd48A^{T-DNA}/Atcd48A^{T-DNA};YFP-AtCDC48A \) transgenic plants (Figure 3 and Supplemental movies 1, 2, 3, and 4) and is consistent with publicly available gene expression data (Figure S4). YFP-AtCDC48A fusion protein localizes to the nucleus and cytoplasm of all cells throughout all tissues of the seedling including leaves, the shoot apical meristem (Figure 3A and Supplemental movie 1), and the root (Figure 3B-D and Supplemental movies 3 and 4). In addition, YFP-AtCDC48A fusion protein was observed in developing ovules, pollen sacs, and pollen in mature plants. In germinating pollen, YFP-AtCDC48A was detected in the vegetative nucleus and surrounding the nuclear periphery but was not detected in the two sperm nuclei. Additionally, YFP-AtCDC48A was localized to the growing tip of the pollen tubes (Figure 3E and Supplemental movie 2).

Upon closer examination in root cells, YFP-AtCDC48A subcellular localization was cell cycle dependent. During mitosis, YFP-AtCDC48A was associated with the mitotic spindle (Figure 3C, arrow) and subsequently with the phragmoplast midzone during cytokinesis (Figure 3C and D arrow head). During karyokinesis, YFP-AtCDC48A
containing vesicle-like structures began to localize and coalesce around the chromosomal material (Figure 3D, F and see Supplemental movie 3). Following cell division, YFP-AtCDC48A was associated with the nuclear envelope, nucleoplasm and nucleolar cavity (Figure 3C, D, F, G and see Supplemental movie 3).

**Phenotypic analysis of transgenic Atcdc48A dominant negative (Atcdc48A<sup>DN</sup>) mutant plants**

Loss-of-function insertion alleles of Atcdc48<sup>T-DNA</sup>-1, 2, and 3 display early pleiotropic developmental phenotypes. To examine the role of AtCDC48A in later stages of plant development, transgenic plants expressing dominant-negative mutant Atcdc48A proteins (Atcdc48A<sup>DN</sup>) under the control of an ethanol inducible promoter system from Aspergillus nidulans (Caddick et al., 1998; Roslan et al., 2001) were generated. Using this system, the function of endogenous AtCDC48A was temporally inactivated during the seedling and later stages of plant development thus circumventing the gametophyte and seedling lethal phenotypes encountered with the Atcdc48<sup>T-DNA</sup> loss-of-function insertion alleles. Previous studies have demonstrated that the incorporation of cdc48/p97 D1 and D2 ATP binding and hydrolysis mutant subunits into endogenous CDC48/p97 hexameric complexes disrupts their function in vivo and in vitro (Lamb et al., 2001; Dalal et al., 2004; Park et al., 2007) and thus function as dominant-negative proteins (subunits).

Ethanol-inducible AtCDC48A (H6T7-WT), Atcdc48A<sup>DN-H</sup> (H6T7-DN-H; ATP hydrolysis [E308Q/E581Q]) and Atcdc48A<sup>DN-B</sup> (H6T7-DN-B; ATP binding [K254A/K572A]) plant expression constructs contained an N-terminal 6 histidine and T7 (H6T7) tandem epitope tag to facilitate immunological detection of the transgene products. In the absence of ethanol, H6T7-tagged wild-type and Atcdc48A<sup>DN-H. B</sup> mutant proteins were not detected by immunoblot analysis (Figure 4A, lanes 1, 4 and 7). Upon the addition of (2%) ethanol to the growth medium, expression of H6T7-tagged WT AtCDC48A and Atcdc48A<sup>DN-H. B</sup> proteins were readily detected after 6 hours (Figure 4A, lanes 2, 5, and 8) and after approximately 24 hrs the levels of H6T7-DN mutant protein (Figure 4A, lanes 3, 6, and 9) were approximately 10 fold higher than H6T7-WT based on densitometric immunoblot analysis. Induction of H6T7-tagged wild-type and Atcdc48A<sup>DN-H. B</sup> mutant proteins did...
not vary over an ethanol application range of 0.5–2% (v/v). In addition, untransformed seedling growth was not affected under any of the induction conditions used.

After 48 hours post ethanol treatment, steady state levels of total AtCDC48A protein (Figure 4B, top panel) are highly elevated in lines expressing H6T7-DN protein (Figure 4B, lanes 4-7). Parallel ethanol-treatment of H6T7-WT protein expressing lines (Figure 4B, lanes 2 and 3) show that total AtCDC48A protein levels are similar to wild-type plants (Figure 4B, lane 1). In lines expressing H6T7-DN proteins, the levels of endogenous AtCDC48 and transgene H6T7-AtCDC48-DN protein products was greatly increased relative to H6T7-WT (Figure 4B, middle anti-T7 panel, compare lanes 2 and 3 to 4-7). DRP1A protein detection was used as a loading control (Figure 4B, lower panel).

To determine if the elevated levels of H6T7-DN proteins, relative to H6T7-WT, were due to altered transcript levels for the transgenes, RT-PCR analysis was performed on total RNA from ethanol treated transgenic seedlings (Figure 4C). Expression of transgenic Atcdc48 mRNA was detected 2 hours after ethanol induction (Figure 4C, Lanes 2, 6 and 10) and increased steadily over the 24 hour time course (Figure 4C, lanes 3-4, 7-8, and 11-12). mRNA expression levels were comparable between wild-type and mutant transgenes.

Transgenic plants expressing wild-type AtCDC48A (H6T7-WT) exhibited no abnormal phenotypes (Figure 5B, D and F, and Figure S5). In contrast, transgenic plants expressing either Atcdc48A<sup>DN-B</sup> or Atcdc48A<sup>DN-H</sup> exhibited aberrant morphological phenotypes in both the aerial and root regions of the plant (Figure 5A, C, E, and G, and Supplemental Figure 5). As shown in Figure 5A, the cotyledons of seedlings expressing H6T7-DN-B stopped expanding and became chlorotic as compared to plants expressing H6T7-WT, the wild-type control (Figure 5B and S5). H6T7-DN-B trichomes were primarily bi-branched and smaller when compared to those from AtCDC48A plants (Figure 5C and 5D, respectively). Roots of H6T7-DN-B plants displayed altered root hair morphology, frequency and distribution (Figure 5E, compared to wild-type right panel Figure 5F). Seedling roots expressing H6T7-DN-B protein showed an enhanced number of epidermal cell root hairs (Figure 5E, G) relative to root expressing H6T7-WT (Figure 5F). In addition, root growth arrested upon induction of H6T7-DN-B and H6T7-DN-H dominant-negative protein expression after ethanol treatment relative to H6T7-WT.
(Figure 5H). *H6T7-DN-H* plants exhibited less severe phenotypes overall than *H6T7-DN-B* plants, including root length (Figure 5H), excessive root hair emergence, and aerial yellowing as observed in the *H6T7-DN-B* plants (Figure S5).

**Analysis of the effects of Atcdc48^{DN-B} expression on root cell development**

To further investigate the morphology of seedlings expressing Atcdc48^{DN-B}, root cross-sections from plants before and after ethanol induction were examined via microscopic analysis. Transverse sections of seedling roots from plants expressing H6T7-WT and H6T7-DN-B were analyzed from the root tip to the differentiation zone (Figure 6). Root cell file organization and differentiation differ according to their longitudinal position (Figure S6). Root cell division occurs predominantly within the zone proximal to the root tip (~100 µm). Within the expansion zone, ~300 µm from the root tip, the root cap cells can be seen in the outermost cell layer and cells start to expand longitudinally. Distal to the region of cell expansion, (~1 mm relative to the root tip) root hairs start to emerge from root epidermal cells (trichoblasts) positioned over the junction of two underlying cortex cells (Dolan et al., 1993).

Prior to the addition of ethanol, *H6T7-DN-B* serial transverse root section analysis indicated no variation in the number of cell files or layers throughout the root length when compared to ethanol induced *H6T7-WT* wild-type roots (Figure 6, columns 1 and 2) (Dolan et al., 1993). Root hairs were observed to emerge from a root trichoblast cell, as presented previously (Figure S6), in both ethanol-induced *H6T7-WT* and uninduced *H6T7-DN-B* mutant roots (Figure 6M and N). In contrast, root hairs of the ethanol-treated *H6T7-DN-B* mutant seedlings were observed immediately above the root cap and emerged from epidermal cells positioned relative to either one or two underlying cortex cells (Figure 6F, black arrowhead, and Figure 5G), thus suggesting cell fate determination defects in the presence of *H6T7-DN-B*. In addition to root hair abnormalities, cell division defects were observed in endodermal and cortex cells (Figure 6, panels C, I, L, and O) of ethanol-treated *H6T7-DN-B* plants. The observed cell division defects manifest as either (1) apparent cytokinesis defect including cell wall stubs (incomplete divisional cell walls) resulting in inappropriate cellular continuity between
neighboring endodermal and/or cortical cell files (Figure 6L and O, black arrows), or (2) inappropriate cell divisions including spurious periclinal divisions in the cortex (Figure 6C, open arrows) and extra anticlinal divisions in the epidermis (Figure 6I, open arrows). These data demonstrate a role for CDC48/p97 in plant cell division, cytokinesis and cell fate determination.

**DISCUSSION**

**AtCDC48A has multiple roles during development**

The AAA ATPase, CDC48/p97, has been shown to function in various pathways including organelle biogenesis and protein degradation (Peters et al., 1992; Acharya et al., 1995; Rabouille et al., 1995). Localization studies and other studies in Arabidopsis implicate a role for AtCDC48A in plant cytokinesis and cell expansion (Feiler et al., 1995; Rancour et al., 2002; Rancour et al., 2004).

To determine if CDC48/p97 function is required for plant cell division, three independent Arabidopsis T-DNA insertion alleles of *Atcdc48A* were identified and characterized. All three *Atcdc48A* alleles were recessive and displayed the same phenotypes including (1) defects in pollen tube germination and expansion, (2) embryo developmental arrest, and (3) seedling lethality (Figure 1 and 2). The nature of the variable expressivity of the mutant phenotypes remains to be determined.

AtCDC48A is a highly abundant protein in Arabidopsis (Rancour et al., 2004). One possible explanation for the variable expressivity of the T-DNA mutant phenotypes is that sufficient levels of paternal and/or maternal levels of AtCDC48A are present during embryogenesis to grant some homozygous embryos the capacity to proceed to the next major AtCDC48A-dependent developmental stage. Another possible explanation is that expression of *AtCDC48B* or *AtCDC48C* may compensate for the loss of AtCDC48A in early stages of development, as AtCDC48B and C share 91% and 95% amino acid sequence identity, respectively, with full-length AtCDC48A (Rancour et al., 2002).
Nevertheless, analysis of homozygous \textit{Atcdc48A}^{T-DNA} mutants demonstrated that \textit{AtCDC48A} is absolutely required for seedling development.

Morphological analysis of \textit{Atcdc48A}^{T-DNA} seedlings has implicated \textit{AtCDC48A} involvement in multiple seedling developmental processes. Root cells in homozygous \textit{Atcdc48A} seedlings were disorganized and morphologically abnormal suggesting roles for \textit{AtCDC48A} in cell proliferation and expansion (Figure 1C) and plasma membrane integrity. CDC48 has been shown to be associated with the plasma membrane in soybean (Shi et al., 1995) and the Arabidopsis SERK1, a plasma membrane-localized LRR receptor-like serine threonine kinase, interacts with \textit{AtCDC48A} (Shah et al., 2002; Rienties et al., 2005). These results indicate that \textit{AtCDC48A} may have a role in plasma membrane biogenesis and signaling.

To circumvent the range of early plant developmental defects observed in the \textit{Atcdc48A}^{T-DNA} mutants, we took an alternative approach, utilizing inducible dominant-negative \textit{Atcdc48A}^{DN-B} gene expression, to examine the \textit{in vivo} role of \textit{AtCDC48A} in plant growth and development. The presence and accumulation of \textit{Atcdc48A}^{DN-B} protein correlated with phenotypes observed throughout the plant including (1) the arrest of leaf growth and expansion as well as early leaf senescence, (2) alterations in the frequency and distribution of root hairs and (3) arrest of root growth with defects in cytokinesis and cell expansion (Figures 5 and 6). The presence of ectopic root hairs in plants expressing \textit{Atcdc48A}^{DN-B} suggests that the chaperone may modulate signal transduction pathways and/or transcription factor levels essential for trichoblast/atrichoblast cell fate determination. More importantly, these \textit{in vivo} dominant negative mutant studies provided direct evidence that \textit{AtCDC48A} is required for plant cytokinesis. Consistent with stereotypical plant cytokinesis defects (Lauber et al., 1997; Waizenegger et al., 2000; Strompen et al., 2002; Falbel et al., 2003; Kang et al., 2003), incomplete cell walls and cell wall stubs were observed in induced transgenic \textit{Atcdc48A}^{DN-B} root endodermal and cortical cells (Figure 7L and O). An intriguing feature of these cytokinesis defects is their prevalence in the endodermal and cortical cell layers and along their interface. The endodermis and cortex arise from a specific longitudinal division of a progenitor cell to give rise to two distinct cell type fates (Dolan et al., 1993). Based on our data, \textit{AtCDC48A} may have a role in the completion of that longitudinal cell division.
work is needed to determine the cell fate identity of the resulting cell types after ethanol treatment in the ethanol-inducible dominant-negative mutant plants.

**YFP-AtCDC48A localization**

The presence of YFP-AtCDC48A signal in regions of cell proliferation and expansion are consistent with our hypothesis that AtCDC48A plays a role in cell division and expansion. In yeast and Xenopus, Cdc48p/p97 have been shown to be associated and to regulate mitotic spindle dynamics (Fröhlich et al., 1991; Cao et al., 2003; Cao and Zheng, 2004; Cheeseman and Desai, 2004). The mitotic spindle localization of AtCDC48A (Fieler et al. 1995) and YFP-AtCDC48A (this study) in plant cells provides additional support that the chaperone may function in plant mitosis.

Following mitosis, AtCDC48A is relocated to the phragmoplast midzone and during late telophase, with the reforming nuclear envelope. Our current hypothesis is that AtCDC48A is required for secretory and nuclear membrane dynamics to complete cell division. Consistent with this CDC48/p97 have been shown to be required for secretory and nuclear membrane fusion and assembly in yeast and mammalian cells (Moir et al., 1982; Fröhlich et al., 1991; Hetzer et al., 2001; Sang and Ready, 2002).

In interphase cells we observed that YFP-AtCDC48A was predominantly associated with the nucleus and, to a lesser extent, cytoplasm. The various specific patterns of nuclear-associated YFP-AtCDC48A localization observed within plant cell roots have not been reported in any other system. YFP-AtCDC48A was observed in discrete foci within the nucleoplasm and within the nucleolar cavity. The nucleoplasmic structures resemble nuclear bodies, such as speckles, which could implicate a function for AtCDC48A in mRNA processing and export (Pendle et al., 2005). The nucleolar cavity has been suggested to be the site of cleavage and modification of pre-rRNAs (Beven et al., 1995; Beven et al., 1996; Shaw and Brown, 2004). However, no detectable changes in 18S and 25S RNA processing were observed in Atcdc48A mutants line indicating that the localization of AtCDC48A within the nucleolar cavity is likely unrelated to rRNA processing. The function of the AtCDC48A containing subnuclear structures remains to be determined.
AtCDC48A protein abundance levels are self-modulated

In addition to its roles in plant growth and development, dominant-negative expression of Atcdc48ADN-B, H suggested that AtCDC48A may regulate its own steady-state protein level (Figure 4). Our previous work supported a model whereby mutant Atcdc48ADN-B, H protomers are incorporated into endogenous wild-type hexamers thus inactivating the chaperone (Park et al., 2007). We have also found that the UBX-containing protein, PUX1, functions to regulate the hexameric structure and ATPase activity of AtCDC48A. However, PUX1 was unable to disassemble nucleotide binding or hydrolysis mutants of AtCDC48A complexes (Park et al., 2007). One model to explain the altered Atcdc48A mutant protein accumulation in planta is the inhibition of ATPase-defective hexamers to be disassembled (Park et al., 2007), a likely prerequisite for degradation. Yeast Cdc48p is poly-ubiquitylated (Mayor et al., 2005), suggesting that disassembly of the CDC48/p97 hexamer into individual subunits is a step in the pathway to its proteasome-dependent degradation. Taken together, these data support a model whereby a certain level or range of AtCDC48A activity is required for cell function and that AtCDC48A protein activity levels are self maintained in order to maintain this homeostasis. Future experiments into how the active pool of AtCDC48A activity is sensed and subsequently balanced remain to be studied.

ATP binding is more important that ATP hydrolysis for AtCDC48A function

One subtle but reaffirmed observation was that plants expressing inducible Atcdc48ADN-B had a more severe phenotype than Atcdc48ADN-H during seedling growth and development. Given that expression levels of the mutant protein forms were comparable, this would suggest that there are functions for nucleotide-substrate bound form of the enzyme that are distinct from those requiring hydrolysis of that nucleotide substrate. A significant conformational change, including rearrangement of the N terminal domains occurs upon nucleotide binding to the CDC48/p97 hexamer (Rouiller et al., 2000). It has been hypothesized that the critical conformational change in the
mammalian homolog of AtCDC48A, p97, is a large rearrangement upon nucleotide binding rather than the smaller changes associated with nucleotide hydrolysis. The location and number of molecules of the adaptor protein p47 molecules bound to p97 varies significantly in the nucleotide-free and ADP-bound states, suggesting that p47 requires ATP to form a tight complex with p97 (Dalal and Hanson, 2001). High affinity binding of substrates in the ATP-bound state has been proposed to be a common feature of AAA proteins (Vale, 2000). Our in vivo data is consistent with this proposal.

We conclude that AtCDC48A is required for many cellular processes necessary for plant growth and development. Our data provide new evidence for AtCDC48A function in plant cytokinesis and cell expansion and the ability of the enzyme to self-modulate its steady-state protein levels.

METHODS

Oligonucleotides Used in This Study

Oligonucleotide sequences shown in Table 1 were synthesized by Integrated DNA Technologies (Coralville, IA). Capitalized sequences represent those complementary to the AtCDC48A locus. Underlined lowercase letters in the oligonucleotide sequences indicate added restriction enzyme sites used for cloning.

Isolation of Atcdc48A T-DNA Insertion Mutants

Three independent Atcdc48A mutant lines containing T-DNA insertions (Atcdc48AT-DNA), SALK_064573, SALK_064893, and SALK_116074 (Alonso et al., 2003), were identified using T-DNA Express search algorithms from the Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress), and seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, at The Ohio State University, Columbus, OH). Atcdc48AT-DNA insertion lines were grown on solidified (0.6% phytoagar) Murashige and Skoog (Murashige and Skoog, 1962) medium (from either Gibco BRL, Rockville, MD or Caisson Laboratories, Inc. Rexburg, ID) and
transplanted into soil (either Superfine Germination Mix, Con-rad Fafard, Agawam, MA or Metro Mix-360, Sun Gro Horticulture, Bellevue, WA) treated with Adept® (Uniroyal Chemical Company Inc., Middlebury, CT) and grown under fluorescent light illumination cycles of 16 hours light and 8 hours dark at 22°C. The mutant lines were screened for $Atcde48A^{T-DNA}$ using oligonucleotide primers SB372, SB400, SB401, SB513, and SB514 (Table 1). DNA sequencing of the PCR-amplified products determined the T-DNA insertion positions within $AtCDC48A$. The genotype of segregating plants was confirmed by PCR using allele-specific primer pairs. For analysis of $AtCDC48A^{T-DNA}$ mutant developing embryos, siliques were cleared in Hoyers solution (0.25g v/w gum Arabic, 3.3 g v/w chloral hydrate, 0.15g v/v glycerol in dH2O) (Meinke, 1994; Liu and Meinke, 1998) and observed with the bright-field light microscopy. To visualize cell organization in seedling roots, tissue was stained with 0.1 mg/ml propidium iodide for 30 seconds, washed 4 times with de-ionized water, and observed using a confocal laser scanning microscopy (Nikon Eclipse TE 2000-U, Japan). Captured images were processed using Image J 1.32 (Wayne Rasband, National Institutes of Health, USA) and Adobe Photoshop and Illustrator (Adobe Systems, San Jose, CA, USA) imaging software on Macintosh computers (Apple Computer, Cupertino, CA, USA).

**In-vitro pollen germination**

Pollen viability and germination was monitored as described (Li et al., 1999). Heterozygote $Atcde48A;qrt$ flowers were harvested and allowed to dehydrate at room temperature for 90 min at 22°C. Pollen grains were transferred to pollen germination media [18% (w/v) sucrose, 0.6% (w/v) phytagar, 0.01% (w/v) boric acid, 1mM MgSO$_4$, 2.5 mM CaCl$_2$, 2.5 mM Ca(NO$_2$)$_2$, pH 7.0] by tapping mature anthers on the surface of the pollen germination medium. Pollen grains were germinated at 28°C for 6 to 24 hours and imaged with a Zeiss Axioskop (Carl Zeiss, Thornwood, NY, USA) equipped with DIC filter sets and a cooled charged-coupled device digital camera containing a 1317 × 1035 pixel array (Micromax, Princeton Instruments, Trenton, NJ, USA). Pollen grains were examined also by epifluorescence microscopy after staining with 4, 6-diamidino-2-
phenylindole (DAPI) (100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.1 % (v/v) TX-100, 0.4 µg ml⁻¹ DAPI) in both the epifluorescence (355 nm emission and 420 nm excitation filter set) and the DIC optics modes. Images were captured using IPLab Spectrum (Signal Analytics, Vienna, VA, USA) and processed using Image J 1.32 and Adobe Photoshop/illustrator imaging software.

**Plant Transformation Vector Construction**

A H6T7 epitope tag was generated as described (Rancour et al., 2004). The cDNA encoding H6T7-tagged AtCDC48A in pPZP211 was PCR amplified using primers SB439 and SB371, TA-cloned into pGEMT-easy (Promega, Madison, WI), and verified by DNA sequence analysis. The AtCDC48A native promoter (754 bp upstream of the AtCDC48A start codon) was amplified by PCR with SB504 and SB656, restriction digested with SalI and NdeI, and subcloned into a SalI and NdeI digested H6T7-AtCDC48A containing pGEMT-easy vector. The AtCDC48A promoter sequence was verified by DNA sequence analysis. The entire construct was subcloned as a SalI and KpnI restriction fragment into pPZP211 (Hajdukiewicz et al., 1994). To generate AtCDC48A promoter-EYFP-AtCDC48A fusion vector, EYFP was amplified with SB695 and SB683 from pCAM-35S-EYFP-C1 (Preuss et al., 2004) to replace the existing H6T7 tag of AtCDC48 in pGEMT-easy. This cassette was then subcloned into the SalI and KpnI sites of pPZP211. Images of plants expressing YFP fused AtCDC48 were taken from confocal laser-scanning microscope (Nikon, Japan) equipped with 40X, 60X, and 90X plan oil-immersion objective using 488 an argon laser and 565 emission filter with 70 nm bandpass (see Supplemental Movies online). Images were processed using Image J 1.32 (Wayne Rasband, National Institutes of Health, USA) and Adobe Photoshop/illustrator (Adobe Systems, San Jose, CA, USA) imaging software on Macintosh computers (Apple Computer, Cupertino, CA, USA)

**Generation of Ethanol Inducible Dominant-Negative Mutants**
The ethanol inducible transcription factor and promoter from the *alc* cassettes of pSRN1 and pACN1 (pSRNACN_bin) (Caddick et al., 1998) were amplified with SB723 and SB737. The amplified fragment was subcloned into the Bsp1201 site of the modified pPZP211 (Bsp1201, compatible with NotI, was engineered into the HindIII site of pPZP211) (Scott Michaels, Indiana University) 5’ to *H6T7-AtCDC48A*. The double ATP hydrolysis and binding dominant-negative *Atcdc48A* (*Atcdc48A*DN) mutants were generated as described (Park et al., 2007). The constructs were introduced into wild-type plants (*Arabidopsis thaliana* Columbia-2 ecotype) by a floral dip transformation method (Clough and Bent, 1998). Transgenic plants were selected on solidified [0.6% (w/v) phytoagar] Murashige and Skoog (Murashige and Skoog, 1962) medium containing 100 µg/mL Kanamycin. Vertically grown T2 generations of ethanol inducible wild-type and dominant-negative *Atcdc48A* seedlings (60 seedlings per (100×100×15 mm) plate were treated with 1 ml of 0.5%, 1%, and 2% ethanol at the bottom of plates 4 days after germination. Ethanol treated plates were placed horizontal for 20 minutes and re-positioned to vertical at 22°C. Root length was measured at 1, 2, and 4 days after ethanol treatment. Average and standard errors were calculated for groups of 24 seedlings. Images of seedling primary leaves and roots were obtained with a stereo microscope (Leica MZ6) equipped with a Leica DFC 480 digital camera (Leica Microsystems Imaging Solutions Lts, Cambridge, UK) 2 hrs, 6 hrs, 1 day, 2 days, and 4 days after ethanol treatment. Four days post ethanol treatment, images of the first true leaves were taken using a Quanta 200 Environmental Scanning Electron Microscopy (FEI, Hillsboro, OR) under a 3.87 Torr vacuum using a 20 kV electron beam. Roots were observed by bright-field microscopy with a Zeiss Axioskop microscope, digital micrograph images were captured with a cooled CCD digital camera, and processed as above.

### Analysis of inducible *H6T7-Atcdc48A* protein, YFP-*AtCDC48A* and mRNA Expression

Total protein extracts were prepared from ethanol inducible transgenic seedling either mock treated or following the specified time post ethanol-treatment and from transgenic lines expressing YFP and YFP-*AtCDC48A*. Four seedlings were homogenized in 100 µL
of 2X SDS-PAGE sample buffer (Laemmli, 1970) and incubated at 65°C for 15 min. The samples were cleared of insoluble debris by centrifugation at 16,000 g for 5 min at room temperature, and 15 μL of the supernatant was resolved on a 12.5% (w/v) SDS-polyacrylamide minigel and analyzed by immunoblotting with anti-AtCDC48A (Rancour et al., 2002), anti-T7 (Novagen, Madison, WI) and anti-YFP antibodies (Rockland, Gilbertsville, PA) (see Supplemental Figure 1). Densitometric analysis of immunoblot film exposures was performed using a flatbed digital scanner and Image J 1.32 software.

Total RNA was isolated from mock treated or at specified times post ethanol-treatment from whole ethanol inducible wild-type and mutant Atcdc48A\textsuperscript{DN} seedlings using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA (2 μg) was treated with RQ DNase (Promega, Madison, WI) to eliminate genomic DNA. First strand cDNA was generated using 1 μg of RQ DNase-treated total RNA as template, oligo-(dT), and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) in a 20 μL reaction mixture. A 1 μL aliquot of a 20-fold dilution of the cDNA was subsequently PCR-amplified (30 cycles) with the oligonucleotide primer pair for \textit{AtCDC48A} (SB439 and SB36) and ubiquitin (SB747 and SB748) as a control.

**Cross-section analysis of wild-type and Atcdc48A\textsuperscript{DN} mutant roots**

The ethanol-inducible wild-type and mutant Atcdc48A\textsuperscript{DN} seedlings, before and four days after 2% ethanol treatment, were vacuum infiltrated and fixed overnight at 4°C with 4% (v/v) gluteraldehyde in 50 mM potassium phosphate buffer, pH 7.0. Tissue was subsequently rinsed with 50 mM potassium phosphate (pH 7.0) buffer and dehydrated through a graded ethanol series (30% - 100%). Tissue was embedded in LR White (EMS, Hatfield, PA). Transverse sections (5 μm) were cut with a Reichert-Jung Ultracut model E microtome (Vienna, Austria) and stained with toluidine blue O as described previously (Kang et al., 2001).
Accession numbers
Sequence data in this article can be found in the GeneBank/EMBL data libraries under accession numbers: AtCDC48A (At3g09840), AtCDC48B (At3g53230) and AtCDC48C (At5g03340)

SUPPLEMENTAL DATA
The following materials are available in the online version of this article

**Supplemental Figure 1.** PCR genotyping of embryos from pale (P) and green (G) developing seed from self-fertilized Atcdc48T-DNA/+ plants.

**Supplemental Figure 2.** PCR verification of native promoter driven YFP-AtCDC48A cDNA complementation of Atcdc48T-DNA mutant.

**Supplemental Figure 3.** Analysis of the expression of YFP-AtCDC48A in wild-type and Atcdc48A mutant plants

**Supplemental Figure 4.** AtCDC48 gene expression.

**Supplemental Figure 5.** Comparative phenotype of inducible ethanol-treated H6T7-WT, H6T7-DN-H, and H6T7-DN-B seedlings.

**Supplemental Figure 6.** Schematic of a wild-type Arabidopsis root

**Supplemental Movie 1.** Native promoter::YFP-AtCDC48A expression and localization in trichomes, leaves, and the shoot apical meristem of homozygous Atcdc48A T-DNA seedlings.

**Supplemental Movie 2.** The vegetative nucleus moves toward the growing tip of the pollen tub.

**Supplemental Movie 3.** YFP-AtCDC48A localizes to the nucleus membrane during karyokinesis.

**Supplemental Movie 4.** YFP-AtCDC48A localization in the nucleus

SUPPLEMENTAL DATA MATERIAL AND METHODS
Embryo DNA Isolation
Embryos were isolated from developing seed from siliques of self-fertilized \(Atcdc48A^{T-DNA}/+\) plants. DNA from individual embryos was isolated according to the following procedure. Isolated embryos were homogenized in 40 \(\mu\)l 250 mM NaOH followed by heating to 100°C for 30 sec. To the sample, 20 \(\mu\)l buffer (500 mM Tris/HCl pH 8.0, 0.25% (v/v) NP-40) followed by 40 \(\mu\)l 250 mM HCl were added, the sample mixed and incubated at 100°C for 2 minutes. Samples were cooled to ambient temperature, centrifuged at ambient temperature for 10 min at 16Kxg. The supernatant was used directly for PCR analysis using primer sets for the gene (SB513 and SB514) and the T-DNA insertion (SB514 and SB372).

Analysis of complementation of \(Atcdc48^{T-DNA}\) mutants with native promoter::YFP-\(AtCDC48A\) cDNA
Complementation constructs were originally transformed into \(Atcdc48^{T-DNA}/+\) plants. DNA from T2 plants initially selected for the YFP-\(AtCDC48A\) complementation construct by fluorescence microscopic analysis was tested by PCR with primers specific for the uninterrupted endogenous gene (gene; primers SB513 and SB514) and the original T-DNA insertion into the \(AtCDC48A\) locus (T-DNA; primers SB514 and SB372). Those plants that tested initially as homozygous for the \(AtCDC48A\) locus T-DNA insertion were verified by PCR and the latter data is presented (Figure S2).

Immunoblot analysis of the expression of YFP-\(AtCDC48A\) in wild-type and \(Atcdc48^{T-DNA}\) mutant plants.
Immunoblot analysis of total protein prepared from 4 day-old seedlings of transgenic lines expressing YFP alone in wild-type (\(AtCDC48A\)) plants, YFP-\(AtCDC48A\) in complemented \(Atcdc48^{T-DNA}/Atcdc48^{T-DNA}\); YFP-\(AtCDC48A\) mutant plants, and untransformed wild-type \(AtCDC48A\) plants was performed. The immunoblots were probed using anti-GFP (Rockland Immunoochemicals, Gilbertsville, PA) and anti-\(AtCDC48\) (Rancour et al., 2002) antibodies and detected via HRP-conjugates and chemiluminescence exposure to film.
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Figure 1. Schematic representation of $Atcd48A^{T-DNA}$ mutant alleles and the phenotypes of $Atcd48A^{T-DNA}$ seedlings, seeds, and embryos

(A) The exon/intron structure of $AtCDC48A$ cds is shown to scale with broad colored boxes representing exons and black inter-exon thin lines representing introns. The colors of the exons corresponds to the DNA coding regions encoding the protein domains of $AtCDC48A$: pink, N-terminus; black, linker 1; green, D1 ATPase; orange, linker 2; red, D2 ATPase; blue, C-terminus. The position and directions of T-DNA inserts with left border sequences are indicated (T$_L$). T-DNAs are not drawn to scale. (Kan: T-DNA neomycin phosphotransferase selectable gene marker). (B) Stereo micrograph of five-day-old wild-type $AtCDC48A$ and homozygous $Atcd48A^{T-DNA}$ mutant seedlings. Homozygous $Atcd48A^{T-DNA}$ plants soon die after this stage of development. (C) Roots of five-day-old wild-type $AtCDC48A$ (WT) and homozygous $Atcd48A^{T-DNA}$ mutant ($mt$) plants stained with propidium iodide and imaged by LSCM. The mutant root image comprises the entire root length (as shown in panel B) while the left image corresponds to only tip of the wild-type. Scale bar = 10 µm. (D) Stereo micrograph of a portion of an immature silique from a self-fertilized heterozygous $Atcd48A^{T-DNA}$ plant approximately 10 days after fertilization. A pale green $Atcd48A$ homozygous seed is indicated by an arrowhead. (E) Sibling embryos of an immature silique (approximately 5 days after fertilization) from a self-fertilized heterozygous $Atcd48A^{T-DNA}$ plant. Mutant embryo development is shown arrested at the heart stage (arrowhead).
Figure 2. Analysis of development and tube germination of pollen from \textit{AtCDC48A} and heterozygous \textit{Atcdc48A} plants

(A) Pollen grains from wild-type (left panel) and heterozygous \textit{Atcdc48A}^{T-DNA} (right panel) plants were examined at onset of desiccation by epifluorescence microscopy after 4, 6-diamidino-2-phenylindole (DAPI) staining. SN, sperm nuclei; VN, vegetative nucleus. The scale bars = 15 µm. (B) Pollen grains from \textit{qrt} (\textit{AtCDC48A};qrt) and (C) heterozygous \textit{Atcdc48A}^{T-DNA};qrt (\textit{Atcdc48A}/+;qrt) plants were germinated on pollen germination media at 28°C for 24 hours and then imaged with DIC optics. Arrows indicate \textit{Atcdc48A} mutant pollen tubes. Asterisk indicates a pollen tube from outside the field of view. The scale bars = 50 µm.
Figure 3. Localization of YFP-AtCDC48A in transgenic homozygous Atcdc48AT-DNA seedlings

Transgenic T₃ seedlings were examined 3 days after germination. (A) Representative confocal fluorescence microscopic images of YFP-AtCDC48A expressed in leaves (L), trichomes (T), and the shoot apical meristem (SAM). Scale bar = 10 µm. The three panels correspond to the first (left), middle and last (right) images of a serial Z-stack set taken down the vertical axis of the plant. See Supplemental Movie 1 online for complete Z-stack series of images. (B) Expression of YFP-AtCDC48A in the root visualized by wide-field fluorescence microscopy. Scale bar = 100 µm. (C) and (D) YFP-AtCDC48A expression in the root division zone visualized by confocal fluorescence microscopy. Scale bar = 10 µm. Division plane localization is indicated by arrowheads. Spindle localization is indicted by arrows. Asterisks indicates vesicle like structures located neighboring developing nuclear membranes. (E) The vegetative nucleus during pollen tube elongation. The three panels correspond to the first (left), middle and last (right) images of a time series taken during pollen tube elongation in vitro. See Supplemental Movie 2 online for complete time series. Arrow indicates the tip of the pollen tube. Scale bar = 10 µm. (F) YFP-AtCDC48A localization during karyokinesis in dividing root cells. The three panels correspond to the first (left), middle and last (right) images of a time series taken during root cell division. See Supplemental Movie 3 online for complete time series. Arrowhead indicates a dividing cell division plane. Scale bar = 10 µm. (G) YFP-AtCDC48A nucleus localization in the primary root elongation zone. The three panels correspond to the first (left), middle and last (right) images of a serial Z-stack set taken perpendicular to the root radial axis. See Supplemental Movie 4 online for complete Z-stacks. Scale bar = 10 µm.
Figure 4. Expression of conditional dominant-negative Atcdc48A mutants

(A) Time course of transgenic ethanol-induced H6T7-Atcdc48 mutant protein expression. Expression of H6T7-AtCDC48A (H6T7-WT, lanes: 1, 2 and 3), H6T7-Atcdc48\textsuperscript{DN-B} (H6T7-DN-B, lanes: 4, 5 and 6), and H6T7-Atcdc48\textsuperscript{DN-H} (H6T7-DN-H lanes: 7, 8 and 9) proteins was monitored by SDS-PAGE and immunoblot analysis using an anti-T7 antibody (upper panel) and an anti-HSP70 antibody as a protein load control (lower panel). Seedling samples were prepared prior to ethanol treatment (lanes 1, 4 and 7), or after 6 hours (lanes 2, 5 and 8) or 24 hours (lanes 3, 6 and 9) post ethanol treatment.

(B) Immunoblot analysis of endogenous AtCDC48 and H6T7-AtCDC48A protein expression levels from untransformed (Col2, lane 1) and independent transgenic ethanol-induced wild-type H6T7-AtCDC48A (H6T7-WT) (lanes: 2 and 3), H6T7-Atcdc48A\textsuperscript{DN-H} (H6T7-DN-H) (lanes: 4 and 5) and H6T7-Atcdc48A\textsuperscript{DN-H} (H6T7-DN-B) (lanes 6 and 7). Panels correspond to probing of samples with anti-AtCDC48A (top), anti-T7 (middle) and anti-DRP1A (bottom, load control). Samples were processed and analyzed at 48 hours post induction.

(C) RT-PCR analysis of \textit{H6T7-AtCDC48A} and \textit{H6T7-Atcdc48A}\textsuperscript{DN} gene expression. cDNA was synthesized from total RNA from H6T7-AtCDC48A (H6T7-WT, lanes: 1-4 H6T7-Atcdc48\textsuperscript{DN-B} (H6T7-DN-B lanes: 5-8) and H6T7-Atcdc48\textsuperscript{DN-H} (H6T7-DN-H lanes: 9-12) isolated prior to ethanol treatment (lanes 1, 5 and 9) or after 2 hours (lanes 2, 6 and 10), 6 hours (lanes 3, 7 and 11) or 24 hours (lanes 4, 8 and 12) post ethanol treatment. cDNA fragments were PCR amplified using primers specific to the transgene (upper panel) or the ubiquitin control (lower panel).
Figure 5. Phenotypic analysis of conditional Atcdc48A$^{DN}$ mutant plants

Five-day-old $H6T7$-Atcdc48A$^{DN-B}$ ($H6T7$-$DN-B$; panels A, C, E, and G) and $H6T7$-AtCDC48A ($H6T7$-$WT$; panels B, D, and F) seedlings were treated with 2% (v/v) ethanol and imaged after four days. Stereo micrographs of the aerial portion of ethanol treated $H6T7$-$DN-B$ (A) and $H6T7$-$WT$ (B) plants. Scanning electron micrographs of primary leaves and trichomes from ethanol treated $H6T7$-$DN-B$ (C) and $H6T7$-$WT$ (D) plants. Scale bar = 200 µm. Stereo micrographs of roots from ethanol treated $H6T7$-$DN-B$ (E) and $H6T7$-$WT$ (F) plants. Scale bar = 0.5 mm. (G) Higher magnification DIC image composite of an ethanol treated $H6T7$-$DN-B$ seedling root. Scale bar = 50 µm. (H) Total seedling root growth for $H6T7$-$WT$ (WT) and $H6T7$-Atcdc48A$^{DN}$ mutants (ATP binding: DN-B, and ATP hydrolysis: DN-H) was measured after 4 days post-ethanol (black bars) or mock (white bars) treatment. The data represents a minimum sample size of 25 plants for each construct. The standard deviation is represented as error bars for each group.
Figure 6. Cell division and trichoblast differentiation are affected in conditional Atcdc48A^{DN-B} mutants

H6T7-AtCDC48A (H6T7-WT; column 1) and H6T7-Atcdc48A^{DN-B} (H6T7-DN-B; columns 2 and 3) seedlings were analyzed 4-days after 2% (v/v) ethanol (columns 1 and 3) or mock treatment (column 2). Sections were stained with toluidine blue O and imaged by bright-field microscopy. Figure rows correspond to similar distances of tissue sections from the root tip. A root guide is available in Supplemental Figure 6. Panels A-C: approximately 100 µm above the root tip, Panels D-F: approximately 300 µm above the root tip corresponding to the division zone, Panels G-I: approximately 350 µm above the root tip corresponding to the division-expansion transition zone, Panels J-L: approximately 400-450 µm above the root tip corresponding to the expansion zone, Panels M-O: approximately 500-1000 µm from the root tip corresponding to the expansion-differentiation transition zone. Filled arrows highlight apparent cytokinesis defects. Open arrows highlight aberrant cell divisions. Filled arrowheads indicate root hairs emerging from inappropriate epidermal cells. Open arrowheads highlight root hair projections. Scale bars = 50 µm.
Table 1: Oligonucleotides used in study.

| Name | Sequence (5’-3’) | Cloning/screening |
|------|------------------|-------------------|
| SB371 | tcagtacagcgccgcAGTAATCCAAAGTAGAG | 3’ H6T7-AtCDC48A cloning; Not I |
| SB372 | CAAAACAGCGTGGGACCCGCTTGGTCGCAACT | Salk T-DNA left border |
| SB400 | ATTTGGAGAAGTAGTGG | 5’ AtCDC48A T-DNA insertion screening |
| SB401 | GTAGAACCACATAAGAAATCC | 5’ AtCDC48A T-DNA insertion screening |
| SB439 | ggaatctagcatcatcatcatcatac | 5’ H6T7-AtCDC48A cloning; Nde I |
| SB504 | agatctgagcatggagattccgaaggttgaag | 5’ AtCDC48A UTR; Sal I |
| SB513 | TGGATTCTTATGTGGTCTAC | 5’ AtCDC48A T-DNA insertion screening |
| SB514 | CATTCAATAAAATTACACTCA | 3’ AtCDC48A T-DNA insertion screening |
| SB656 | cattgGATTGAGTTTGAGATTAACCGAAGAG | 3’ AtCDC48A UTR; Nde I |
| SB723 | gcggccgcCATGGAGTCAAAGATTCAAATAG | 5’ ethanol inducible cassette cloning; Not I |
| SB737 | gcggccgcTCCTTAGCTCTGAAAATCTCGAC | 3’ ethanol inducible cassette cloning; Not I |
SUPPLEMENTAL DATA FIGURE LEGENDS

Figure S1. PCR genotyping of embryos from pale (P) and green (G) developing seed from self-fertilized Atcdc48T-DNA/+ plants. DNA from isolated embryos from pale (samples A and B; lanes 1, 2, 4, and 5) and green (sample C; lanes 3 and 6) seed was tested by PCR with primers specific for the uninterrupted gene (primers SB513 and SB514; lanes 1-3) and the T-DNA insertion (primers SB514 and SB372; lanes 4-6).

Figure S2. PCR verification of native promoter driven YFP-AtCDC48A cDNA complementation of Atcdc48AT-DNA mutant. DNA from F 2 plants selected for the YFP-AtCDC48A complementation construct (DNA samples I-VI) were tested by PCR with primers specific for the uninterrupted endogenous gene (gene; primers SB513 and SB514; lanes 1-7) and the original T-DNA insertion into the AtCDC48A locus (T-DNA; primers SB514 and SB372; lanes 8-14). Untransformed Col2 plant DNA (DNA sample C) was used as a control (Lanes 7 and 14).

Figure S3. Analysis of the expression of YFP-AtCDC48A in wild-type and Atcdc48AT-DNA mutant plants. Immunoblot analysis of total protein prepared from 4 day-old seedlings of transgenic lines expressing YFP alone in wild-type (AtCDC48A) plants (lane 1), YFP-AtCDC48A in complemented homozygous Atcdc48A mutant plants (lane 2 and 4) and untransformed wild-type AtCDC48A plants (lane 3). The immunoblots were probed using anti-GFP and anti-AtCDC48 antibodies. Open arrowhead indicates YFP-AtCDC48A. Filled arrow indicates YFP protein and asterisk indicates proteins that non-specifically interacted with antibody. AtCDC48A* indicates possible cross-interaction of AtCDC48B and AtCDC48C in homozygous Atcdc48A seedlings.

Figure S4. AtCDC48 gene expression. Composite tissue and cell-type specific gene expression array data for AtCDC48A (At3g09840), AtCDC48B (At3g53230), and AtCDC48C (At5g03340). Data was obtained from Genevestigator (Zimmerman, P., Hirsch-Hoffman M., Hennig, L., and Gruissem, W. (2004) GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox. Plant Physiol. 136: 2621-2632;
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Figure S5. Comparative phenotype of inducible ethanol-treated H6T7-WT, H6T7-DN-H and H6T7-DN-B seedlings. Dissecting microscope images of ethanol treated inducible H6T7-WT (left panel), H6T7-DN-H (middle panel) and H6T7-DN-B (right panel) seedlings. Dashed lines represent length of root at time of ethanol application. H6T7DN-H mutants show an intermediary root hair proliferation phenotype between that observed with expression of H6T7-WT, a wild-type phenotype, and H6T7-DN-B, a hyper-proliferative root hair phenotype (see figure 4). Asterisks highlights chlorotic leaves observed after ethanol treatment of H6T7-DN-B. The chlorotic leaf phenotype is seldom observed in H6T7-DN-H and never observed in H6T7-WT.

Figure S6. Schematic of a wild-type Arabidopsis root. The image was adapted from (Schiefelbein et al., 1997). Transverse sections of root division/expansion and differentiation zones are shown on the right. Distinct cell layers are indicated by color. Blue represents lateral root cap, Brown; epidermal cell, Green; cortex cell, Yellow; endodermis, Red; pericycle/stele. DIV; Division zone, EXP; Expansion zone, DIFF; Differentiation zone. An approximate scale relative to the root tip of sections imaged in Figure 6 is shown on the left side of the root schematic (see the text for detail).

Supplemental Movie 1. Native promoter::YFP-AtCDC48A expression and localization in trichomes, leaves, and the shoot apical meristem of homozygous Atcdc48A<sup>T-DNA</sup> seedlings. Serial confocal fluorescence micrograph sections (0.5 μm sections) of transgenic seedling along the long axis of the plant. Observed are cross-sections of a leaf (L) to the left and horizontal sections of shoot apical meristem (SAM) to the right. Several trichomes (T) are observed during the optical sectioning. YFP-AtCDC48A appears to localize to the cytoplasm and nucleus of all cells observed. Scale bar = 10 μm.
Supplemental Movie 2. The vegetative pollen nucleus moves toward the growing tip of the pollen tube. Localization of YFP-AtCDC48A in Arabidopsis growing pollen tube visualized with confocal laser scanning microscopy. Pollen grains were germinated on pollen germination media at 28°C. Actual duration of movie was approximately 30 min. Play back is at 4 frames per second. Scale bar = 10 μm.

Supplemental Movie 3. YFP-AtCDC48A localizes to the nucleus membrane during karyokinesis. YFP-AtCDC48A was expressed in homozygous Atcdc48A plants under control of its native promoter. Division zone of root cells was visualized with confocal laser scanning microscopy. Actual duration of movie was approximately 30 min. Play back is at 4 frames per second. Scale bar = 10 μm.

Supplemental Movie 4. YFP-AtCDC48A localization in the nucleus. Localization of YFP-AtCDC48A in elongation zone of root cell visualized with confocal laser scanning microscopy. Optical sections were taken in steps of 0.5 μm. Images are displayed sequentially. Play back is at 2 frames per second. Scale bar = 10 μm.
Figure 1. Schematic representation of Atc4848A<sup>T-DNA</sup> mutant alleles and the phenotypes of Atc4848A<sup>T-DNA</sup> seedlings, seeds, and embryos

(A) The exon/intron structure of AtCDC48A cds is shown to scale with broad colored boxes representing exons and black inter-exon thin lines representing introns. The colors of the exons corresponds to the DNA coding regions encoding the protein domains of AtCDC48A: pink, N-terminus; black, linker 1; green, D1 ATPase; orange, linker 2; red, D2 ATPase; blue, C-terminus. The position and directions of T-DNA inserts with left border sequences are indicated (T<sub>L</sub>). T-DNAs are not drawn to scale. (Kan: T-DNA neomycin phosphotransferase selectable gene marker). (B) Stereo micrograph of five-day-old wild-type AtCDC48A and homozygous Atc4848A<sup>T-DNA</sup> mutant seedlings. Homozygous Atc4848A<sup>T-DNA</sup> plants soon die after this stage of development. (C) Roots of five-day-old wild-type AtCDC48A (WT) and homozygous Atc4848A<sup>T-DNA</sup> mutant (mt) plants stained with propidium iodide and imaged by LSCM. The mutant root image comprises the entire root length (as shown in panel B) while the left image corresponds to only tip of the wild-type. Scale bar = 10 μm. (D) Stereo micrograph of a portion of an immature silique from a self-fertilized heterozygous Atc4848A<sup>T-DNA</sup> plant approximately 5 days after fertilization. Pale green Atc4848A homozygous seed is indicated by an arrowhead. Mutant embryos arise from mature silique (approximately 5 days after fertilization) from a self-fertilized heterozygous Atc4848A<sup>T-DNA</sup> plant. Mutant embryo development is shown arrested at the heart stage (arrowhead).
Figure 2. Analysis of development and tube germination of pollen from AtCDC48A and heterozygous Atcdc48A plants

(A) Pollen grains from wild-type (left panel) and heterozygous Atcdc48A T-DNA (right panel) plants were examined at onset of desiccation by epifluorescence microscopy after 4, 6-diamidino-2-phenylindole (DAPI) staining. SN, sperm nuclei; VN, vegetative nucleus. The scale bars = 15 μm. (B) Pollen grains from qrt (AtCDC48A;qrt) and (C) heterozygous Atcdc48A T-DNA ;qrt (Atcdc48A/+;qrt) plants were germinated on pollen germination media at 28°C for 24 hours and then imaged with DIC optics. Arrows indicate Atcdc48A mutant pollen tubes. Asterisk indicates a pollen tube outside the field of view. The scale bars = 50 μm.
Figure 3. Localization of YFP-AtCDC48A in transgenic homozygous Atcdc48AT-DNA seedlings

Transgenic T3 seedlings were examined 3 days after germination. (A) Representative confocal fluorescence microscopic images of YFP-AtCDC48A expressed in leaves (L), trichomes (T), and the shoot apical meristem (SAM). Scale bar = 10 \mu m. The three panels correspond to the first (left), middle and last (right) images of a serial Z-stack set taken down the vertical axis of the plant. See Supplemental Movie 1 online for complete Z-stack series of images. (B) Expression of YFP-AtCDC48A in the root visualized by wide-field fluorescence microscopy. Scale bar = 100 \mu m. (C) and (D) YFP-AtCDC48A expression in the root division zone visualized by confocal fluorescence microscopy. Scale bar = 10 \mu m. Division plane localization is indicated by arrowheads. Spindle localization is indicted by arrows. Asterisks indicates vesicle like structures located neighboring developing nuclear membranes. (E) The vegetative nucleus during pollen tube elongation. The three panels correspond to the first (left), middle and last (right) images of a time series taken during pollen tube elongation in vitro. See Supplemental Movie 2 online for complete time series. Arrow indicates the tip of the pollen tube. Scale bar = 10 \mu m. (F) YFP-AtCDC48A localization during karyokinesis in dividing root cells. The three panels correspond to the first (left), middle and last (right) images of a time series taken during root cell division. Arrowhead indicates a dividing cell division plane. Scale bar = 10 \mu m. (G) YFP-AtCDC48A visual localization in the primary root elongation zone. The three panels correspond to the first (left), middle and last (right) images of a serial Z-stack set taken perpendicular to the root radial axis. See Supplemental Movie 4 online for complete Z-stacks. Scale bar = 10 \mu m.
Figure 4. Expression of conditional dominant-negative Atcdc48A mutants
(A) Time course of transgenic ethanol-induced H6T7-Atcdc48A mutant protein expression. Expression of H6T7-AtCDC48A (H6T7-WT, lanes: 1, 2 and 3), H6T7-Atcdc48A^{DN-B} (H6T7-DN-B, lanes: 4, 5 and 6), and H6T7-Atcdc48A^{DN-H} (H6T7-DN-H lanes: 7, 8 and 9) proteins was monitored by SDS-PAGE and immunoblot analysis using an anti-T7 antibody (upper panel) and an anti-HSP70 antibody as a protein load control (lower panel). Seedling samples were prepared prior to ethanol treatment (lanes 1, 4 and 7), or after 6 hours (lanes 2, 5 and 8) or 24 hours (lanes 3, 6 and 9) post ethanol treatment. (B) Immunoblot analysis of endogenous AtCDC48 and H6T7-Atcdc48A protein expression levels from untransformed (Col2, lane 1) and independent transgenic ethanol-induced wild-type H6T7-AtCDC48A (H6T7-WT) (lanes 2 and 3), H6T7-Atcdc48A^{DN-B} (H6T7-DN-B) (lanes 4 and 5) and H6T7-Atcdc48A^{DN-H} (H6T7-DN-H) (lanes 6 and 7). Panels correspond to propping of samples with anti-AtCDC48A (top), anti-T7 (middle) and anti-DRP1A (bottom, load control). Samples were processed and analyzed at 48 hours post induction. (C) RT-PCR analysis of H6T7-AtCDC48A and H6T7-Atcdc48A^{DN} gene expression. cDNA was synthesized from total RNA from H6T7-AtCDC48A (H6T7-WT, lanes: 1-4 H6T7-Atcdc48A^{DN-B} (H6T7-DN-B lanes: 5-8) and H6T7-Atcdc48A^{DN-H} (H6T7-DN-H lanes: 9-12) isolated prior to ethanol treatment (lanes 1, 5 and 9) or after 2 hours (lanes 2, 6 and 10), 6 hours (lanes 3, 7 and 11) or 24 hours (lanes 4, 8 and 12) post ethanol treatment. cDNA fragments were PCR amplified using primers specific to the transgene (upper panel) or the ubiquitin control (lower panel).
Figure 5. Phenotypic analysis of conditional Atcdc48A\textsuperscript{DN} mutant plants

Five-day-old H6T7-Atcdc48A\textsuperscript{DN-B} (H6T7-DN-B; panels A, C, E, and G) and H6T7-AtCDC48A (H6T7-WT; panels B, D, and F) seedlings were treated with 2\% (v/v) ethanol and imaged after four days. Stereo micrographs of the aerial portion of ethanol treated H6T7-DN-B (A) and H6T7-WT (B) plants. Scanning electron micrographs of primary leaves and trichomes from ethanol treated H6T7-DN-B (C) and H6T7-WT (D) plants. Scale bar = 200 \textmu m. Stereo micrographs of roots from ethanol treated H6T7-DN-B (E) and H6T7-WT (F) plants. Scale bar = 0.5 mm (G) Higher magnification DIC image composite of an ethanol treated H6T7-DN-B seedling root. Scale bar = 50 \textmu m. (H) Total seedling root growth for H6T7-WT (WT) and H6T7-Atcdc48A mutants (ATP binding: DN-B, and ATP hydrolysis: DN-H) was measured at 10 days post treatment (black bars) or mock (white bars) treatment. The data represents a minimum sample size of 25 plants for each construct. The standard deviation is represented as error bars for each group.
Figure 6. Cell division and trichoblast differentiation are affected in conditional Atcdc48A^{DN-B} mutants

H6T7-AtCDC48A (H6T7-WT; column 1) and H6T7-Atcdc48A^{DN-B} (H6T7-DN-B; columns 2 and 3) seedlings were analyzed 4-days after 2% (v/v) ethanol (columns 1 and 3) or mock treatment (column 2). Sections were stained with toluidine blue O and imaged by bright-field microscopy. Figure rows correspond to similar distances of tissue sections from the root tip. A root guide is available in Supplemental Figure 6. Panels A-C: approximately 100 μm above the root tip, Panels D-F: approximately 300 μm above the root tip corresponding to the division zone, Panels G-I: approximately 350 μm above the root tip corresponding to the division-expansion transition zone, Panels J-L: approximately 400-450 μm above the root tip corresponding to the expansion zone, and Panels M-O: approximately 500-1000 μm from the root tip corresponding to the expansion zone.

Filled arrows highlight apparent cytokinesis defects. Open arrowheads indicate root hairs emerging from inappropriate epidermal cells. Open arrowheads highlight root hair projections. Scale bars = 50 μm.