Isolation, characterization and functional analysis of a *cdc48* homologue from tobacco BY-2 cells*

CHEN Zhiling[1]**, YU Yi[2]**, LIU Lina[1] and XIA Guixian[2]***

(1. College of Life Science, Capital Normal University, Beijing 100037, China; 2. Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China)

Accepted on July 6, 2006

Abstract The fission yeast *Schizosaccharomyces pombe* was used to identify genes from tobacco BY-2 cells that may play roles in cell cycle regulation. A cDNA encoding a protein homologous to the yeast CDC48 was isolated and the gene was designated as *NtCDC48*. The cDNA contains an open reading frame coding for a predicted protein of 808 amino acids which comprises of two typical ATPase modules (aa 245—374 and aa 518—646). Overexpression of *NtCDC48* in tobacco BY-2 cells led to an increase in the mitotic index as well as to the formation of diffused mitotic spindles. NtCDC48-GFP fusion proteins are distributed ubiquitously through G1 to M phases, yet their subcellular localization varied regularly along with the cell cycle progression. These results indicate that NtCDC48 may play an important role in the regulation of cell cycle in BY-2 cells.

Keywords: tobacco BY-2 suspension cells, *NtCDC48*, spindle, cell cycle.

CDC48/p97 is a family of proteins that exist ubiquitously in eukaryotic cells and play important roles in various cellular processes. The CDC48/p97 proteins contain one or two typical ATPase modules, one substrate/receptor binding site and belong to the category of AAA-ATPase that is associated with diverse physiological activities. In mammalian and yeast cells, CDC48/p97 proteins have been shown to participate in the cellular processes including spindle assembly or disassembly, endoplasmic reticulum-associated degradation and membrane fusion[1—4]. To date, very few plant CDC48 homologues have been identified and little is known about their functions[5—7]. Moreover, although CDC48 homologues from animal, yeast and plant share a high sequence homology, their functions appeared divergent. For example, Vlosin-containing protein (VCP), a porcine CDC48 homologue, could not complement *Saccharomyces cerevisiae CDC48* mutants[8]. Therefore, continuous isolation and characterization of plant *cdc48* genes are required in order to gain more insights into the *in vivo* functions of their gene products.

Tobacco BY-2 suspension cells are *in vitro* cultivated cell line. BY-2 cells have unique characteristics such as high growth rate (only 13 hours are needed to complete the entire cell cycle), high homogeneity and readily transformation via *Agrobacterium tumefaciens*. More importantly, these cells can be synchronized to a relatively high degree. All these features make BY-2 cells an ideal model system to study plant cell division.

Present study reports the isolation of *NtCDC48* cDNA from BY-2 cells and expression pattern of *NtCDC48* in different phases of the cell cycle. The functional analysis of NtCDC48 in cell cycle regulation was also investigated and the results suggest that NtCDC48 may play a critical role in the process of spindle dissociation in BY-2 cells.

1 Materials and methods

1.1 Yeast strain and growth

The fission yeast *Schizosaccharomyces pombe* (*leu1-32*, *h-*) was grown and manipulated according to the methods described by Moreno et al.[10].

1.2 Cell culture and synchronization

Tobacco BY-2 cells were maintained as described by Nagata et al.[9]. The culture was kept at 27℃ in darkness and shaked at 130 r/min in LS medium (pH

* Supported by the National Basic Research Priorities Program (Grant No. 2002CCA03000) and National Natural Science Foundation of China (Grant No. 30100091)

** These authors contributed equally to this work

*** To whom correspondence should be addressed. E-mail: guixianx@yahoo.com

5.8). The synchronization protocol was based on the method of Nagata et al.[9]. The stationary culture was transferred to the fresh medium (1:10) containing 5 $mg \cdot L^{-1}$ aphidicolin. After 24 h of incubation, cells were washed extensively with fresh medium to remove aphidicolin completely. For determination of cell mitotic index, samples of wild type and transgenic cells were taken at 1 h interval after the cell synchronization and stained with DAPI to determine the mitotic index.

1.3 BY-2 cDNA library construction and transformation

Total RNA was extracted from lag, log and stationary phases of BY-2 cells and reversely transcripted to cDNA. The BY-2 cDNAs were constructed in pREP5N (a *S. pombe/Escherichia coli* shuttle vector) under the control of a thiamine repressible promoter *nmt*-1. The cDNA library was transformed into *S. pombe* cells by electroporation and the screening of the transformants showing aberrant cell division was based on the method described by Xia et al.[11].

1.4 Construction of 35S: *NtCDC48* and *NtCDC48*: *GFP*

The *NtCDC48* cDNA was inserted into pPZP111 vector under the control of 35S promoter. To construct the *NtCDC48*: *GFP* expression vector, a pair of primers (sense: 5′ GGATTCACGTATCAACTTCTGCAAAAATC 3′; antisense: 5′ CCGCGGCCACTATACAGGTCATCTTCATC 3′) were used to amplify the coding sequence of *NtCDC48*. The obtained PCR product was inserted into pPZP-GFP vector to express the NtCDC48-GFP fusion protein. All constructs were transformed into BY-2 cells by *Agrobacterium*-mediated transformation.

1.5 RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from BY-2 cells using TRI Reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. Single-stranded cDNA was synthesized from 5 μg total RNA. *Actin7* was used to normalize each reaction. Primers to detect *NtCDC48* were: 5′ GTGTATGTTTACCTGCTTGC 3′ (forward) and 5′ AACTCGAACCTAGTCTAACAG 3′ (reverse). Primers to amplify *actin7* were: 5′ TGGAATGGTGAAGGCTGGTTT 3′ (forward) and 5′ CTGTTGGAAGGTGCTGAGGGA 3′ (reverse).

1.6 Cell staining and observation

Equal volumes of tobacco BY-2 cells expressing *NtCDC48-GFP* and a solution containing 2.0 mol/L SYTO64, 100 mmol/L Pipes, 0.1% DMSO, 0.4 mol/L mannitol were mixed, 10 min later, cells were placed onto a slide and immediately examined using a Lecia TCS SP2 confocal laser microscope.

Microtubules localization in *NtCDC48* overexpressing cells was examined as described by Hasezawa[12].

2 Results

2.1 Isolation and characterization of *NtCDC48* cDNA

A cDNA library was constructed using the total RNA extracted from lag, log and stationary phase tobacco BY-2 cells and transformed into *S. pombe* cells by electroporation. After induction of the plant cDNAs by removing thiamine (VB1) from the medium, colonies showing severe morphological changes compared to the regular shape of the control cells were selected for further studies. To confirm that the morphological changes were resulted from the expression of the plant cDNAs, plasmids were isolated from the selected clones and retransformed into original *S. pombe* cells. Around 120 colonies were obtained and the sequences of the plant cDNAs were determined.

A cDNA clone encoding for the yeast CDC48 homologue was selected for more detailed analysis. The cDNA was 2729 bp in length containing a 74 bp 5′ untranslated region (UTR), a 2427 bp open reading frame (ORF) and a 228 bp 3′ UTR. The ORF encodes for a peptide of 808 amino acids (Fig. 1). Blast analysis indicates that the predicated protein shares a high sequence homology with CDC48 from many other organisms and is 92% identical to AtCDC48 of Arabidopsis. In addition, the protein contains an N-terminal domain (aa: 27—113) and two ATPase modules (aa: 245—374 and aa: 518—646), each containing perfect Walker A and B motifs (Fig. 2). These structural features suggest that the putative protein belongs to AAA ATPase family (ATPase associated with a variety of cellular activities). Thus, we designate the gene as *NtCDC48*.

```
1    TTTGGCCTCGAGGTCGACCCACGCGTCCGCAAAATCAGAAACTCTTCAGAGACGTATCAACTTCTGCAAAAATCATGACTAACAAAGCTG
                                                                          M  T  N  K  A
91   AATCCTCCGATTCGAAAGGGACAAAGCGGGACTATAGTACGGCGATATTGGAGAGGAAGAAGTCGCCGAATCGGCTTGTTGTTGATGAGG
     E  S  S  D  S  K  G  T  K  R  D  Y  S  T  A  I  L  E  R  K  K  S  P  N  R  L  V  V  D  E
181  CAATCAACGATGACAACTCTGTTGTTGCTCTTCACCCTGATACTATGGAGAAGCTTCAGCTTTTTCGTGGTGACACTATCTTGATCAAGG
     A  I  N  D  D  N  S  V  V  A  L  H  P  D  T  M  E  K  L  Q  L  F  R  G  D  T  I  L  I  K
271  GTAAGAAGAGAAAAGATACAATCTGCATAGCTCTTGCTGATGACACCTGTGATGAGCCGAAGATCAGGATGAACAAGGTTGTCAGAAATA
     G  K  K  R  K  D  T  I  C  I  A  L  A  D  D  T  C  D  E  P  K  I  R  M  N  K  V  V  R  N
361  ACCTAAGGGTTCGACTTGGTGATGTTGTCTCTGTGCATCAGTGTCCTGATGTCAAGTATGGCAAACGTGTACACATTCTTCCCATTGATG
     N  L  R  V  R  L  G  D  V  V  S  V  H  Q  C  P  D  V  K  Y  G  K  R  V  H  I  L  P  I  D
451  ATACCATTGAAGGGGTCACTGGGAATCTCTTTGATGCTTACTTAAAACCCTATTTCCTTGAAGCATACAGACCGGTGAGGAAGGGTGATC
     D  T  I  E  G  V  T  G  N  L  F  D  A  Y  L  K  P  Y  F  L  E  A  Y  R  P  V  R  K  G  D
541  TTTTCTTGGTAAGGGGAGGGATGAGAAGTGTAGAGTTCAAGGTTATTGAAACTGATCCTCCTGAATACTGTGTTGTAGCCCCTGATACGG
     L  F  L  V  R  G  G  M  R  S  V  E  F  K  V  I  E  T  D  P  P  E  Y  C  V  V  A  P  D  T
631  AGATATTTTGTGAGGGTGAACCTGTGAGTAGGGAAGACGAGAATAGGCTAGATGAAATCGGTTATGATGATGTTGGGGGCGTGCGTAAAC
     E  I  F  C  E  G  E  P  V  S  R  E  D  E  N  R  L  D  E  I  G  Y  D  D  V  G  G  V  R  K
721  AAATGGCTCAAATACGGGAGCTTGTTGAGCTTCCACTAAGGCACCCACAACTCTTCAAATCTATTGGTGTCAAACCTCCTAAAGGAATTC
     Q  M  A  Q  I  R  E  L  V  E  L  P  L  R  H  P  Q  L  F  K  S  I  G  V  K  P  P  K  G  I
811  TGTTGTATGGACCTCCTGGATCAGGAAAGACTTTAATAGCCCGAGCAGTTGCAAATGAGACTGGTGCGTTCTTCTTCTGTATTAATGGTC
     L  L  Y  G  P  P  G  S  G  K  T  L  I  A  R  A  V  A  N  E  T  G  A  F  F  F  C  I  N  G
901  CAGAGATCATGTCAAAATTGGCTGGAGAAAGTGAAAGCAATCTTAGGAAGGCATTTGAGGAAGCTGAAAAGAATGCACCATCAATCATTT
     P  E  I  M  S  K  L  A  G  E  S  E  S  N  L  R  K  A  F  E  E  A  E  K  N  A  P  S  I  I
991  TTATCGATGAAATTGACTCAATAGCTCCTAAACGTGAGAAGACACATGGAGAGGTTGAGAGGAGGATTGTCTCCCAGCTTTTGACATTGA
     F  I  D  E  I  D  S  I  A  P  K  R  E  K  T  H  G  E  V  E  R  R  I  V  S  Q  L  L  T  L
1081 TGGATGGACTCAAATCACGTGCCCATGTAATTGTTATGGGTGCCACTAATCGCCCCAACAGCATTGACCCTGCCCTAAGAAGGTTTGGTA
     M  D  G  L  K  S  R  A  H  V  I  V  M  G  A  T  N  R  P  N  S  I  D  P  A  L  R  R  F  G
1171 GATTTGATAGGGAAATAGACATTGGTGTTCCAGATGAAGTGGGGCGTCTCGAGGTGCTTCGTATCCATACGAAGAACATGAAGCTTGCTG
     R  F  D  R  E  I  D  I  G  V  P  D  E  V  G  R  L  E  V  L  R  I  H  T  K  N  M  K  L  A
1261 AAGAAGTTGATTTAGAAAGAATTGGCAAGGACACACATGGTTATGTCGGTGCTGATTTAGCAGCTTTGTGTACCGAGGCTGCTCTTCAAT
     E  E  V  D  L  E  R  I  G  K  D  T  H  G  Y  V  G  A  D  L  A  A  L  C  T  E  A  A  L  Q
1351 GCATCAGAGAGAAGATGGACGTGATTGATTTGGAGGATGAGACCATTGATGCAGAGATACTGAACTCTATGGCTGTGACAAATGAGCACT
     C  I  R  E  K  M  D  V  I  D  L  E  D  E  T  I  D  A  E  I  L  N  S  M  A  V  T  N  E  H
1441 TCCAAACTGCTCTTGGAACGAGCAATCCCTCTGCCTTGCGTGAAACTGTTGTTGAAGTTCCCAATGTTTCCTGGGAGGATATTGGAGGCC
     F  Q  T  A  L  G  T  S  N  P  S  A  L  R  E  T  V  V  E  V  P  N  V  S  W  E  D  I  G  G
1531 TTGAGAATGTCAAGCGTGAGCTCCAAGAGACTGTTCAATATCCAGTGGAACATCCTGAGAAATTCGAGAAGTTTGGTATGTCTCCGTCAA
     L  E  N  V  K  R  E  L  Q  E  T  V  Q  Y  P  V  E  H  P  E  K  F  E  K  F  G  M  S  P  S
1621 AGGGAGTCCTGTTCTACGGCCCACCTGGATGTGGGAAAACTTTGCTCGCGAAGGCCATTGCAAATGAATGCCAGGCCAACTTCATCAGTG
     K  G  V  L  F  Y  G  P  P  G  C  G  K  T  L  L  A  K  A  I  A  N  E  C  Q  A  N  F  I  S
1711 TTAAGGGTCCAGAATTGCTCACCATGTGGTTTGGAGAGAGTGAAGCCAATGTTAGAGAAATATTTGACAAGGCTCGACAGTCTGCTCCAT
     V  K  G  P  E  L  L  T  M  W  F  G  E  S  E  A  N  V  R  E  I  F  D  K  A  R  Q  S  A  P
1801 GTGTCCTATTCTTTGATGAACTGGATTCCATCGCCACACAGAGAGGAAGTAGTGTGGGAGATGCTGGGGGAGCTGCTGATAGGGTATTGA
     C  V  L  F  F  D  E  L  D  S  I  A  T  Q  R  G  S  S  V  G  D  A  G  G  A  A  D  R  V  L
1891 ATCAACTCCTTACTGAAATGGATGGAATGAATGCTAAGAAGACTGTATTCATTATTGGTGCAACCAACAGGCCTGACATTATTGATCCTG
     N  Q  L  L  T  E  M  D  G  M  N  A  K  K  T  V  F  I  I  G  A  T  N  R  P  D  I  I  D  P
1981 CACTTCTACGGCCTGGTCGTCTTGATCAATTGATTTATATTCCTCTCCCTGATGAAGACTCTCGTCACCAAATTTTCAAGGCGTGTCTAC
     A  L  L  R  P  G  R  L  D  Q  L  I  Y  I  P  L  P  D  E  D  S  R  H  Q  I  F  K  A  C  L
2071 GAAAGTCACCCCTCTCTAAGGATATCGATTTAAGAGCTCTAGCGAAGTACACACAGGGCTTCAGTGGAGCTGACATTACAGAAATCTGTC
     R  K  S  P  L  S  K  D  I  D  L  R  A  L  A  K  Y  T  Q  G  F  S  G  A  D  I  T  E  I  C
2161 AACGTGCTTGCAAATACGCTATCAGAGAAAACATTGAGAAAGACATTGAGAGGGAGAAAAGGAGAAGCGAGAATCCTGAGGCCATGGAGG
     Q  R  A  C  K  Y  A  I  R  E  N  I  E  K  D  I  E  R  E  K  R  R  S  E  N  P  E  A  M  E
2251 AAGACGTTGATGATGAGGTAGCCGAGATCAAGCCTGCTCATTTTGAGGAATCAATGAAGTATGCTAGGAGGAGTGTTAGTGACGCAGATA
     E  D  V  D  D  E  V  A  E  I  K  P  A  H  F  E  E  S  M  K  Y  A  R  R  S  V  S  D  A  D
2341 TTCGCAAGTACCAGGCTTTTGCTCAGACGTTGCAGCAGTCTAGAGGTTTTGGTACTGAATTCCGATTCTCAGAGACAAGCACGGCAGGAG
     I  R  K  Y  Q  A  F  A  Q  T  L  Q  Q  S  R  G  F  G  T  E  F  R  F  S  E  T  S  T  A  G
2431 GGACAACTGGAACTGCTGACCCCTTCGCAACTTCAGCTGGTGGAGCAGATGAAGATGACCTGTATAGTTAGCTTGTCAGAGATTAATTTT
     G  T  T  G  T  A  D  P  F  A  T  S  A  G  G  A  D  E  D  D  L  Y  S  *
2521 CTTGTCTTACATTGCAACCCTGTAAAATGGACTAATTACTCGGCTCGAAATCCCTCCTTTGCTGTCAGTTTATAATTTATATTTGGATCT
2611 TCTATAGCTATTAATTATAAGATTTATTTTGTTCTATTATTCTCTATATATGGCTTTGATGGGGTAAGTTTCCTCCTTGTGGGGAGCTCT
2701 GATACCTGCTGCTGATAAATGATTAAGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 1. *NtCDC48* cDNA and deduced amino acid sequence.

```
                    ***** ***
NtCDC48      FKSIGVKPPK GILLYGPPGS GKTLIARAVA NETGAFFFCI NGPEIMSKLA GESESNLRKA 293
AtCDC48      .......... .......... .......... .......... .......... .......... 292
yeast CDC48  ..A..I...R .V.M.....T ....M..... ........L. ....V...M. .......... 299
mouse CDC48  ..A......R .......... ........L. .......... .......... 289

                    ########
NtCDC48      FEEAEKNAPS IIFIDEIDSI APKREKTHGE VERRIVSQLL TLMDGLKSRA HVIVMGATNR 353
AtCDC48      .......... .......... .......N.. .......... .......... .......... 352
yeast CDC48  .........A .......... ....D..N.. ....V..... .....M.A.S N.V.IA.... 359
mouse CDC48  .........A ......L.A. .......... .......... .......Q.. .....A.... 349

NtCDC48      PNSIDPALRR FGRFDREIDI GVPDEVGRLE VLRIHTKNMK LAEEVDLERI GKDTHGYVGA 413
AtCDC48      .......... .......... .....I.... .......... ...D...... S......... 412
yeast CDC48  .......... .......V.. .I..AT.... .......... ..DD....AL AAE....... 419
mouse CDC48  .......... .......V.. .I..AT.... I.Q....... ..DD....QV ANE...H... 409

NtCDC48      DLAALCTEAA LQCIREKMDV IDLEDETIDA EILNSMAVTN EHFQTALGTS NPSALRETVV 473
AtCDC48      .......... .......... .....DS... .......... ...H....N. .......... 472
yeast CDC48  .I.S..S... M.Q......L ...DEDE... .V.D.LG..M DN.RF...N. .......... 479
mouse CDC48  ......S... ..A..K...L .......... .VM..L...M DD.RW..SQ. .......... 469

                                                ** ******
NtCDC48      EVPNVSWEDI GGLENVKREL QETVQYPVEH PEKFEKFGMS PSKGVLFYGP PGCGKTLLAK 533
AtCDC48      .......N.. .......... .......... .......... .......... .......... 532
yeast CDC48  .SV..T.D.V ...DEI.E.. K...E...L. .DQYT...L. .......... ..T....... 539
mouse CDC48  ...Q.T.... ....D..... ..L....... .D..L....T .......... .......... 529

                                                ####### #
NtCDC48      AIANECQANF ISVKGPELLT MWFGESEANV REIFDKARQS APCVLFFDEL DSIATQRGS- 592
AtCDC48      .......... .......... .......... .......... .......... .......GG 592
yeast CDC48  .V.T.VS... .........S ..Y....S.I .D......AA ..T.V.L... ....KA..G- 598
mouse CDC48  .......... ..I....... .......... .........A .......... ....KA..G- 588

NtCDC48      SVGDAGGAAD RVLNQLLTEM DGMNAKKTVF IIGATNRPDI IDPALLRPGR LDQLIYIPLP 652
AtCDC48      .G..G..... .......... .......... .......... ..S....... .......... 652
yeast CDC48  .L......S. ..V....... .......N.. V........Q ....I..... .....V.... 658
mouse CDC48  NI..G..... ..I..I.... ...ST..N.. .......... ....I..... .......... 648
```

Fig. 2. ATPase module comparison in the peptide sequences of NtCDC48, *Arabidopsis thaliana* CDC48 (NP_187595), *S. pombe* CDC48 (P25694) and mouse VCP (NP_035529). The ATPase modules are shown in black box. Walker A and B motifs are marked with *** and # # #, respectively. Same amino acid sequences are indicated as ···. GenBank accession number of NtCDC48: DQ515925.

Overexpression of *NtCDC48* in yeast resulted in the formation of abnormal cells. As shown in Fig. 3, when VB1 was depleted from the medium, *S. pombe* cells harboring pREP5N-*NtCDC48* became elongated and the nuclei appeared enlarged compared with those cells containing the empty pREP5N vector, indicating that cell division was affected in the transgenic yeast cells[13].

2.2 Effects of *NtCDC48* overexpression on tobacco BY-2 cell cycle

In order to test the effect of *NtCDC48* overexpression on tobacco BY-2 cell cycle, *NtCDC48* cDNA was cloned into plant expression vector pPZP111 in the sense orientation and transformed into tobacco BY-2 cells. Several homozygous transgenic lines were generated and expression of the transgene was analyzed by RT-PCR (Fig. 4). Three independent lines (N1, N3, N4) which showed relatively high levels of *NtCDC48* transcripts were chosen for further detailed analysis.

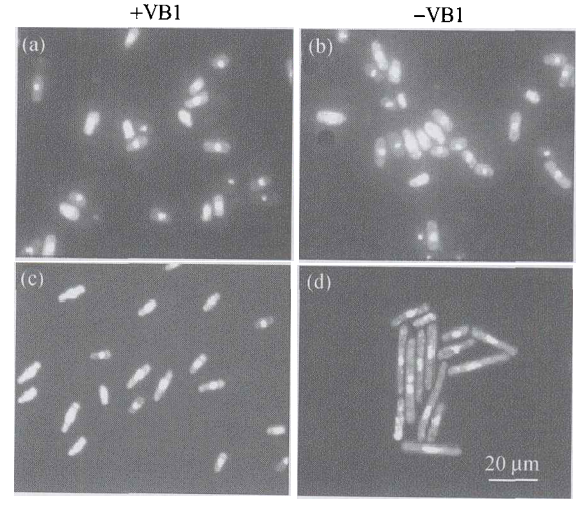

Fig. 3. Effects of *NtCDC48* overexpression on *S. pombe* morphology. (a) Morphology of *S. pombe* cells harboring pREP5N cultured for 18 h in medium with VB1; (b) morphology of *S. pombe* cells harboring pREP5N cultured for 18 h in medium without VB1; (c) morphology of *S. pombe* cells harboring pREP5N-*NtCDC48* cultured for 18 h in medium with VB1; (d) morphology of *S. pombe* cells harboring pREP5N-*NtCDC48* cultured for 18 h in medium without VB1.

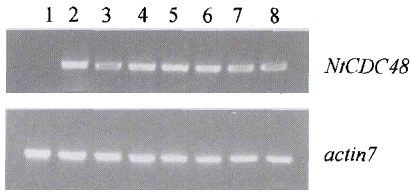

Fig. 4. RT-PCR analysis of *NtCDC48* expression in the transgenic cells. 1, Wild type cells; 2—8, transgenic cell lines (N1, N2, N3, N4, N5, N6 and N7).

As revealed in Fig. 5, the wild type cells showed a peak mitotic index of 15% 9 h after aphidicolin releasing, while the transgenic cells had a peak mitotic index of 20% 10 h after aphidicolin releasing. Although the time span taken for transgenic cells to reach peak value was slightly different from that of wild type, the M phase duration was similar (about 4 h). These results indicated that *NtCDC48* overexpression could accelerate cell division.

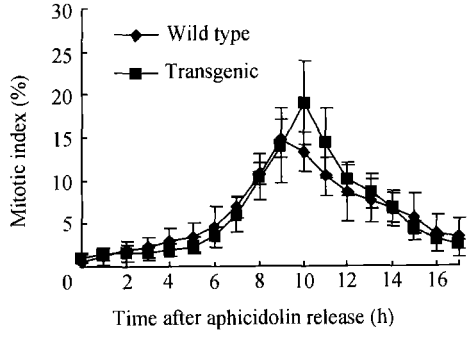

Fig. 5. Mitotic index of the wild-type and transgenic By-2 cells.

2.3 *NtCDC48* overexpression altered spindle organization

During mitosis, spindle microtubule arrays are responsible for chromosomes segregation. However, spindles failed to disassemble properly in CDC48 mutants and inhibited cell division in yeast[3]. To see if *NtCDC48* expression had an impact on the spindle structure in BY-2 cells, microtubule localization in *NtCDC48* transgenic cell lines was examined through indirect immunofluorescence staining. As shown in Fig 6, specific immunofluorescence signals concentrated on the spindle microtubule arrays in metaphase wild type cells, and a dark zone appeared in the equatorial region where the chromosomes aligned. Compared with wild type cells, microtubule arrays of metaphase transgenic cells were not able to form a typical spindle structure. The two poles were not evident and the polar microtubules appeared dispersed. In addition, the distance between the poles was shorter. These results imply that NtCDC48 may play a key role in spindle organization.

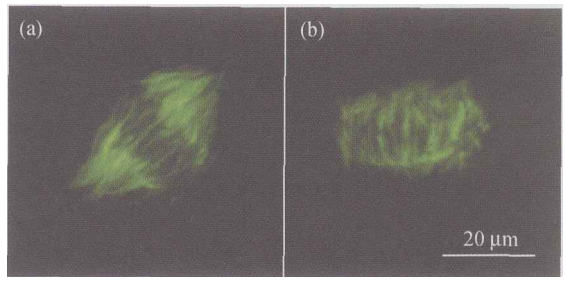

Fig. 6. Effects of *NtCDC48* overexpression on spindle structure. (a) Control cell, showing spindle microtubule arrays with focused poles; (b) transgenic cell, showing spindle microtubule arrays with diffused poles.

2.4 Subcellular localization of NtCDC48-GFP during M phase

To investigate the subcellular localization of NtCDC48-GFP during M phase, the recombinant plasmid pPZP111-*NtCDC48-GFP* was transformed into tobacco BY-2 suspension cells. Three stable transgenic lines which exhibited relatively high levels of fluorescence were selected for visualizing distribution of the fusion proteins. SYTO64, a cell-pervasive nuclear acids marker, was applied to indicate different cell cycle phases. When the nuclear membrane was broken-down in prophase, NtCDC48-GFP was distributed throughout the nucleus and cytoplasm (Fig. 7(a)). In metaphase cells, the chromosomes aligned orderly at the equatorial region, while NtCDC48-GFP

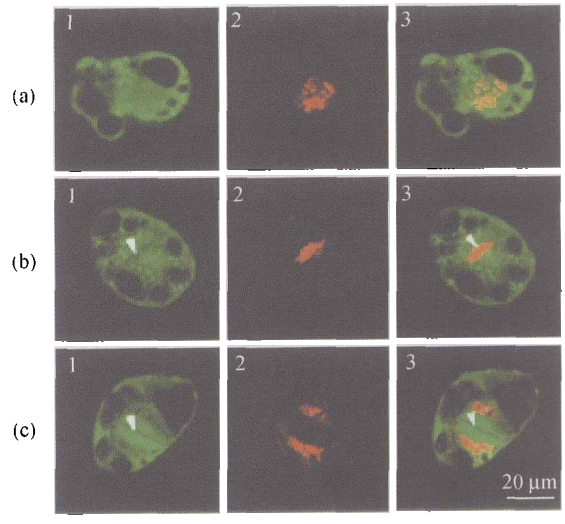

Fig. 7. NtCDC48-GFP localization in M phase. 1, NtCDC48-GFP; 2, SYTO labeled nuclei; 3, co-localization. (a) Prophase: NtCDC48-GFP mainly distributed around the nucleus and in the cytoplasm; (b) metaphase: fluorescent dots appeared in the cytoplasm far away from the metaphase plate; (c) telophase: NtCDC48-GFP restricted mainly to the two sides of division plane (arrow head indicates the cell plate).

distribution pattern changed. No green fluorescence was detected at the equatorial region, whereas fluorescent dots were detected in the cytoplasm far away from the equatorial plate. These regions were speculated to be the sites where the spindle poles would emerge (Fig. 7(b)). In cells undergoing cytokinesis, NtCDC48-GFP restricted mainly to the two sides of division plane where the phragmoplast resided (Fig. 7(c)). It has been established that phragmoplast arrays were organized following spindle arrays disassembly. Hence, these results suggest that NtCDC48, like CDC48 homologues in mammals, may function in regulating spindle disassembly[14].

In the control cells transformed with pPZP-GFP vector, green fluorescence was found in the whole cell without obvious phase specificity (data not shown).

3 Discussion

NtCDC48 cDNA was cloned from tobacco BY-2 cells whose overexpression gave rise to a clear morphological change to *S. pombe* cells. Amino acid analysis of NtCDC48 indicates that it shares high homology with *Arabidopsis* AtCDC48 and yeast CDC48. All these CDC48 homologues contain two ATPase domains, each including the Walker A and Walker B motifs. This structural feature is the typical characteristics of AAA-ATPase family, implying that NtCDC48 is a member of this super family.

CDC48 homologues are found ubiquitously in eukaryotic cells and have been shown to participate in various cellular processes. During recent years, increasing evidence demonstrated that CDC48 and its homologue VCP/97 functioned in various cellular activities through binding to different anchor proteins. Rabouille et al. reported that p97—p47 could mediate cisternal regrowth through interacting with t-SNARE and Syntaxin5[15]; Cao et al. showed that CDC48 participated in endoplasmic reticulum-associated protein degradation, nuclear membrane assembly and spindle disassembly via forming Cdc48-Ufd1-Npl4 complex[14,16].

The correct assembly and disassembly of spindle is one of the key steps during cell cycle transition. Many proteins are involved in this complex process, including microtubule associating proteins (MAPs), kinesins and protein kinases[17,18]. In mammalian cells, it has been shown that MAPs like TPX2, NuMA and XMAP215 play an important role in spindle assembly[19—21], and p97-Ufd1-Npl4 complex could regulate the spindle disassembly at the end of mitosis by associating with XMAP215 and TPX2[14]. Compared to the mammalian cells, microtubules in plant cells appear more dynamic. During cytokinesis, disassembly of spindle gives rise to construction of phragmoplast where cell plate will form, thus completing the cell cycle. These findings suggest that spindle disassembly in plant cells may have unique features. Several microtubules related proteins have been identified from plant cells and some of them are shown to be co-localizing with spindle[22,23]. Yet, the mechanisms of spindle disassembly remain unclear. The present study showed that overexpression of *NtCDC48* in BY-2 cells could enhance the diffusion of the polar spindle microtubules, implying that the function of NtCDC48 was related to spindle disassembly. As the process of spindle disassembly is a complex procedure and requires many coordinate proteins, further study of the role played by NtCDC48 in this process is needed.

Imamura et al. cloned a CDC48 homologue from zebrafish and found that overexpression of this gene in cultured embryonic cells of the fish could promote cell proliferation under cold conditions. They considered that the accumulation of CDC48 proteins in cold acclimated cells could compensate for the defect of membrane fusion function at M phase of the cell cycle[24]. In plant cells, Rancour et al. found that PUX1 (plant UBX-containing protein 1) regulated AtCDC48 activity through promoting the disassembly of hexameric AtCDC48 and regarded PUX1 as the negative regulator of AtCDC48. They further showed that *pux1* mutant plants had more cells in the root division zone[6]. In this study, we observed that overexpression of *NtCDC48* in BY-2 cells could increase the mitotic index and interfere the structure of spindle. Based on these results and the subcellular localization of NtCDC48, we make two speculations on the cellular roles of NtCDC48: (1) Cell division requires the spindle disassembly and high expression level of *NtCDC48* may accelerate this process; (2) NtCDC48 proteins change their subcellular localization regularly and may bind to different proteins at different cell cycle phases to regulate the cell division. In contrast to their function in BY-2 cells, overexpression of *NtCDC48* in *S. pombe* resulted in the interruption of M-G1 progression and in the formation of elongated cells containing a larger and diffused nucleus. This may be caused by an inhibition effect of

NtCDC48 on spindle disassembly in yeast cells. The above results indicate that NtCDC48 functions differently in BY-2 and fission yeast cells, further suggesting that the mitotic regulation mechanisms may be different between plant and yeast cells.

In summary, how NtCDC48 functions in BY-2 cells deserves further investigation. In the future, we will focus our work on identifying proteins interacting with NtCDC48 and meanwhile extend the research on this gene's application in plant cell engineering.

Acknowledgement We would like to thank Dr. K. Maundrell for providing us with pREP plasmids.

References

1 Moir D., Stewart B. C., Osmond B. C. et al. Cold sensitive cell division cycle mutants of yeast: isolation, properties, and pseudoversion studies. *Genetics*, 1982, 100: 547—563.

2 Kondo H., Rabouille C., Newman R. et al. p47 is a cofactor for p97-mediated membrane fusion. Nature, 1997, 388: 75—78.

3 Frohlich K. U., Fries H. W., Rudiger M. et al. Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a members of protein family involved in secretion, peroxisome formation, and gene expression. J. Cell Biol., 1991, 114: 443—453.

4 Braun S., Matuschewski K., Rape M. et al. Role of the ubiquitin-selective CDC48 (UFD1/NPL14) chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J., 2002, 21: 625—621.

5 Rancour D. M., Dickey C. E., Park S. et al. Characterization of Atcdc48. Evidence for multiple membrane fusion mechanisms at the plane of cell division in plants. Plant Physiol., 2002, 130: 1241—1253.

6 Rancour D. M., Park S., Knight S. D. et al. Plant UBX domain-containing protein 1, PUX1, regulates the oligomeric structure and activity of Arabidopsis cdc48. J. Biol. Chem., 2004, 279 (52): 54264—54274.

7 Prakish A. P., Kush A., Lakshmanan P. et al. Cytosine methylation occurs in a CDC48 homologue and a MADS-box gene during adventitious shoot induction in *Petunia* leaf explants. J. Exp. Bot., 2003, 54(386): 1361—1371.

8 Feiler H. S., Desprez T., Santoni V. et al. The higher plant Arabidopsis thaliana encodes a functional cdc48 homologue which is a highly expressed in dividing and expanding cell. EMBO J., 1995, 14: 5626—5637.

9 Nagata T., Nemoto Y. and Hasezawa S. Tobacco BY-2 cell line as the "Hela" cell line in the cell biology of higher plant cells. Int. Rev. Cytol., 1992, 132: 1—30.

10 Moreno S., Klar A. and Nurse P. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Meth. Enzymol., 1991, 194: 795—823.

11 Xia G. X., Ramachandran S., Hong Y. et al. Identification of plant cytoskeletal, cell cycle-related and polarity-related proteins using *Schizosaccharomyces pombe*. Plant J., 1996, 10(4): 761—769.

12 Hasezawa S., Marc J. and Palevitz B. A. Microtubule reorganization during the cell cycle in synchronized BY-2 tobacco suspensions. Cell Motil. Cytoskeleton, 1991, 18: 94—106.

13 Porceddua A., Veyldera L. D., Hayles J. et al. Mutational analysis of two Arabidopsis thaliana cyclin-dependent kinases in fission yeast. FEBS Letters, 1999, 446: 182—188.

14 Cao K., Nakajima R., Meyer H. H. et al. The AAA-ATPase Cdc48/p97 regulates spindle disassembly at the end of mitosis. Cell, 2003, 115: 355—367.

15 Rabouille C., Levine T. P., Peters J. M. et al. An NSF-like ATPase, p97, and NSF mediate cisternal regrowth from mitotic Golgi fragments. Cell, 1995, 82: 905—914.

16 Woodman P.G. p97, a protein coping with multiple identities. J. Cell Sci., 2003, 116: 4283—4290.

17 Gruss O. J. and Vernos I. The mechanism of spindle assembly: functions of Ran and its target TPX2. J. Cell Biol., 2004, 166: 945—955.

18 Ambrose J. C., Li W. X., Marcus A. et al. A minus-end-directed kinesin with plus-end tracking protein activity is involved in spindle morphogenesis. Mol. Biol. Cell., 2005, 16: 1584—1592.

19 Wittmann T., Wilm M., Karsenti E. et al. TPX2, a novel Xenopus MAP involved in spindle pole organization. J. Cell Biol., 2000, 149: 1405—1418.

20 Merdes A., Ramyar K., Vechio J.D. et al. A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. Cell, 1996, 87: 447—458.

21 McNally F. Microtubules dynamics: new surprises from an old MAP. Curr. Biol., 2003, 13: R597—R599.

22 Liu B., Cyr R. and Palevitz B. A. A kinesin-like protein, KatAp, in the cells of Arabidopsis and other plants. Plant Cell, 1996, 8: 119—132.

23 Smirnova E., Reddy A.S.N., Bowser J. et al. Minu end-directed kinesin-like motor protein, KCBP, localizes to anaphase spindle poles in *Haemanthus* endosperm. Cell Motil. Cytoskeleton, 1998, 41: 271—280.

24 Imamura S., Ojima N. and Yamashita M. Cold-inducible expression of the cell division cycle gene CDC48 and its promotion of cell proliferation during cold acclimation in Zebrafish cells. FEBS Letters, 2003, 549: 14—20.