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The higher plant *Arabidopsis thaliana* encodes a functional *CDC48* homologue which is highly expressed in dividing and expanding cells

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We have identified an *Arabidopsis thaliana CDC48* gene which, unlike the putative mammalian homologue valosin-containing protein (VCP), functionally complements *Saccharomyces cerevisiae cdc48* mutants. *CDC48* is an essential gene in *S.cerevisiae* and genetic studies suggest a role in spindle pole body separation. Biochemical studies link VCP function to membrane trafficking and signal transduction. We have described the *AtCDC48* expression pattern in a multicellular eukaryote; the zones of cell division, expansion and differentiation are physically separated in higher plants, thus allowing the analysis of *in situ* expression patterns with respect to the state of cell proliferation. *AtCDC48* is highly expressed in the proliferating cells of the vegetative shoot, root, floral inflorescence and flowers, and in rapidly growing cells. *AtCDC48* mRNA and the encoded protein are up-regulated in the developing microspores and ovules. *AtCDC48* expression is down-regulated in most differentiated cell types. AtCDC48p was primarily localized to the nucleus and, during cytokinesis, to the phragmoplast, a site where membrane vesicles are targeted in the deposition of new cell wall materials. This study shows that the essential cell division function of *CDC48* has been conserved by, at least, some multicellular eukaryotes and suggests that in higher plants, *CDC48* functions in cell division and growth processes.

Keywords: *Arabidopsis thaliana*/ATPase/CAD/CDC48/VCP

Introduction

The *CDC48* gene of *Saccharomyces cerevisiae* encodes a highly conserved ATPase which is a member of the recently defined superfamily of ATPases known as the conserved ATPase domain (CAD) or ATPases associated with diverse cellular activities (AAA) superfamily (Kunau *et al.*, 1993). The CDC48 protein (CDC48p) contains two copies of the highly conserved nucleotide-binding domain which defines this superfamily (Fröhlich *et al.*, 1991). This 'ATPase module', which spans ~230 amino acids, includes the Walker type-A and -B motifs which are important in ATP binding and hydrolysis, respectively (Walker *et al.*, 1982; Koonin, 1993a), and a second region of homology (SRH) which contains DNA/RNA helicase-like motifs (for reviews, see Gorbalenya *et al.*, 1989; Schmid and Linder, 1992; Fuller-Pace, 1994). A number of putative CDC48 homologues have been identified by sequence homology, including mammalian valosin-containing protein (VCP) (Koller and Brownstein, 1987; Egerton *et al.*, 1992), the *Xenopus laevis* p97ATPase (Peters *et al.*, 1990), a soybean VCP-like protein (sVCP; Shi *et al.*, 1995) and two archaebacterial proteins, the *Sulfolobus acidocaldarius* SAV protein (Confalonieri *et al.*, 1994) and the *Halobacterium salinarium cdcH* protein (GenBank accession number X79560). *Saccharomyces cerevisiae* CDC48p (ScCDC48p), mammalian VCP, sVCP and the *Xenopus laevis* p97ATPase are highly conserved over their entire amino acid sequence. ScCDC48p and VCP are 84% similar throughout and 96% similar within the first copy of the ATPase module, the region of highest homology. Despite this high similarity, porcine VCP is incapable of functionally complementing *cdc48* yeast mutants (K.-U.Fröhlich, personal communication).

Genetic analysis of *CDC48* has shown that the gene is essential for cell cycle progression in *S.cerevisiae* and suggests a role in spindle pole body (SPB) duplication or separation. Conditional mutants grown at the restrictive temperature arrest in the medial nuclear division stage of the cell division cycle with a large bud and microtubules spreading abberantly into the cytoplasm from an unreplicated or unseparated SPB (Fröhlich *et al.*, 1991). This G_2 phase arrest occurs because of the absence of an intact mitotic spindle at the critical checkpoint. In contrast, mammalian VCP has been implicated in signal transduction events and membrane trafficking. Murine VCP is phosphorylated on tyrosine residues in response to T cell activation (Egerton *et al.*, 1992; Egerton and Samelson, 1994). A subset of the VCP in mammalian cells stoichiometrically binds clathrin, a structural protein involved in receptor-mediated endocytosis and Golgi sorting (Pleasure *et al.*, 1993) and biochemical reconstitution studies suggest that mammalian VCP is associated with, and involved in, the budding of transition vesicles from the endoplasmic reticulum (Zhang *et al.*, 1994). CDC48p (and its putative homologues) lack membrane-spanning regions and biochemical fractionation studies have localized ScCDC48p and p97ATPase to the soluble protein fractions from both the nucleus and the cytoplasm, where, in the latter case, it appears to be loosely bound to vesicular structures (Peters *et al.*, 1990; Fröhlich *et al.*, 1991).

Detailed biochemical and structural analysis of p97ATPase and VCP have shown that six of the 97 000 M_r subunits form a soluble, ring-like hexameric complex (Peters *et al.*, 1990, 1992; Zhang *et al.*, 1994). This homo-oligomeric complex is a highly active, *N*-ethylmaleimide (NEM)-inhibitable, Mg^{2+}-dependent ATPase with a characteristically high pH optimum of 9.0 *in vitro* (Peters

et al., 1990; Zhang *et al.*, 1994). Similar structural and biochemical characteristics have also been noted for the CAD proteins involved in mediating the protein–protein interactions of membrane vesicle fusion with Golgi compartments, Sec18p and its mammalian homologue, NEM-sensitive fusion protein (NSF) (Block *et al.*, 1988). A potential role for CDC48p in mediating protein–protein interactions involved in membrane transport processes has been proposed (Fröhlich *et al.*, 1991; Pleasure *et al.*, 1993; Zhang *et al.*, 1994) but currently little is known about the biochemical function of the ubiquitous and sometimes highly abundant VCP/CDC48 proteins.

We present here the characterization of the *Arabidopsis thaliana CDC48* (*AtCDC48*) gene, its expression pattern and a functional analysis of its encoded protein. In addition, the molecular characterization and subcellular localization of AtCDC48p will be presented. This work demonstrates that a plant CDC48 homologue, unlike the putative mammalian homologue, can functionally complement conditional *cdc48* yeast mutants, allowing mitotic spindle assembly and, therefore, cell division to occur without arrest. These results suggest that CDC48 is involved in similar cell division cycle processes in both yeasts and higher plants.

Results

Characterization of the AtCDC48 gene and its translated sequence

A full-length *A.thaliana CDC48* clone (YAY020) was isolated in the process of randomly sequencing *A.thaliana* cDNAs (Höfte *et al.*, 1993). This 2637 bp clone was sequenced on both strands and found to contain a single frameshift mutation 757 bases from the putative AUG initiation codon. This frameshift was due to the loss of a single G, as determined by comparison with the sequences of partial clones previously obtained. The full-length clone was corrected by the removal of the 426 bp *Eco*RI–*Bam*HI fragment containing the deletion and replacement with the same fragment from a partial *AtCDC48* clone which was identical except for the single base deletion. The corrected sequence contains an open reading frame of 2427 bases (Figure 1). The presumed AUG initiation codon sequence in *AtCDC48* (AAUC**AUG**UC) is similar to the consensus calculated for plant sequences (AACC**AUG**GC) (Lütcke *et al.*, 1987). Variability of the site of poly(A) tail addition was observed in the *AtCDC48* clones isolated, as is not uncommon for plant cDNAs (Dean *et al.*, 1986). Clone YAY020 terminated with a poly(A) tail 108 bases after the stop codon (Figure 1). Putative polyadenylation signal sequences, AUUACU and a G/T cluster (GTGTTTTT), were positioned 46 bp and 13 bp upstream of the poly(A) tail, respectively (Dean *et al.*, 1986). The extended 3′ untranslated region found in some of the partial clones (Figure 1, last line) contained alternative putative polyadenylation signal sequences (AUUCUU, AAUCAA), the former of which is positioned 31 bp before a G/T cluster. As in the *S.cerevisiae CDC48* sequence, a T-rich region follows the translation termination codon in *AtCDC48* (Fröhlich *et al.*, 1991).

The *AtCDC48* gene encodes a protein of 809 amino acids (aa) with a predicted molecular weight of 88 963 (Figures 1 and 2). Analysis of the predicted amino acid sequence revealed that the protein is highly similar to ScCDC48p and VCP: AtCDC48p is 68% identical (83% similar) to ScCDC48p and 77% identical (87% similar) to VCP. Within the first ATPase module, the region of highest homology, these values rise to 84% identical (94% similar) and 86% identical (93% similar) for ScCDC48p and VCP, respectively (Figure 2). AtCDC48p is 92% identical to the sVCP (Shi *et al.*, 1995). AtCDC48p contains two copies of the ATPase module (aa 207–414 and aa 481–691), each with perfect Walker A (GPPGSGKT and GPPGCGKT) and B motifs (DEID and DELD) (Walker *et al.*, 1982; Gorbalenya and Doonin, 1989) and an SRH containing a modified 'sAT' region and a basic amino acid signature (VMGATN...RFGRFDR and IIGATN...RPGRL), motifs commonly found in the DNA/RNA helicases (Gorbalenya *et al.*, 1989; Koonin, 1993b; Fuller-Pace, 1994). Like ScCDC48p and VCP, AtCDC48p is hydrophilic in nature with acidic clusters flanking the core region containing the duplicated ATP-binding module.

The N-terminal region of AtCDC48p contains two putative nuclear localization signals [KK.(8X).RKK and KGKKRKD] (Robbins *et al.*, 1991), the former of which is conserved in a modified form in ScCDC48p and the latter of which is conserved in both ScCDC48p and VCP (Figure 2). Interestingly, the putative bipartite nuclear localization signal is adjacent to a cyclin-dependent kinase (cdk) phosphorylation consensus sequence in AtCDC48p (SPNR) (Nigg, 1993). A second, conserved, putative cdk phosphorylation consensus site [(S/T)PSK] lies just upstream of the Walker A motif of ATP-module II. In addition, 12 putative casein kinase II phosphorylation sites (S/TXXD/E) are found in AtCDC48p (TPAE, SKKD, TCEE, TNGE, TLMD, TVVE, SWND, SGGD, TEMD, SGAD, SVSD and SDAD), eight of which are conserved in ScCDC48p and VCP. Also of interest, is the conserved, negatively charged C-terminal sequence, DDDLY(N/G/S). This motif is also found at the C-terminus of eukaryotic, non-organellar heat shock proteins, some of which have quaternary structures reminiscent of p97ATPase (Mian, 1993). The tyrosine residue found within this motif in VCP is phosphorylated in response to T cell activation (Egerton and Samelson, 1994). Another region of potential interest is a 30 amino acid stretch found at the end of the first ATPase module (aa 386–415). This region is 67% similar to the terminus of the clathrin coat protein encoded by *APS1* (Phan *et al.*, 1994). Aps1p is a component of a large, distinct protein complex which is peripherally associated with membranes and involved in a subset of clathrin functions at the Golgi apparatus. The evidence that a fraction of the VCP in mammalian cells is associated with clathrin suggests that this conserved region may be of functional importance.

Functional complementation of S.cerevisiae CDC48 mutants

Complementation tests were conducted to determine if the protein encoded by *AtCDC48* could functionally substitute for the putative yeast homologue (Figure 3). Two *S.cerevisiae cdc48* conditional lethal mutants, the cold-sensitive (cs) strain DBY2030 and the temperature-sensitive (ts) strain KFY189, were transformed with the yeast expression vector pFL61 (Minet *et al.*, 1992) containing *AtCDC48*

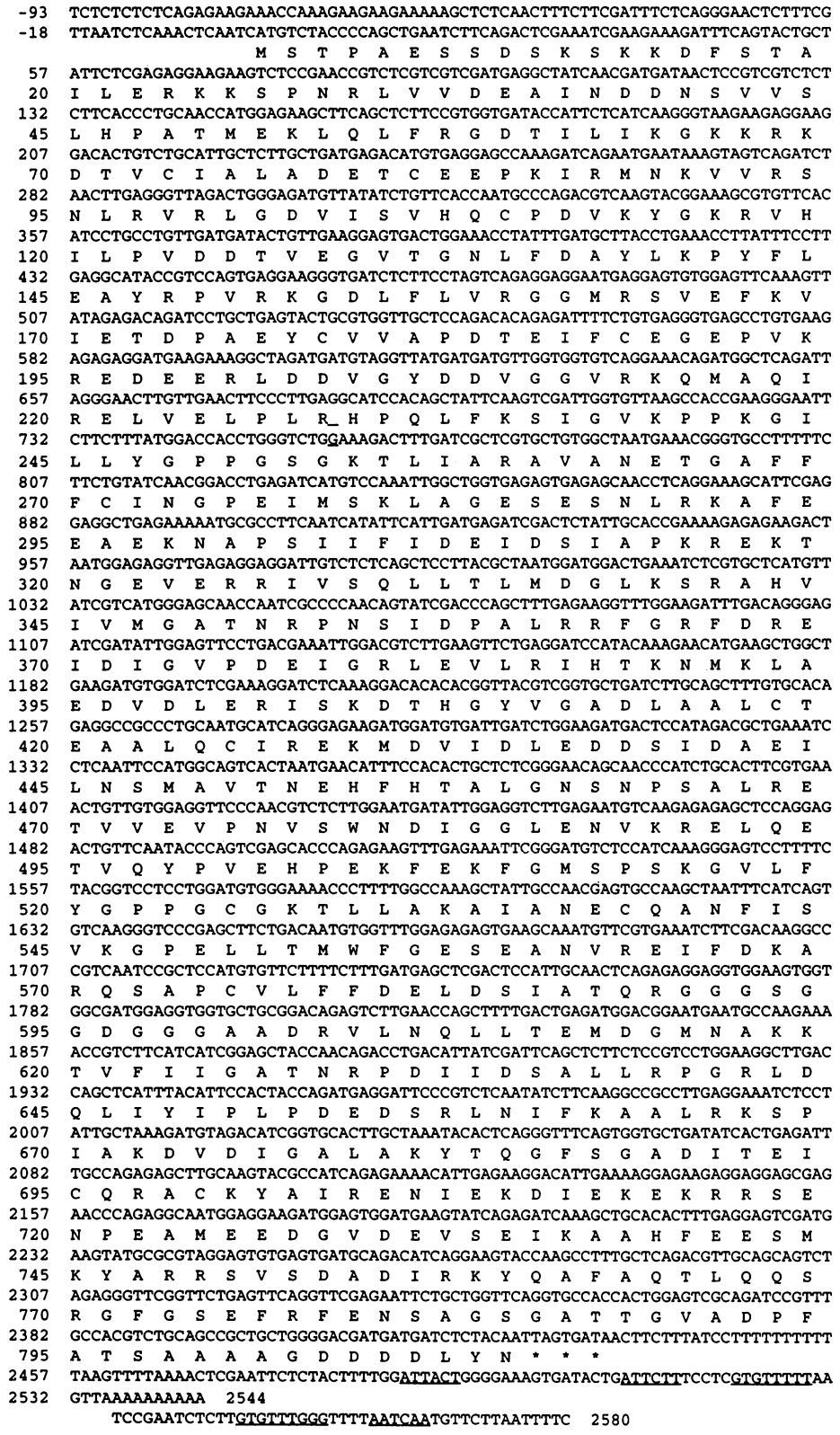

Fig. 1. The nucleotide sequence of *AtCDC48* and predicted amino acid sequence. The nucleotide missing from the YAY020 clone at bp 757 is under- and overlined. The regions corresponding to putative polyadenylation signal sequences are underlined. A longer 3′ untranslated region was found in the partial clone, VCV017, and is shown in the last line at the bottom. The GenBank accession number of this sequence is U37587.

in the sense (pAtcdc48s) or antisense (pAtcdc48a) orientations, or an empty vector control. Ura^+ transformants were selected on minimal medium without uracil at 28°C and eight transformants carrying each construct type were re-streaked and challenged at the non-permissive temperatures of 36°C and 37°C for KFY189 or 16°C for DYB2030. Functional complementation was observed for all transformants expressing *AtCDC48* in the sense orienta-

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        1                                    {---------pep725--------}              70
AtCDC48 ..........  MSTPAESSDS KSKKDFSTAI LERKKSPNRL VVDEAINDD. .NSVVSLHPA TMEKLQLFRG
Pig VCP ..........  ...MASGADS KG.DDLSTAI LKQKNRPNRL IVDEAINED. .NSVVSLSQP KMDELQLFRG
ScCDC48 ....MGEEH KPLLDASGVDP REEDKTATAI LRRKKKDNML LVDDAINDD. .NSVIAINSN TMDKLELFRG
    SAV  MRINILRVEY HSKTAHSILL QLDLFKKVKV ICHMSQSIKF RVTEARQRDV GKKVARISET SMRKLNVEAG

     71  DTILIKGKKR KDTVCIALAD ETCEEPKIRM NKVVRSNLRV RLGDVISVHQ CPDVKYGKRV HILPVDDTVE
         DTVLLKGKKR REAVCIVLSD DTCSDEKIRM NRVVRNNLRV HLGDVISIQP CPDVKYGKRI HVLPIDDTVE
         DTVLVKGKKR KDTVLIVLID DELEDGACRI NRVVRNNLRI RLGDLVTIHP CPDIKYATRI SVLPIADTIE
         DYIEIIGQDG NSALAQVMPA YDISDDEIRI DGYIRKSIKV GIGDDVTVRK .TNVSPASKV .VLAPTQPIR

    141  GVTGNLFDAY LKPYFLEAYR PVRKGDLFLV RGGMRSVEFK VIETDPAEYC VVAPDTEIFC EGEPVKRED.
         GITGNLFEVY LKPYFLEAYR PIRKGDIFLV RGGMRAVEFK VVETDPSPYC IVAPDTVIHC EGEPIKREDE
         GITGNLFDVF LKPYFVEAYR PVRKGDHFVV RGGMRQVEFK VVDVEPEEYA VVAQDIIIHW EGEPINREDE
         ..FDNSFVEY VKDTLMD..K PLAKGETLPI PIYTGTLELT VVNTQPSNYV YVTGSTNIEI REEPVK..ES
                   [                                                  ____A___
    211  EERLDDVGYD DVGGVRKQMA QIRELVELPL RHPQLFKSIG VKPPKGILLY GPPGSGKTLI ARAVANETGA
         EESLNEVGYD DIGGCRKQLA QIKEMVELPL RHPALFKAIG VKPPRGILLY GPPGTGKTLI ARAVANETGA
         ENNMNEVGYD DIGGCRKQMA QIREMVELPL RHPQLFKAIG IKPPRGVLMY GPPGTGKTLM ARAVANETGA
         SLAYPKVSWE DIGDLEEAKQ KIREIVEWPM RHPELFQRLG IDPPKGILLY GPPGTGKTLL ARALRNEIGA
                                                 ____B____
    281  FFFCINGPEI MSKLAGESES NLRKAFEEAE KNAPSIIFID EIDSIAPKRE KTNGEVERRI VSQLLTLMDG
         FFFLINGPEI MSKLAGESES NLRKAFEEAE KNAPAIIFID ELDAIAPKRE KTHGEVERRI VSQLLTLMDG
         FFFLINGPEV MSKMAGESES NLRKAFEEAE KNAPAIIFID EIDSIAPKRD KTNGEVERRV VSQLLTLMDG
         YFITVNGPEI MSKFYGESEQ RIREIFKEAE ENAPSIIFID EIDAIAPKRE DVTGEVEKRV VAQLLTLMDG
              *********_____SRH ######
    351  LKSRAHVIVM GATNRPNSID PALRRFGRFD REIDIGVPDE IGRLEVLRIH TKNMKLAEDV DLERISKDTH
         LKQRAHVIVM AATNRPNSID PALRRFGRFD REVDIGIPDA TGRLEILQIH TKNMKLADDV DLEQVANETH
         MKARSNVVVI AATNRPNSID PALRRFGRFD REVDIGIPDA TGRLEVLRIH TKNMKLADDV DLEALAAETH
         IKGRGRVIVI GATNRPDAID PALRRPGRFD REIEIRPPDT KGRKDILQVH TRNMPITDDV DLDKLAEMTY
              ]
    421  GYVGADLAAL CTEAALQCIR EKMD..VIDL EDDSIDAEIL NSMAVTNEHF HTALGNSNPS ALRETVVEVP
         GHVGADLAAL CSEAALQAIR KKMD..LIDL EDETIDAEVM NSLAVTMDDF RWALSQSNPS ALRETVVEVP
         GYVGADIASL CSEAAMQQIR EKMD..LIDL DEDEIDAEVL DSLGVTMDNF RFALGNSNPS ALRETVVESV
         GYTGADLAAL AKEAAIYALR RFVDEKKLNL DQPTIPAEII KELKVSMNDF LNALKSIQPS LLREVYVEVP
              [              {---------pep200----------}____A ___
    491  NVSWNDIGGL ENVKRELQET VQYPVEHPEK FEKFGMSPSK GVLFYGPPGC GKTLLAKAIA NECQANFISV
         QVTWEDIGGL EDVKRELQEL VQYPVEHPDK FLKFGMTPSK GVLFYGPPGC GKTLLAKAIA NECQANFISI
         NVTWDDVGGL DEIKEELKET VEYPVLHPDQ YTKFGLSPSK GVLFYGPPGT GKTLLAKAVA TEVSANFISV
         KVNWNDIGGL DNVKQQLREA VEWPLRFPEL FTKSGVTPPK GILLFGPPGT GKTMLAKAVA TESGANFIAV
                                          ____B___
    561  KGPELLTMWF GESEANVREI FDKARQSAPC VLFFDELDSI ATQRGGGSGG DGGGAADRVL NQLLTEMDGM
         KGPELLTMWF GESEANVREI FDKARQAAPC VLFFDELDSI AKARGGNI.G DGGGAADRVI NQILTEMDGM
         KGPELLSMWY GESESNIRDI FDKARAAAPT VVFLDELDSI AKARGGSL.G DAGGASDRVV NQLLTEMDGM
         RGPEILSKWV GESEKAIREI FRKARQAAPT VIFFDEIDSI APIRG...LS TDSGVTERIV NQLLAEMDGI
              *********_____SRH ######
    631  NAKKTVFIIG ATNRPDIIDS ALLRPGRLDQ LIYIPLPDED SRLNIFKAAL RKSPIAKDVD IGALAKYTQG
         STKKNVFIIG ATNRPDIIDP AILRPGRLDQ LIYIPLPDEK SRVAILKANL RKSPVAKDVD LEFLAKMTNG
         NAKKNVFVIG ATNRPDQIDP AILRPGRLDQ LIYVPLPDEN ARLSILNAQL RKTPLEPGLE LTAIAKATQG
         VPLNKVVIIA ATNRPDILDP ALLRPGRFDR LIYVPPPDKT ARFEILKVHT KNVPLAEDVS LEDIAEKAEG
              ]
    701  FSGADITEIC QRACKYAIRE NIEKDIEKEK RR........ ....SENPEA MEEDGVDEVS EIKAAHFEES
         FSGADLTEIC QRACKLAIRE SIESEIRRER ER........ ....QTNPSA MEVEEDDPVP EIRRDHFEEA
         FSGADLLYIV QRAAKYAIKD SIEAHRQHEA EKEVKVEGED VEMTDEGAKA EQEPEVDPVP YITKEHFAEA
         YTGADLEALV REATINAMR. SIYSMCDKQS RDECKGNMEC Y......QKH IKECMNKTSF KVSKEDFEKA

    771  MKYARRSVSD ADIRKYQAFA QTLQQSRG.F GS.EFRFENS AGSGATTGVA DPFATSAAA. .....AGDDD
         MRFARRSVSD NDIRKYEMFA QTLQQSRG.F GSFRFPSGNQ GGAGPSQGSG GGTGGSVYT. .....EDNDD
         MKTAKRSVSD AELRRYEAYS QQMKASRGQF SNFNFNDAPL GTTATDNANS NNSAPSGAGA AFGSNAEEDD
         LNVVKASLTQ ADIQRYERFS KELKRAIA.. .......... .......... .......... ..........

    841  DLYN
         DLYG
         DLYS
         ....
```

Fig. 2. Comparison of the predicted amino acid sequences of AtCDC48p, *Sus scrofa* (pig) VCP (M30143), *S.cerevisiae* CDC48p (X56956) and *Sulfolobus acidocaldarius* SAV (L17042). The ATPase modules are delineated in brackets; the Walker A and B nucleotide binding motifs are overlined. The SRH is overlined with the modified 'sAT' region (*) and a 'basic' amino acid signature (#) delineated. Putative nuclear localization signals are in italics. The peptides to which antibodies pAb725 and pAb200 were made are noted (---).

tion at the non-permissive temperatures of 36°C for KFY189 and 16°C for DBY2030 (examples shown in Figure 3A and B, respectively). Interestingly, AtCDC48p was able to functionally complement KFY189 at 36°C but not at 37°C. The mutant strains KFY189, DBY2030 and transformants carrying the control constructs (empty pFL61 vector or *AtCDC48* in the antisense orientation) did not grow at any of the non-permissive temperatures tested (examples shown in Figure 3A and B). Two experiments were conducted to verify that expression of *AtCDC48* resulted in functional complementation. All transformants grown in the presence of 5-fluoro-orotic acid

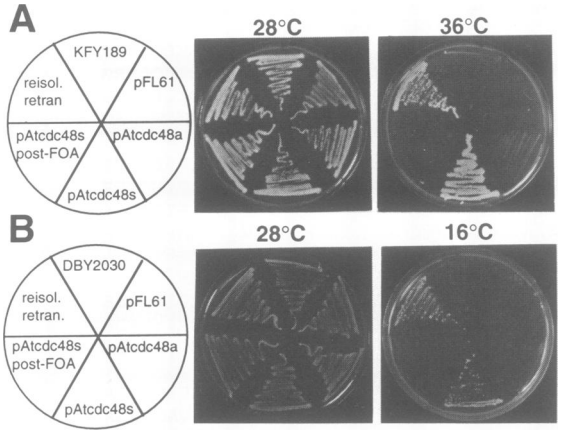

Fig. 3. Temperature-sensitive growth of conditional *S.cerevisiae cdc48* mutants KFY189 (ts) (**A**) and DBY2030 (cs) (**B**) at the non-permissive temperatures of 36°C and 16°C, respectively. Untransformed mutants (top sector) and mutants transformed with the following constructs were analysed (clockwise): empty pFL61, pAtCDC48 in the antisense orientation (pAtcdc48a) and pAtCDC48 in the sense orientation (pAtcdc48s). Examples of the following controls are included (continuing clockwise): pAtcdc48s transformants cured of their plasmids on FOA and mutants transformed with pAtCDC48s which was isolated from transformants showing putative complementation.

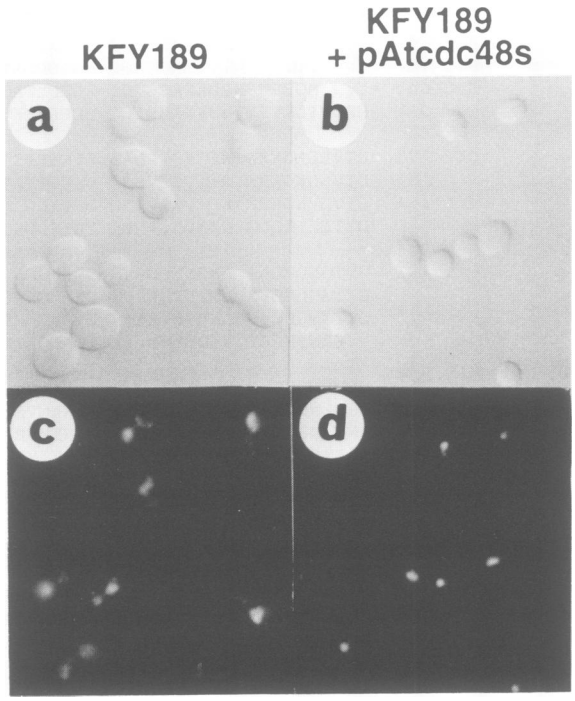

Fig. 4. Temperature-sensitive phenotypes of *cdc48* mutant KFY189 and KFY189 transformed with pAtcdc48s grown at 36°C for 5 h. Cells are visualized with Nomarski optics (**a** and **b**) and DNA by DAPI fluorescence (**c** and **d**).

(FOA), which causes selective loss of *URA3*-containing constructs (Boeke *et al.*, 1984), lost the ability to grow at the restrictive temperatures (examples shown in Figure 3). In addition, plasmids were re-isolated from four putative complemented transformants (two from each mutant strain), amplified in *Escherichia coli* and transformed back into the *S.cerevisiae cdc48* mutants. Enzymatic digestion patterns of all plasmids obtained matched those of pFL61 containing *AtCDC48* in the sense orientation. After transformation of these plasmids back into the original *cdc48* mutant strains (KFY189 and DBY2030) ten transformants of each type were challenged at the corresponding non-permissive temperature and all, again, displayed functional complementation (examples shown in Figure 3). These results verify that AtCDC48p functionally complements both *S.cerevisiae cdc48* conditional mutants, allowing growth and cell division at the non-permissive temperatures.

The phenotypes of the *S.cerevisiae cdc48* ts mutant (KFY189) and transformants expressing *AtCDC48* were compared after growth at the permissive or non-permissive temperatures. Cultures of KFY189 and KFY189 transformed with pAtcdc48s, when grown at the permissive temperature, contained cells of all stages of the cell cycle as determined by DAPI staining and microscopy (data not shown). At the non-permissive temperature of 36°C, >95% of the KFY189 cells displayed a medial nuclear division cell cycle block; the larger than normal cells arrested with large buds and nuclei wedged between the mother cell and the bud (Figure 4a and c). In contrast, cultures of transformants expressing *AtCDC48* did not block at the non-permissive temperature and were indistinguishable from those grown at the permissive temperature (Figure 4b and d). These results clearly show that the AtCDC48p can replace the essential cell division cycle function of the endogenous *S.cerevisiae* CDC48 protein in this yeast.

Expression patterns of AtCDC48

Southern blot analysis suggests that *AtCDC48* is a single copy gene in the *A.thaliana* genome (data not shown). Other CAD proteins exist in *A.thaliana*; many clones encoding CAD proteins have been identified in the *A.thaliana* EST collection and multiple bands are observed on Southern blots probed with *AtCDC48* and washed under low stringency condition. Therefore, the *AtCDC48* gene and 3′ and 5′ portions (which contained untranslated regions and exclude the region encoding the highly conserved ATPase modules) were used, in separate experiments, to make digoxigenin-labelled antisense RNA probes in order to qualitatively analyse *AtCDC48* expression in higher plant tissues. All probes tested consistently gave the same results when used for *in situ* hybridization analysis and recognized a single mRNA of ~2.5 kb on Northern blots of total RNA isolated from *A.thaliana* flower buds (example in Figure 5a) or young plants.

In situ hybridization experiments were conducted on whole mount plant preparations and sections of young plants, inflorescence shoots and flowers of all stages (Figure 5b–h). In the vegetative shoot apex, high, uniform levels of *AtCDC48* expression were detected in the regions which contain morphologically undifferentiated cells undergoing division or elongation: the meristematic zone, leaf primordia and stipules (Figure 5b and c). The vascular tissue of the hypocotyl and cotyledons was the only differentiated tissue to express high levels of *AtCDC48* in the vegetative shoot (Figure 5b and c). High, uniform levels of *AtCDC48* expression were also detected in the meristematic and elongation zones of the root (Figure 5b) and in lateral root primordia. Expression was not observed in the differentiated cells behind the elongation zone of the root. In the inflorescence shoot, high levels of expres-

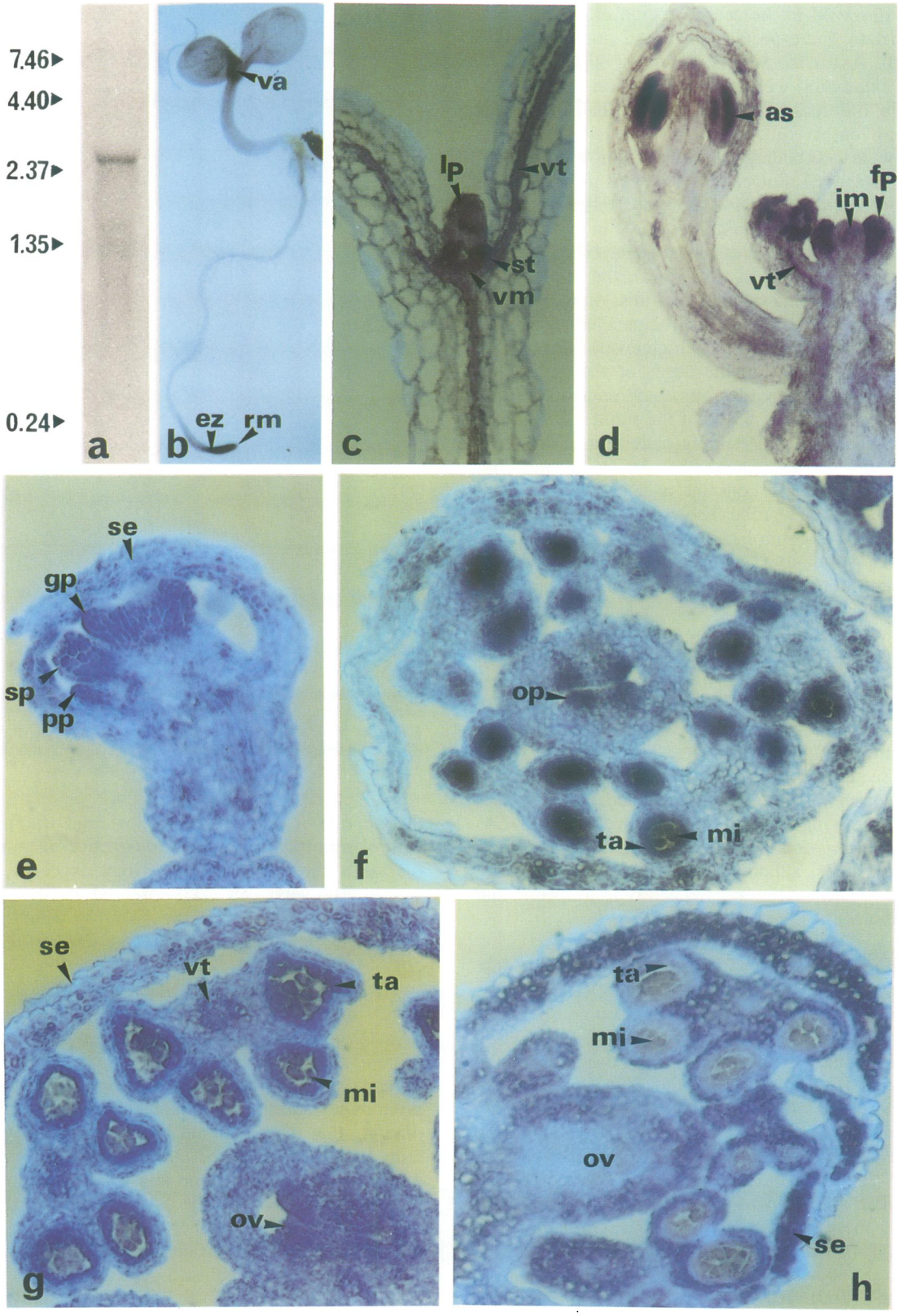

Fig. 5. Northern blot (**a**) and *in situ* analysis of *AtCDC48* (**b–g**) and *CAB2* (**h**) expression in *A.thaliana* seedlings, inflorescence shoot and floral buds. (b) Whole-mount *in situ* pattern of *AtCDC48* expression in a 7-day-old seedling. Longitudinal sections of (c) a vegetative shoot apex from a 5-day-old seedling, (d) inflorescence shoot with stage 2 flower primordia and stage 5 and 8 flowers and (e) a stage 6 flower are shown. Transverse sections of flowers at stage 9 (f) and 10/11 (g and h) are shown. Abbreviations: va, vegetative shoot apex; ez, root elongation zone; rm, root meristem; lp, leaf primordium; vm, vegetative meristem; st, stipules; vt, vascular tissue; im, inflorescence meristem; fp, flower primordium; as, anther sac; gp, gynoecial primordium; sp, stamen primordium; pp, petal primordium; se, sepals; op, ovule primordium; ta, tapetum; mi, microspores; ov, ovules.

sion were observed in the central region of the inflorescence meristem, and especially in stage 2 floral primordia (Figure 5d). As in the vegetative shoot, high levels of *AtCDC48* expression were also observed in the vascular tissue of the inflorescence shoot. In floral buds, a similar pattern was observed; *AtCDC48* expression was up-regulated in the cells undergoing cell division or expansion. In young (stage 6) flowers, high levels of *AtCDC48* transcript were distributed uniformly throughout the developing organ primordia (Figure 5e). In older (stage 9) flowers *AtCDC48* expression remained high in the ovule primordia (arising in four rows), the microspores (which are undergoing meiosis at this time) and the tapetum within the anther sac, and the petal edges (Figure 5f). In older flowers (stage 10/11) high levels of *AtCDC48* expression were limited to the edges of the petals (not shown), the elongating ovules, separated microspores, the tapetum and the vascular tissue of the stamenal filaments (Figure 5g). The uniform staining pattern in tissues containing dividing cells, suggests that *AtCDC48* transcript levels remain relatively constant over the cell division cycle. *AtCDC48* sense probes showed no hybridization in these tissues. As a control, the expression pattern of the chlorophyll a/b binding protein gene (*CAB2*) was analysed in different sections of the same samples (Figure 5h). The *CAB2* pattern was distinct, as expected, and the inverse of that noted for *CDC48*. In the anther, *CAB2* strongly labelled only the outer 'green' cell layer of the anther, but not the stomium/septum region; *CAB2* labelling was absent from the anther sac where *CDC48* transcripts were abundant. These data show that specific staining patterns were obtained with our analysis. Therefore, in whole plants and sections, the highest levels of *AtCDC48* expression were observed uniformly distributed in the tissues containing growing and/or dividing cells. *AtCDC48* expression was down-regulated in morphologically differentiated cells, although high transcript levels were maintained in a few differentiated cell types (tapetum and vascular tissues).

Immunoblot analysis and localization of AtCDC48p

Antibodies raised against two conserved, VCP peptides were obtained from M.Egerton, L.Samelson and K.Koller (Koller and Brownstein, 1987; Egerton *et al.*, 1992). Polyclonal antibody 725 (pAb725) was raised against the 21 aa peptide KNRPNRLIVDEAINEDNSVVS (pep725: aa residues 20–40 of the murine VCP) (Egerton *et al.*, 1992). Polyclonal antibody 200 (pAb200) was raised against the valosin peptide VQYPVEHPDKFLKFGMTPSKGVLFY (pep200: aa residues 493–517 of the porcine VCP) (Koller and Brownstein, 1987). The amino acid residues of these peptides were identical or similar to those found in AtCDC48p unless underlined (Figure 2). These antibodies were used to immunostain two-dimensional (2-D) IEF/SDS–PAGE protein blots of *A.thaliana* proteins isolated from young etiolated seedlings (Figure 6). PAb725 was raised against a peptide located near the N-terminus and therefore outside of the ATPase modules and not strongly conserved in the other CAD proteins. PAb725 recognizes *A.thaliana* proteins which migrate on IEF/SDS–PAGE at a molecular weight of 106 kDa with a p*I* of ~5.7 (Figure 6A). This signal is completely blocked by preincubation of the antibody with

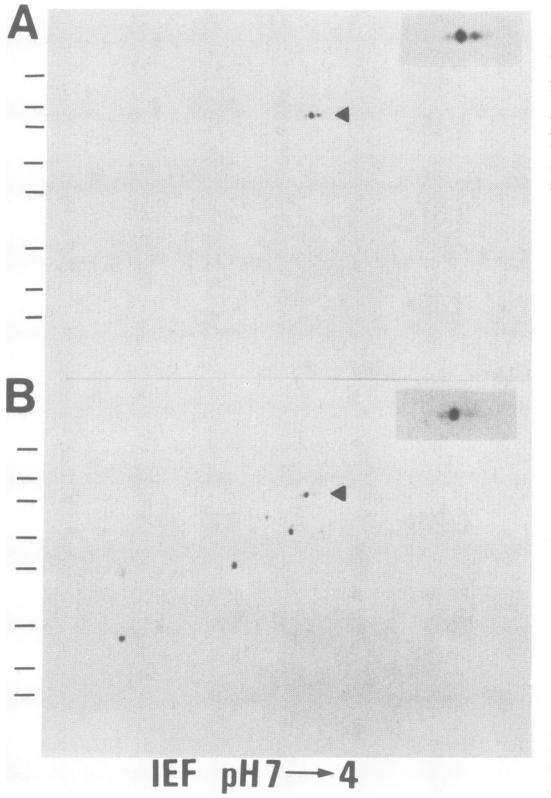

Fig. 6. Two-dimensional IEF/SDS–PAGE immunoblot patterns of *A.thaliana* proteins recognized by anti-CDC48 peptide antibodies pAb725 (**A**) and pAb200 (**B**). The immunoreactive protein(s) delineated with arrows are shown enlarged to the upper-right in each panel. Molecular weight markers in each panel, from top to bottom, were: 200, 116, 97.4, 66, 45, 31, 21.5 and 14.5 kDa.

the peptide it was raised against and the preimmune control serum for pAb725 was negative on immunoblots (data not shown). The higher than expected molecular weight (106 versus 89 kDa) observed on SDS–PAGE has also been noted for VCP and CDC48p which migrate at 100 and 115 kDa, respectively (Peters *et al.*, 1990; Fröhlich *et al.*, 1991).

The primary 106 kDa protein, as well as a few other lower molecular weight proteins, are recognized by pAb200 on *A.thaliana* immunoblots of etiolated seedling proteins (Figure 6B). While non-specific staining of plant proteins by the pAb200 serum cannot be ruled out, as the preimmune serum was unavailable for analysis, it is possible that the additional lower molecular weight proteins recognized by pAb200 are breakdown or processing products of the 106 kDa protein which have lost their N-terminus and therefore the pAb725 epitope (Koller and Brownstein, 1987). Another possibility is that the lower molecular weight proteins are other CAD family members; PAb200 was raised against a peptide that falls within the ATPase module (see Figure 2) which is highly conserved in the members of this superfamily.

Immunolocalization of AtCDC48p was conducted by staining semi-thin sections of paraffin-embedded floral buds and fixed root tip cells of *A.thaliana* with pAb725 (Figures 7 and 8, respectively). Immunostaining of sections showed that the pattern of protein expression in plant tissues was similar to that observed for *AtCDC48* mRNA. In floral buds, AtCDC48p was localized to the developing

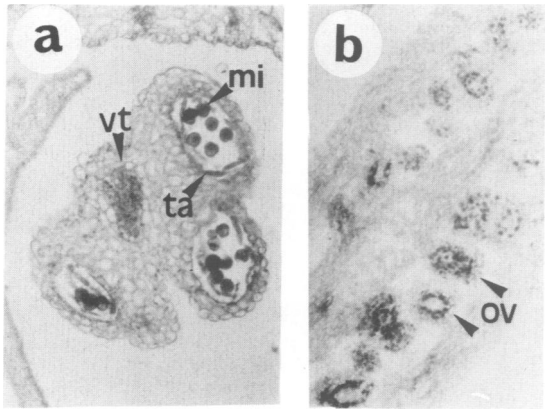

Fig. 7. Immunolocalization showing the expression of AtCDC48p in semi-thin sections of *A.thaliana* floral bud tissues. Immunostaining patterns of an anther (**a**, transverse section) and an ovary with developing ovules (**b**, longitudinal section) are shown. Stained cell types are labelled as follows: vt, vascular tissue; ta, tapetum; mi, microspores; ov, ovules. These expression patterns are identical to those observed for *AtCDC48* RNA (Figure 5).

microspores, tapetum and vascular tissue of the anther, and to the expanding ovules in the ovary (Figure 7a and b, respectively); this pattern is identical to the *AtCDC48* mRNA expression pattern (Figure 5g). Controls with the preimmune serum and secondary antibody alone gave low, uniform staining or no staining on sections, respectively (data not shown).

At the subcellular level, AtCDC48p was primarily localized as diffuse staining in the nucleoplasm, that was absent from the nucleolus, in interphase cells and cells containing a preprophase band (late G_2) (Figure 8a and c). Cortical and punctate staining of the cytoplasm was also observed in some cells (Figure 8a and e). In metaphase cells, the condensed chromatin was devoid of staining and a slight concentration of staining could be observed surrounding the chromosomes (Figure 8e). In some metaphase cells the staining was noticeably absent from the ends of the spindle (Figure 8g). In cells undergoing cytokinesis, AtCDC48p staining was observed in the reformed nuclei and at the phragmoplast; the site where new plasma membrane and cell wall are constructed (Figure 8i and k). No nuclear staining and only a weak, punctate stain of the cytoplasm was observed with preimmune serum (Figure 8m). Various fixation and treatment conditions were tested. The nucleoplasm staining was consistently observed in preparations that were extracted with 0.1% NP-40. When extraction and air-drying were avoided, however, the nucleoplasm staining was obscured and large spots, in addition to the punctate staining, were observed in the cytosol (data not shown). The spotty cytosolic staining pattern was variable, however, and not very stable. These results suggest that AtCDC48p is a soluble protein which is located in the nucleus and the cytoplasm and associates with newly deposited plasma membrane and, possibly, certain cytosolic structures.

Discussion

AtCDC48 is the first member of the CAD superfamily to be described in the higher plant *A.thaliana*. Extreme conservation of the amino acid sequence is observed with respect to the yeast and putative metazoan homologues and is especially high within the core of the protein (aa 207–691) which contains the two copies of the ATPase module. The ATPase modules contain perfect Walker type-A and -B motifs, which are important in ATP binding and hydrolysis, respectively and the SRH contains a modified 'sAT' region and a 'basic' amino acid signature, motifs generally found in ATPases with DNA/RNA helicase activity. In addition, the spacing between these motifs and the motifs themselves, as they are found in AtCDC48p and its homologues, are identical to those found in the 'ABC domain' in the DNA-dependent ATPases (Koonin, 1993b). The significance of this similarity is unknown but it appears to represent a genuine ancestral relationship.

The developmental organization of higher plants allows gene expression patterns to be analysed with respect to the state of cell proliferation as the meristematic zones, which contain dividing and expanding cells, and the zones of cell differentiation are morphologically distinct and physically separated from each other. We have described the *in situ* expression pattern of *AtCDC48* and the results obtained are consistent with the hypothesis that *CDC48* plays a role in cell division, and also suggest that it functions in expanding cells but not generally in morphologically differentiated cells in higher plants. The patterns of *AtCDC48* expression are very similar, if not identical, to those observed for certain other genes implicated in cell division cycle competence or growth, such as the cdk *CDC2* (Martinez *et al.*, 1992) and a type-1 phosphatase (Arundhati *et al.*, 1995). Like those genes, *AtCDC48* expression does not vary with the stages of the cell division cycle and is up-regulated in the meristematic and growing tissues of the hypocotyl, root, inflorescence meristems and flowers. This pattern was especially clear in flowers where, initially, high levels of expression were observed throughout the undifferentiated organ primordia and later only in the edges of the petals, the microspores, the tapetum and developing ovules. *AtCDC48* expression was down-regulated in morphologically differentiated cells, although high transcript levels were observed in a few differentiated cell types. Expression in the tapetum, a floral bud tissue which is mitotically and metabolically very active and secretory in function, was especially high throughout development; even after the cessation of cell division in this tissue. This study shows that *AtCDC48* expression does not correlate strictly with cells actively undergoing cell division but that *AtCDC48* transcripts are abundant in morphologically undifferentiated cells which maintain meristematic capabilities and/or are growing.

We have demonstrated that a higher plant *CDC48* homologue can indeed functionally complement *S.cerevisiae cdc48* conditional mutants, allowing mitotic spindle assembly and cell division to proceed. Interestingly, the porcine VCP homologue does not functionally complement *cdc48* mutants despite amino acid conservation comparable with AtCDC48p (K.-U.Fröhlich, personal communication). The dissimilarities between the plant and the mammalian proteins are so rare (87% similar) that it may be possible to determine a functional domain by sequence comparison. Indeed, one difference between the VCP, AtCDC48 and ScCDC48 protein sequences is that a putative nuclear localization signal exists at the same position in the N-terminus in the plant and yeast sequence

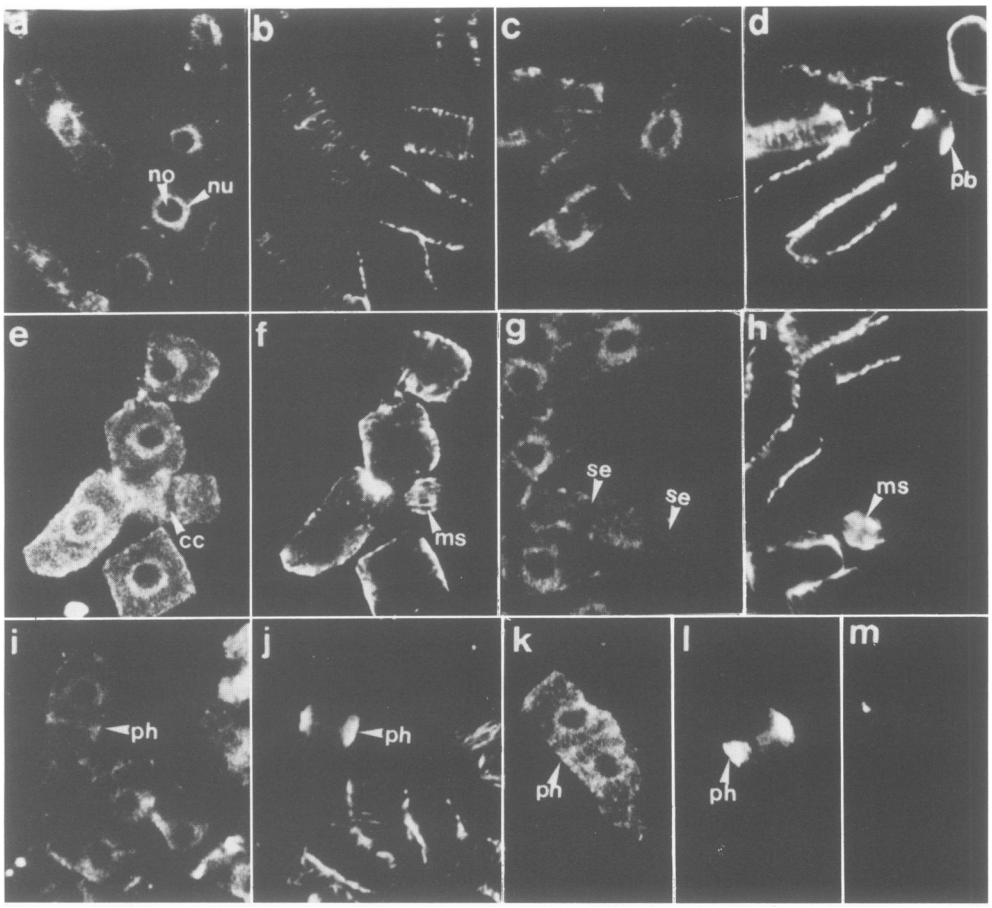

Fig. 8. Double-labelling immunofluorescence microscopy showing the localization of AtCDC48p (**a**, **c**, **e**, **g**, **i** and **k**) and α-tubulins (**b**, **d**, **f**, **h**, **j** and **l**) in formaldehyde-fixed cells from *A.thaliana* root tips. The tubulin labelling follows the CDC48p labelling for each pair. Interphase cells (a and b), a late G_2 stage cell with a preprophase band (c and d), metaphase cells with mitotic spindles (e–h), cells undergoing cytokinesis with phragmoplasts (i–l), and pAb725 preimmune serum control (**m**) labelling are shown. Abbreviations: nu, nucleus; no, nucleolus; pb, preprophase band; cc, condensed chromatin; ms, mitotic spindle; se, spindle 'ends'; ph, phragmoplast.

[KK.(8X).RKK and RRKKK, respectively] but this putative signal has been lost in VCP. This signal may be important in the import of CDC48 proteins into the nucleus in yeast, possibly because the nuclear envelope does not degenerate during the cell division cycle. These proteins may be transported between the nucleus and the cytoplasm and function in both places. We have shown that AtCDC48p lacks membrane-spanning regions and immunostaining patterns suggest that it is a soluble protein which localizes to the nucleoplasm and the cytoplasm, as do VCP and ScCDC48p (Peters *et al.*, 1990; Fröhlich *et al.*, 1991).

While the manner in which CDC48p is regulated is currently unknown, AtCDC48p immunoblot patterns and specific sequence motifs suggest a testable hypothesis. The three-spot pattern observed on 2-D immunoblots may represent post-translational modification of a single, 106 kDa protein. Mammalian VCP migrates on IEF/SDS–PAGE with a similar molecular weight, p*I* and pattern (Peters *et al.*, 1990) although multiple, distinct spots were not clearly resolved and instead a smear was observed, possibly as a result of using ampholines as opposed to the immobilized pH gradients utilized in this study. Post-translational modifications which could result in the pattern observed include phosphorylation. The phosphorylation of VCP on tyrosine residues in response to T-cell activation and other residues has previously been demonstrated (Egerton *et al.*, 1992). We have identified putative cdk and casein kinase II phosphorylation sites in AtCDC48p, some of which are conserved in ScCDC48p and VCP. One cdk phosphorylation consensus site is of particular interest as it immediately follows the putative bipartite nuclear localization signal, discussed previously. The combination of a nuclear localization signal adjacent to either a cdk phosphorylation site or a casein kinase II phosphorylation site has been shown to be an important method of regulating protein function by facilitating import into the nucleus at specific times during the cell cycle (Moll *et al.*, 1991). Genetic evidence supports the hypothesis that some of the CAD proteins are activated by the *S.cerevisiae* cdk encoded by *CDC28* (Ghislain *et al.*, 1993). In yeast, the CDC28 kinase, and other kinases, play an important role in spindle pole body duplication (Rose *et al.*, 1993), the process in which CDC48 appears to function. In HeLa cells, the cdk $p34^{cdc2}$ localizes to the centrosomes during duplication (Bailly *et al.*, 1989) and in maize, $p34^{cdc2}$ localizes primarily to the nucleus in interphase and early prophase cells (Colasanti *et al.*, 1993), as does CDC48p. It will be important to determine if AtCDC48p is phosphorylated by cdks or other kinases, possibly linking its activity or localization with cell cycle progression.

It has been suggested that CDC48/VCP, like the CAD-ATPase NSF, functions in some aspect of membrane vesicle transport or fusion (Fröhlich *et al.*, 1991; Pleasure *et al.*, 1993). This may also be the case in higher plants. Shi *et al.* (1995) have shown that sVCP is recognized by an antibody raised against a crude soybean plasma membrane fraction. In this study, we have observed the localization of a portion of the AtCDC48p in the cell at the cortex and in the phragmoplast; particularly in the region where membrane vesicles fuse to form new plasma membrane and cell wall during cytokinesis. It will be important to determine if CDC48 has a role in membrane trafficking and, if so, if this activity is linked to the cell division phenotype observed in yeast.

In conclusion, we have shown that the *A.thaliana* genome encodes a CDC48 homologue which is very similar in sequence to the *S.cerevisiae* CDC48p and mammalian VCP, but, unlike VCP, can functionally complement conditional *CDC48* yeast mutants. These results show that the essential function of CDC48 with respect to cell division processes has been conserved in at least some multicellular eukaryotes and suggest that CDC48 has a similar function in higher plants. The observation that *AtCDC48* is highly expressed in meristematic and expanding cells but not generally in morphologically differentiated cells would support this hypothesis and suggests that CDC48 also functions in cell growth processes. Further study of CDC48 may help elucidate the processes of mitotic spindle formation and membrane vesicle trafficking in higher plants, subjects of which very little is currently understood.

Materials and methods

AtCDC48 cDNA isolation, sequencing and characterization

AtCDC48 cDNA clones, one partial (clone VCV017-22492) and one full-length (clone: YAY020), were obtained from the Expressed Sequence Tag Project (INRA-Versailles, CNRS-Gif-sur-Yvette). Double-stranded nested deletions were conducted as described by the manufacturer (Pharmacia) with the addition of a Klenow polishing step before recircularization. Double-stranded DNA sequencing was conducted with an ABI373A sequencer and dye labelled T7 and T3 primers (Applied Biosystems; Foster City, CA). DNA and protein sequences were analysed with GCG package programs (SEQED, LINEUP and PILEUP).

Functional complementation of S.cerevisiae cdc48 mutants

Two *cdc48* conditional *S.cerevisiae* mutants were kindly provided by K.-U.Fröhlich (Physiologisch-chemisches Institut der Universität Tübingen; Tübingen, Germany): DBY2030 (*MAT***a** ade2-101 lys2-801 ura3-52 cdc48-1 cs) and KFY189 (*MAT***a** lys2 leu2 ura3 cdc48-8 ts). *AtCDC48* was cloned in the sense and antisense orientations into the *Not*I sites of pFL61 (Minet *et al.*, 1992), a yeast expression vector which gives constitutive expression from the phosphoglycerate kinase promoter. The *Not*I sites were added to the *AtCDC48* sequence by moving the *Hin*dIII–*Spe*I fragment from YAY020 into a modified pBluescript vector containing *Not*I sites flanking these sites. Standard electroporation techniques (Becker and Guarente, 1991) were used to transform each mutant with three constructs: the vector pFL61, pFL61 expressing the sense mRNA from *AtCDC48* (pAtcdc48s) and pFL61 expressing the antisense mRNA strand from *AtCDC48* (pAtcdc48a). Ura^+ transformants were selected on synthetic medium containing 2% glucose, but without uracil (SD) at 28°C. Transformants were subsequently re-streaked onto rich medium (1% yeast extract, 2% bacto-peptone and 2% glucose; YEPD) for analysis of functional complementation at the non-permissive temperatures of 16°C for DBY2030 and 36 and 37°C for KFY189, respectively. Control experiments were conducted as follows. Plasmid DNA was isolated from yeasts transformed with Atcdc48s and showing a complemented phenotype as previously described (Hoffman and Winston, 1987) with a few modifications: (i) the extraction buffer consisted of 2 M Tris–HCl, pH 8, 0.2 M ethylenediaminetetra-acetic acid (EDTA), 10% (w/v) Nonidet P-40 (NP-40), 15% (w/v) lithium lauryl sulfate and 10% (w/v) sodium deoxycholate; and (ii) the plasmids were further purified by ethanol/ammonium acetate precipitation. The plasmids were transformed by electroporation (Dower *et al.*, 1988) and amplified in *E.coli* strain DH5α, repurified with Qiagen TIP20 columns (Qiagen Inc.), analysed by endonuclease restriction enzyme digests and subsequently retransformed into the mutant yeast strains. In addition, yeasts transformed with pAtcdc48s were streaked onto SD containing 0.1% uracil and 0.1% 5-fluoro-orotic acid (FOA; Sigma) for selective loss of the plasmids expressing *URA3* (Boeke *et al.*, 1984). Colonies that grew were subsequently streaked onto YEPD and challenged at the non-permissive temperature.

Phenotypic analysis was conducted by splitting fresh, log-phase cultures of KFY189 and KFY189 transformed with Atcdc48s in half and shifting a portion of each culture to the non-permissive temperature of 36°C. After 5 h, the cells were harvested, fixed for 2 h with 4% paraformaldehyde in 35 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.9, 0.5 mM $MgSO_4$ buffer, washed in this buffer and applied to poly-L-lysine treated slides. The cells were allowed to settle for 10 min, air-dried and the DNA subsequently stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 μg/ml. The preparations were examined with a Nikon FXA microscope equipped with Nomarski and epifluorescence optics.

Northern blot and in situ analysis of AtCDC48 expression

Antisense RNA probes were prepared by *in vitro* transcription with digoxigenin-dUTP (Boehringer-Mannheim) according to the manufacturer's instructions, except where noted. RNA probes corresponding to the full-length *AtCDC48* clone (YAY020) and a subclone containing the 5′ terminal 370 bp (up to the *Bgl*II site in YAY020) were linearized with *Sal*I and the partial *AtCDC48* clone (VCV017) which contains the 3′ terminal 1.2 kb region was linearized with *Xho*I for antisense probe synthesis. All RNA probes were synthesized with the T3 polymerase from 5 μg of linearized plasmid template; the DTT and RNAsin (Boehringer-Mannheim) concentrations were increased to 15 mM and 1 U/μl, respectively. The RNA probes corresponding to the full-length *AtCDC48* clone (YAY020) and the partial *AtCDC48* clone (VCV017) were hydrolysed for 45 min in the presence of 100 mM carbonate buffer, pH 10.2, at 60°C. An equal volume of 0.2 M sodium acetate (pH 6, with acetic acid) was used to stop the hydrolysis reactions. The short RNA probe corresponding to the 5′ terminus was not hydrolysed. The samples were incubated with DNase I (0.2 U/ml) for 10 min at 37°C, extracted with phenol:chloroform:isoamyl alcohol and the RNA probes precipitated with 400 mM LiCl and 3 vols of ethanol. The RNA pellet was solubilized in 100 μl *in situ* hybridization buffer [2× SSC, 10% (w/v) dextran, 1% (v/v) Denhardt's and 0.1 mg/ml salmon sperm DNA].

For Northern blots, total RNA was isolated from *A.thaliana* plants (5 days old, grown in culture medium) and flower buds of all stages (var. Columbia) (Verwoerd *et al.*, 1989). Total RNA (10 μg/lane) was separated on a formaldehyde/agarose gel, blotted onto Hybond-N nylon membrane (Amersham) by capillary transfer using 10× SSC and UV-cross-linked to the membrane (Stratalinker, Stratagene). Digoxigenin-labelled probes (10 μl), synthesized as described above, were heated at 80°C for 5 min and added to blots in fresh prehybridization buffer [50% (v/v) formamide, 5× SSC, 0.1% (w/v) *N*-lauroyl sarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS) and 5% Blocking Reagent (Boehringer-Mannheim)]. After hybridization overnight at 65°C the blots were subsequently washed in: (1) 2× SSC, 0.1% SDS for 30 min at 23°C; (2) 20 μg/ml RNase A in NTE (0.5 M NaCl, 10 mM Tris–HCl, pH 7.5, and 1 mM EDTA) for 30 min at 37°C; and (3) 0.1× SSC, 0.1% SDS for 60 min at 50°C. Hybridized probes were immunologically detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-DIG) and nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/XP) substrates. The blots were rinsed with B1 (100 mM Tris–HCl, pH 7.5, and 150 mM NaCl), blocked in B1 containing 3% bovine serum albumin (BSA) and 0.5% Tween-20, and incubated with Anti-DIG (Boehringer-Mannheim) diluted 1:10 000 in B1 containing 1% BSA. The blots were extensively washed with B1 containing 0.1% BSA. After rinsing in NBT/XP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) the blots were incubated with the NBT (340 μg/ml) and XP (175 μg/ml) substrates until a positive signal was obtained.

Whole mount *in situ* analysis was conducted as previously described (Ludevid *et al.*, 1992) with one modification in that 10% (v/v) dimethyl sulfoxide was added to the fixative. For *in situ* analysis on semi-thin sections, plant material was prepared, embedded in methacrylate resin (TAAB; UK) and sectioned essentially as previously described (Baskin

et al., 1992; Kronenberger *et al.*, 1993). Briefly, young seedlings and flower buds were fixed in a PME buffer [100 mM PIPES, pH 6.9, 10 mM $MgSO_4$, 10 mM ethylene glycol-*bis*(b-aminoethyl ether) *N,N,N',N'*-tetra-acetic acid (EGTA)] containing 1 mM DL-dithiothreitol (DTT) and 8% paraformaldehyde, under vacuum for 10 min and then put into fresh fixative overnight at 4°C. After further washing, the samples were dehydrated in an ethanol series, infiltrated with a series of ethanol: methacrylate (4 parts butyl: 1 part methyl methacrylate containing 10 mM DTT), the tissues were embedded in methacrylate and polymerized in the presence of 0.5% (w/v) benzoin ethyl ether by UV light. Sections (6 mm) were cut on a Reichert ultramicrotome (Heidelberg) and deposited onto sterile poly-L-lysine coated slides. The sections were air-dried, the resin removed with acetone, and the sections air-dried again. Treatment of the sections before hybridization, hybridization and washing of the sections was conducted essentially as previously described (Coen *et al.*, 1990; Jackson, 1991). The sections were hybridized at 50°C overnight with 10 µl of the digoxigenin-labelled RNA probe which had been diluted to 100 µl with *in situ* hybridization buffer and heat denatured. The sections were washed in 4× SSC, 2× SSC, RNase A (20 µg/ml) in NTE at 37°C for 30 min, 0.1× SSC at 50°C for 15 min and PBS (130 mM NaCl and 10 mM sodium phosphate, pH 7). After blocking the sections in B2 (B1 plus 0.5% Blocking Reagent (Boehringer-Mannheim) and in B3 (B1 plus 1% BSA and 0.3% Triton X-100), the sections were incubated with Anti-DIG (diluted 1:2500 in B3). The sections were washed extensively in B3 and rinsed in NBT/XP buffer. The chromogenic reaction, conducted as described above, was carried out for ~16 h. The sections were counterstained with DAPI and Calcofluor white M2R (Sigma) and examined with a Nikon FXA microscope equipped with epifluorescence optics.

Protein immunoblot analysis

The pAb725 antibody which was raised against the VCP peptide corresponding to aa 20–40 of the porcine VCP sequence (KNRPN-RLIVDEAINEDNSVVS) was kindly provided by M.Egerton (The Bancroft Centre, Brisbane, Queensland) and L.Samelson (NIH, Bethesda, MD, USA). The pAb200 antibody which raised against the anti-valosine peptide (aa 493–517; VQYPVEHPDKFLKFGMTPSKGVLFY) was obtained from K.Koller (Affymax Research Institute, Palo Alto, CA, USA). The underlined residues in the peptide sequence indicate residues which are not identical or similar in the *A.thaliana* sequence.

Protein extraction, IEF/SDS–PAGE and semi-dry electrophoretic blotting were conducted as previously described (Santoni *et al.*, 1994). Briefly, IEF/SDS–PAGE was performed horizontally with the MultiphorII apparatus according to the manufacturer's instructions (Pharmacia LKB Biotechnology; Uppsala, Sweden). Total proteins (15 µg) were focused on preformed immobilized pH gradients (pH 4–7) and subsequently separated by molecular weight on SDS–PAGE conducted horizontally on an excelGel™ (Pharmacia). The proteins were transferred to Immobilon™ and the protein blots were blocked with TBS (25 mM Tris–HCl, pH 8, 137 mM NaCl and 3 mM KCl) containing 0.5% Tween-20 and 2% BSA. The blots were incubated with diluted antibodies (1:1000 for pAb725 or 1:500 for pAb200). The blots were extensively washed in TBS and incubated with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma) which had been diluted 1:15 000. The blots were washed with TBS and the signal detected using ECL reagents (Dupont–NEN).

Immunolocalization

Floral buds were fixed and dehydrated as above for *in situ* analysis. For inclusion into paraffin the samples were infiltrated with the following series: 50% ethanol/50% xylene, 100% xylene, 50% xylene/50% paraffin at 40°C for 6 h with three changes and 100% paraffin at 60°C for 2 days with multiple changes. Sections (6 µm) were placed on poly-L-lysine-coated slides and after removal of the paraffin with xylene and rehydration through an ethanol/NaCl series, the sections were post-fixed with 4% paraformaldehyde in 100 mM PIPES, pH 6.9. After thorough washing in TBS, the sections were immunostained with pAb725 as described above for the immunoblots except that a goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma) was used at a dilution of 1:1000 and detected with NBT/XP chromogenic reagents.

Arabidopis thaliana plants (5 days old) were fixed in PME buffer containing 4% paraformaldehyde for 60 min and washed in PME overnight at 4°C. The next day the samples were re-washed with PME and treated with 2% Driselase (Sigma) in PME for 10 min. After further washes in PME and then water, the root tips were squashed onto poly-L-lysine-treated slides, air-dried, extracted with 0.2% (v/v) NP-40 in PME for 10 min and washed extensively with PME. The samples were blocked in PME containing 0.5% (w/v) BSA (PMEB) and then incubated in a mixture of anti-VCP (pAb725) and anti-tubulin antibodies for 60 min at 37°C and then 60 min at 23°C. The pAb725 or the preimmune serum was diluted 1:150 in PMEB and anti-tubulin antibodies, YOL1/34 and YL1/2 (Sera-Lab), were diluted 1:50. After extensive washing in PME the samples were incubated with a goat anti-rabbit TRITC-labelled antibody (diluted 1:500 in PMEB) and an anti-mouse FITC-labelled antibody (diluted 1:100 in PMEB). The samples were then washed (PMEB), stained with DAPI, mounted in Citifluor (Oxford Instruments Sarl) and examined on a Zeiss confocal scanning laser microscope using a 100× objective. An argon laser provided the source of excitation light. The images were recorded using the software provided with the Zeiss system. Photographs were taken from a high-resolution, flat screen monitor.

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