Characterization of a novel cell cycle-related gene from Arabidopsis

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Abstract

Cell division is a fundamental biological process sharing conserved features and controls in all eukaryotes. The cell cycle is usually divided into four phases: G1, S, G2, and M. Regulated gene expression is an important mechanism for controlling cell cycle progression and genes involved in cell division-related processes often show transcriptional regulation dependent on cell cycle position. In the present report, a novel cell cycle-related gene (AtCPR) from Arabidopsis thaliana was isolated and characterized. Sequence analysis revealed that the deduced amino acid sequence of AtCPR showed 53.2% identity with p38-2G4, a mouse G1-to-S cell cycle specifically modulated and proliferation-associated nuclear protein. Assay of expression of AtCPR in partially synchronized cells suggested that AtCPR mRNA was expressed in the G1-to-S phase. In the AtCPR transgenic plants, no apparent phenotypic change was observed. By fusing a GFP tag to the AtCPR protein, it was found that AtCPR was mainly located in the nucleus. However, AtCPR does not have any transcriptional activation ability. cDNA microarray analysis showed that a total of 17 and 30 genes were identified as up-regulated and down-regulated, respectively.

Key words: Cell cycle-related, G1-to-S phase, microarray, synchronized cells.

Introduction

Cell division is one of the most fundamental biological processes and conspicuous features of life. The cell cycle consists of the alternating phases of DNA replication (S phase) and chromosome separation (M phase) interrupted by gaps known as G1 (interval between M and S phases) and G2 (interval between S and M phases) (den Boer and Murray, 2000). Since the cell theory was proposed by Schleiden and Schwann in 1838, many approaches have been taken to elucidate how cells divide, but insight into the molecular basis of division control only developed after the genetic analysis of cell division was initiated in yeast 34 years ago, which led to the identification of key molecules (CDKs, Cyclin-Dependent Kinases) of the regulatory complex: cdc2 gene in Schizosaccharomyces pombe and CDC28 in Saccharomyces cerevisiae (Hartwell et al., 1970). Another key molecule (cyclins) was first identified in marine invertebrates as proteins whose levels oscillated during the cell cycle and that, when injected into frog oocytes, could induce meiosis (Balter and Vogel, 2001). The observation that the S. pombe cdc13 gene, which encodes a cyclin, genetically interacts with cdc2 established a tight functional link between the components of the cell-cycle engine (Doerner, 1994). Since then, an increasing number of cyclins and CDKs have been identified in yeast and animal (Lew and Reed, 1992). It is generally agreed that the basic control mechanisms that regulate the progression through the cell cycle in species as diverse as yeast and animals are remarkably well conserved through evolution, both structurally and functionally. The main drivers are CDKs, a class of highly conserved serine/threonine kinases. CDK activity is strictly regulated in a complex mechanism including association with cyclin subunits, reversible phosphorylation, and association with other regulatory factors to maintain the correct temporal ordering of critical cell-cycle events.

The molecular analysis of cell division and its regulation in plants lags far behind such studies in yeast and animals. The first indication that this commonality might extend to
the plant kingdom (Mironov et al., 1999) came with the identification of a plant protein immunologically related to the CDKs (John et al., 1989), a finding that was followed by the cloning of a cDNA fragment encoding a CDK-like protein from pea (Feiler and Jacobs, 1990). Intensive cloning efforts over the past decade have identified a large number of key molecules associated with the control of cell proliferation in diverse plant species (for a review, see Stals and Inzé, 2001). The majority of these are homologues of, or contain domains homologous to, yeast and animal genes which are known to have a role in the regulation of cell division (Potuschak and Doerner, 2001). Some of them, at least, are pertinent to the understanding of cell division control (Hemerly et al., 1995; Doerner et al., 1996; Mironov et al., 1999).

Regulation of gene expression in different phases, especially in the critical control points as cells move from G1 to S phase, and from G2 into M phase, is proposed to be an important mechanism for control of progression through the cell cycle. Expression of many genes involved in cell division-related processes such as cyclin-dependent kinases (CDKs), CDK inhibitor genes, cyclins, retinoblastoma (Rb) proteins, E2F, and many other genes showed cell cycle phase-dependent behaviour (Menges et al., 2002). In yeast and mammalian cells, around 800 genes have been identified by microarray analysis as potentially cell cycle-regulated (Spellman et al., 1998; Cho et al., 2001; Whitfield et al., 2002). In the plant, Menges et al. (2002) conducted a wide-ranging analysis of regulated gene expression in Arabidopsis cell cycle-synchronized cultures and demonstrated that a large number of plant genes involved in a wide range of cellular processes, including cell cycle control, cytoskeleton, transcription, proteolysis, phosphorylation, signal transduction, biosynthesis, carbon and amino acid metabolism, hormone response, and organelle function, were likely to show cell cycle-dependent regulation of their expression. Nearly 500 genes displayed significant fluctuation in expression. In the present study, a novel cell cycle-related gene designated AtCPR was isolated and characterized from Arabidopsis, which showed homology to a cell cycle-specifically modulated and proliferation-associated nuclear protein in mouse. Expression analysis showed that AtCPR was regulated in a cell cycle phase-dependent manner. cDNA microarray analysis identified 17 and 30 genes as up-regulated and down-regulated, respectively. Subcellular localisation and transcriptional activation activity of AtCPR were also investigated.

Materials and methods

Plant materials, growth condition and stress treatments

Seeds of Arabidopsis thaliana ecotype Columbia were surface-sterilized by soaking in 70% ethanol (v/v) for 5 min and in 15% bleach (v/v) for 15 min, and then rinsed three times with sterile distilled water, and plated on solid Murashige and Skoog medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, with pH adjusted to 5.8 using 1 N KOH (Murashige and Skoog, 1962). After vernalization at 4 °C for 72 h, the seeds were germinated at 23 °C under continuous illumination. Ten-day-old seedlings were transferred from the plate and subjected to various treatments with: 200 mM NaCl, 5 μM NAA, 15% PEG 6000, 150 mM CaCl2; or at 4 °C for cold stress, respectively. For the collection of plant parts for organ-specific expression analysis, wild-type plants were grown in soil until flowering. Samples were harvested at the various times indicated and quickly frozen in liquid nitrogen and stored at −70 °C for total RNA isolation.

AtCPR gene cloning

While using a cDNA fragment encoding a conserved DNA binding domain of AtDREB2A (Liu et al., 1998) as a probe to screen a salt-treated cDNA library of Atriplex hortensis, a cDNA of 1.5 kb was isolated under low stringency condition. This 1.5 kb gene from Atriplex hortensis was designated as Cell-Proliferation-Related gene (AtCPR). The homologous gene of Arabidopsis AtCPR was cloned by the reverse transcription-PCR (RT-PCR) method using cDNAs synthesized from RNA isolated from Columbia wild-type plants as templates. The specific primer pairs used were: 5′-TTTACCATTTCCCTCTTTTCCA-3′ and 5′-ATAAGAGAATGAGATAAGATGTAG-3′. The full-length sequence of AtCPR has been deposited in GenBank databases under the accession number AY056616.

RNA isolation and RNA gel-blot analysis

Total RNA was isolated following the description by Zhang et al. (1996). Briefly, the samples were powdered in liquid nitrogen, extracted with guanidinium–thiocyanate–chloroform, precipitated with ethanol, and purified with LiCl. Total RNA was suspended in RNase-free water and stored at −20 °C after quantification. Total RNA (25 μg) was denatured and size-fractionated on a 1.0% agarose gel containing 2.2 M formaldehyde, and subsequently capillary-transferred to Hybond-N+ nylon membranes using 20× SSC. RNA hybridization was performed overnight at 65 °C by using α-32P-dCTP-labelled AtCPR cDNA as a probe. Membranes were washed with 2× SSC, 0.1% SDS, and 1× SSC, 0.1% SDS for 15 min at 45 °C respectively. After stripping of the probe, the same blots were reprobed with 18S rRNA gene to examine the equal loading. The membranes were then autoradiographed by using a phosphoimaging system (Amersham Pharmacia).

Subcellular localization of AtCPR

The complete AtCPR open reading frame sequence was amplified by PCR using the original plasmid as a template with the gene-specific primer pair: 5′-AAATCAGGAGATGGTCTCGAGGATGAGACGCTTC-3′ and 5′-TTTACCATTTTCTTTTCCA-3′ and 5′-ATAAGAGAATGAGATAAGATGTAG-3′. The PCR product was cloned into pGEM-T Easy vector (Promega) to get T-easy-AtCPR (SacII/SalI) and sequenced. Another specific primer pair, 5′-ATTGTCAGACTGAGTAAAAGGAAAGCT-3′ and 5′-AAAGACCTCTATTGATGTTGATCA-3′, was used to amplify the open reading frame sequence of the green fluorescence protein (GFP) reporter gene. GFP gene sequence was ligated to the 3′ terminal of the AtCPR gene through the SacII site to get T-easy-AtCPR-GFP (SacII/SalI) and sequenced. AtCPR-GFP was finally subcloned into the Smal and SacI sites of a binary vector pBI121 (Clontech) to obtain AtCPR-GFP overexpression constructs under the control of the 35S promoter of Cauliflower mosaic virus using pBluescript vector as an intermediate vector. The construct was transformed into Agrobacterium tumefaciens strain GV 3101 and then transformed into Arabidopsis Columbia wild-type using a vacuum-infiltration method. The transgenic plants were selected on MS media containing 50 mg l−1 kanamycin and confirmed further.
by PCR. The transgenic lines were observed under a confocal microscope (Olympus FV500) for green fluorescence (Xie et al., 2003). Two independent Arabidopsis lines expressing the GFP gene under the control of the 35S promoter were used as controls (Dellaire et al., 2003).

Analysis of AtCPR transcriptional activation activity

The yeast strains YRG-2 (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-358 lys2::UGA/SALG-TATG/GAL3 HIS3 URA3::UGA/SALG/TATG/GAL3-TATA/TCTC-lacZ) containing the HIS3 and lacZ reporter genes was used as an assay system (Stratagene) following previous description (Wang et al., 2003). The complete AtCPR open reading frame was obtained by PCR with the specific primer pairs: 5'-ATTGTCGAGCTTGGAGGATGAGAG-3' and 5'-TATGTCGACTCTTCTGAGCATTACT-3'. The PCR product was in-frame fused to the GAL4 DNA binding domain vector to generate the plasmid pBD-AtCPR. Plasmid pBD-AtCPR was digested by PstI and ligated to generate a C-terminal amino acid sequence-deletion plasmid pBD-AtCPR-N. The GAL4 is a transcription factor involved in the expression of galactose-induced genes. According to the manufacturer's protocol (Stratagene), the plasmids of pBD (control), pGAL4 (control), pBD-AtCPR, and pBD-AtCPR-N were introduced into the yeast YRG-2, respectively. The transformed yeast cells were then streaked on the plates YAPD or SD-His medium (without His) to check the transcriptional activation activity according to their growth status.

Construction of AtCPR plant overexpression vector

DNA fragment corresponding to the complete AtCPR open reading frame was amplified by PCR from cDNAs synthesized using RNA isolated from Columbia wild-type plants. The AtCPR-specific primer pairs containing Smal and SacI sites at the termini for construction of the sense expression vector are: 5'-ATTGTCGAGCTTGGAGGATGAGAG-3' and 5'-TATGTCGACTCTTCTGAGCATTACT-3'. The PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced. Full-length cDNAs were subcloned into the Smal and SacI sites of the binary vector pBI121 (Clontech) to obtain the construct harbouring the AtCPR gene in the sense orientation under the control of the 35S promoter of Cauliflower mosaic virus. The construct was introduced into Agrobacterium tumefaciens strain GV 3101, which was used to transform flower mosaic virus Easy vector (Promega) and sequenced. Full-length cDNAs were isolated from Columbia wild-type plants. The construct was introduced into the binary vector pBI121, which was used to transform flower mosaic virus Easy vector (Promega) and sequenced. Full-length cDNAs were isolated from Columbia wild-type plants. The construct was introduced into the binary vector pBI121, which was used to transform flower mosaic virus Easy vector (Promega) and sequenced.

Culture and synchronization of Arabidopsis cell suspension and RT-PCR analysis of marker genes

Seeds of Arabidopsis thaliana ecotype Columbia were surface-sterilized, plated, and vernalized as described above. Callus formation was induced in continuous darkness at 23 °C by placing root explants excised from 15-d-old seedlings onto solid callus induction medium (MS supplemented with 3% w/v sucrose, 0.5 mg l⁻¹ 2,4-D, 0.05 mg l⁻¹ kinetin, 0.8% w/v agar, with pH adjusted to 5.8 using 1 N KOH). Subculture was carried out every month. To establish suspension cultures, rapidly dividing, friable, white callus was inoculated into 1 × MS medium containing 3% w/v sucrose, 0.5 mg l⁻¹ 2,4-D, 0.05 mg l⁻¹ kinetin, and incubated on a rotary shaker at 110 rpm. Cell suspension cultures were subcultured every 7 d and small, fast-growing cell aggregates and single cells were selected with rigorous attention.

To achieve synchronized, cell suspension (7 d after previous subculture) was washed by vacuum-assisted filtration, resuspended with callus induction medium lacking sucrose (MS, 0.5 mg l⁻¹ 2,4-D, 0.05 mg l⁻¹ kinetin) and incubated to conduct sucrose starvation. After the cell suspension was starved for 18 h, cycloheximide (dissolved in 100% ethanol) was added to the medium to a final concentration of 50 μM. Another 6 h later, the cell suspension was washed with callus induction medium twice by vacuum-assisted filtration and resuspended in 13 flasks. Individual flasks were processed as a single sample. Samples were collected every 2 h.

Total RNA (5 μg) from each sample was digested with DNase I (Promega) to get rid of any DNA contamination and then used to synthesize the first-strand cDNA using the cDNA synthesis kit according to the manufacturer’s instruction (Promega). One μl of the cDNA mix was used as a template in a 20 μl PCR reaction volume to examine expressions of AtCPR and other marker genes. The PCR condition was 94 °C, 3 min and 30 cycles of 94 °C, 30 s; 56 °C, 1 min; 72 °C, 1 min, with a final extension of 10 min at 72 °C. PCR Products were separated on 1% agarose gel containing ethidium bromide and photographed. The accession number and amplified fragment size of each gene, and the specific primer pairs used were: CycD3;1, X83371, 949 bp, 5'-AGATCCTGTTACTCATCTTCT-3' and 5'-TTATGAGTGGCTACGATTG-3'; Histone H4, AY142651, 312 bp, 5'-ATGTCAGGAAAGGAAAGG-3' and 5'-TCAACCACCAAATCTATA-3'; CDKB1;1, NM_115278, 930 bp, 5'-ATGGAAGTACGAGAAGCT-3' and 5'-TCGAAGTACGACTTGTCAAA-3'; ACTIN7, X57839, 885 bp, 5'-ATGGTCAGATCAGGAAGAAG-3' and 5'-CTACTGACATGTTCACAG-3'; AtCPR, AT056616, 1080 bp, 5'-TTACAGGTGTTTATAGCTGA-3' and 5'-TACATTCTTGGACATTCTACT-3'; 5'-CATGCTACCTTGTTAGATGA-3' and 5'-CTGACTCATCGTACCT-3'.

cDNA microarray analysis

Total RNA was extracted from the seedlings of Arabidopsis Columbia wild-type plants and AtCPR transgenic line (S2) using Qiagen RNeasy Plant Mini Prep Kit. A microarray containing 9216 Arabidopsis ESTs, which was estimated to represent ~6120 unique genes, was used to analyse the expression profiles of genes. To minimize the inherent variability of the microarray assay and to ensure the reliability of results, at least two microarray slides (four replicates) were used to analyse the mRNA abundance of each sample pair. Each glass slide contained two copies of the entire array, each of which consisted of 16 subarrays with 24 rows and columns. And two independent RNA preparations were made for each biological sample and were used to prepare labelled probes. Microarray preparation, fluorescent labelling of probe, hybridization, washing, and scanning were performed by Xing-Wang Deng’s laboratory (Department of Molecular, Cellular, and Developmental Biology, Yale University). The hybridization signals from each of the replicate ESTs were averaged and used for analysis (Ma et al., 2001). Four replicated data sets of each experiment were pooled. A number of quality control procedures were performed before data points from the four replicates of two independent biological sample sets were averaged. Only those spots, whose signals were higher than the background for both channels and were 2-fold higher than the background at least for one channel, were considered for further data analysis as described by Ma et al. (2001). An average of 2-fold change of gene expression from the four replications was used as a threshold to select those up-regulated or down-regulated genes.

DNA sequencing and data analysis

DNA sequences were determined using the Taq Dye Primer Cycle Sequencing Kit (Amersham) and ABI 373A automatic sequencer. The nucleotide and amino acid sequences were compared with those released in GenBank databases by using the GAPPED BLAST analysis program. The alignment report was produced by software DNASTAR.
Results

Isolation and structural analysis of AtCPR cDNA

To identify putative regulatory molecules involved in the salt-stress response in higher plants, a 204 bp cDNA fragment encoding the conserved DNA binding domain of AtDREB2A (Liu et al., 1998) was used as a probe to screen a cDNA library constructed from the salt-treated halophyte *Atriplex hortensis*. Besides a cDNA encoding a DRE-binding protein (Shen et al., 2003), another cDNA of 1.5 kb was also isolated under the same low stringency condition. Database Blast analysis identified several homologous cDNA sequences showing 42.0% to 78.3% amino acid identity to this 1.5 kb sequence from species as diverse as yeast, human, *C. elegans*, fly, mouse, and plants (Fig. 1). The homologous gene from yeast *S. pombe* encoded a protein that preferentially bound to a synthetic curved DNA sequence (Yamada et al., 1994). The homologous

![Fig. 1. Comparison of the deduced amino acid sequence of AtCPR (AY056616) with homologous sequences from *Mus musculus* p38-2G4 (X84789), from *Homo sapiens* PA204 (U59435), from *Schizosaccharomyces pombe* (NP_593397), from *Caenorhabditis elegans* (AAF39984), from *Drosophila melanogaster* (AAF50751), from *Oryza sativa* probable DNA-binding protein GBP16 (T02069), and from *Atriplex hortensis* AhCPR (AAF91445). Amino acids identical in at least five proteins are highlighted in black. Gaps are introduced to maximize the alignment. The underlined amino acids indicate the putative nuclear localization signal.](https://academic.oup.com/jxb/article-abstract/56/413/807/550019)
gene from mouse encoded a cell cycle-specifically modified and proliferation-associated nuclear protein p38-2G4 (Radomski and Jost, 1995). The gene from human defined a protein highly homologous to the mouse cell cycle protein p38-2G4, with 99.4% identity at the amino acid level (Lamartine et al., 1997). Therefore, the 1.5 kb gene from Atriplex hortensis was designated as Cell-Proliferation-Related gene (AhCPR). In order to understand the function of this kind of gene in plants, homologous genes from the model plant Arabidopsis were isolated using the RT-PCR method, designated as AtCPR and studied further.

The AtCPR was 1472 bp in length with an open reading frame (ORF) of 1179 bp flanked by 96 bp 5'-untranslated region (UTR) and 197 bp 3'-UTR. The ORF encoded a putative protein of 395 amino acids with a calculated molecular mass of 43 kDa. Computational analysis predicted a putative nuclear localization signal between amino acid 357 and 367 (Fig. 1). Alignment of the cDNA and genomic DNA sequences revealed that the AtCPR gene consisted of 9 exons and 8 introns. Database searches and Southern analysis (data not shown) revealed that AtCPR was a single-copy gene and located on chromosome III.

The amino acid sequence of the AtCPR was further analysed using the Prosite programs. Fourteen putative phosphorylation sites (S/TxxD/E) for the casein kinase II, four phosphorylation sites (S/TxK/R) for the protein kinase C, one cAMP and cGMP-dependent protein kinase phosphorylation site (amino acids 160–163, KKNT) and one tyrosine kinase phosphorylation site (amino acids 246–253, KKDESVNY) were identified. Two N-glycosylation sites (amino acids 86–89, NNTV; amino acids 325–328, NGSD), three amidation site (amino acids 135–138, SGRK; 158–161, PGKK; 362–365, GGKK) and one N-myristoylation site (amino acids 289–294, GLVEC V) were also identified. In addition to these sites with a high probability of occurrence, the Prosite analysis also revealed other domains in the AtCPR. A fibronectin type III domain (FN3) (amino acids 146–243), with a low confidence level, was recognized. Fibronectin is a dimeric glycoprotein involved in cell adhesion, cell morphology, thrombosis, cell migration, and embryonic differentiation (Hynes, 1990). Several portions of the AtCPR also showed similarity to the peptidase M24 (Rawlings and Barrett, 1995). Moreover, the amino acid sequence from 235 to 301 also showed homology to the winged helix DNA-binding domain (Gajiwala and Burley, 2000). Whether these various domains have any functions remains to be investigated.

**AtCPR gene expression**

Organ-specific expression pattern of AtCPR was examined using RNA isolated from the flowered wild-type plants (Fig. 2). The results showed that the AtCPR mRNA accumulated in an organ-specific manner with the strongest expression in roots and flowers, comparatively lower expression in stems and siliques, and hardly detectable expression in mature leaves. AtCPR was also strongly expressed in callus.

Many abiotic stress-responsive genes have been studied in this laboratory (Zhang et al., 1996, 1999; Xie et al., 2002; Wang et al., 2003; Shen et al., 2003; He et al., 2004). It was interesting to know whether the AtCPR gene was responsive to different abiotic stresses. The 10-d-old Arabidopsis seedlings were subjected to cold, NaCl, PEG 6000 treatments and expression of the AtCPR gene was examined. It was found that the transcription level of the AtCPR gene did not change significantly (data not shown), indicating that the expression of AtCPR was not influenced by these treatments.

**Subcellular localization of the AtCPR protein**

The deduced amino acid sequence of AtCPR protein contained the KKKKGKKKK motif, characterized by a core peptide enriched in lysine, which was the potential nuclear localization signal (NLS). In order to know the subcellular distribution of AtCPR in plant cells, a construct harbouring a fusion gene for the AtCPR and green fluorescence protein (GFP) fusion protein was introduced into Arabidopsis by a vacuum infiltration method. The transgenic plants were selected on MS media containing kanamycin, and GFP-specific fluorescence was visualized under a confocal microscope. The result showed that the AtCPR-GFP fusion protein was detected in the guard cells, especially in the area of nucleus of the guard cells, whereas the GFP control protein was mainly localized in the cytoplasm as well as in the nucleus of the guard cells (Fig. 3). This result indicated that the AtCPR was mainly a nuclear protein. In other cell types, the AtCPR-GFP fluorescence was not consistently or clearly observed, probably due to the instability of the AtCPR-GFP proteins in these cells (data not shown). Alternatively, the observed transgenic plants may be not at the stage when the fusion protein was highly expressed.
Transcriptional activation activity of AtCPR

Because AtCPR was mainly localized in the nucleus, and with the identification of a putative DNA binding domain, the AtCPR may function as a transcription factor. It was further examined to see if AtCPR possessed any transcriptional activation ability. A yeast system was used to test whether an activation domain was present in AtCPR. The fusion plasmids pBD-AtCPR and pBD-AtCPR-N were transformed into the yeast strain YRG-2 containing a reporter gene \( \text{HIS3} \) plus upstream activating sequences (UAS). The UAS can regulate the expression of the reporter gene \( \text{HIS3} \) (Fig. 4A). If the reporter gene \( \text{HIS3} \) is activated, the yeast harbouring the plasmid can grow on the medium without histidine (SD-His), indicating that there is a transcriptional activation domain in the AtCPR. As seen in Fig. 4B, all the transformants containing the plasmid pGAL4 (the positive control), pBD-AtCPR, and pBD-AtCPR-N grew well on the YAPD medium. Whereas on SD-His medium, only the yeast cells harbouring the positive control plasmid were able to survive. The yeast cells containing the other three plasmids did not grow in the same medium (Fig. 4B). These results indicated that the pBD-AtCPR and pBD-AtCPR-N fusion protein did not bind to UAS of the \( \text{HIS3} \) reporter gene and could not activate its expression. Therefore, the AtCPR protein did not have the transcriptional activation ability.

Cell cycle phase-dependent AtCPR expression

The identified homologous gene of mouse encoded a cell cycle-specifically modified and proliferation-associated nuclear protein p38-2G4. In order to investigate the possibility that AtCPR expression is regulated during the cell cycle, synchronized \textit{Arabidopsis} cell suspensions were used for analysis because synchronized cells were powerful and effective tools for identifying the expression timing and activity of candidate cell-cycle-related genes, and thereby for inferring potential roles in cell-cycle progression (Menges and Murray, 2002). \textit{Arabidopsis} suspension cultures were established using callus generated from the root explants of 15-d-old seedlings. Sucrose starvation combined with cycloheximide treatment, which caused cell cycle arrest at \( \text{G}_1 \), were used to achieve synchronization. RT-PCR was performed to analyse the expression of four
cell cycle marker genes and the AtCPR gene. The result is shown in Fig. 5.

The CDKA;1 gene (originally cdc2a;At, Hirayama et al., 1991), which encodes an archetypal CDK containing the PSTAIRE sequence and is the CDK partner of D-type cyclins (Healy et al., 2001), is constantly expressed in synchronized culture cells. By contrast, expression of CDKB1;1 with the variant PPTALRE motif, increased gradually and reached a peak at 14 h after treatment and then declined after 16 h. The constitutive expressed gene Actin7 from Arabidopsis was used as a control that was not regulated by the cell cycle. The histone H4 gene, whose mRNA accumulation was induced in parallel to DNA synthesis at the G1/S transition (Reichheld et al., 1995), was strongly expressed before 12 h and declined after that. The mRNA level of the D-type cyclin CYCD3;1 was low after the treatments, and then increased within 8 h. The transcript of AtCPR increased steadily between 2 h and 6 h, reached a peak at 8 h, and then decreased. This result showed that expression of AtCPR was regulated during the cell cycle and mainly expressed in the G1-to-S phase.

**Microarray analysis of AtCPR-regulated genes**

To characterize the function of AtCPR in planta, the AtCPR gene in the sense orientation was inserted into the binary vector pBI 121 under the control of the CaMV 35S promoter and transferred into Arabidopsis by vacuum infiltration. Sixteen sense transgenic lines were obtained after selection on kanamycin media and homozygous plants were used for phenotype examination. However, when grown either on vertical agar plates or in soil, none of the independent transgenic lines showed a meiotically stable abnormal phenotype compared with wild-type plants grown under identical conditions (data not shown).

To investigate whether AtCPR can regulate the expression of other genes, microarray analysis was performed using RNA isolated from the sense transgenic line S2 and wild-type plants. At least two independent RNA preparations for each sample were made and used for probe synthesis. Generation of the 9.2 K array, fluorescent labelling of probes, hybridization, and scanning were performed by Deng’s laboratory (Department of Molecular, Cellular, and Developmental Biology, Yale University). The hybridization signals from each of the replicates were averaged and used for analysis. By using 2-fold change (S2/WT) of gene expression as a threshold, a total of 17 and 30 genes were identified as up-regulated and down-regulated, respectively. These genes were classified into different functional groups (Table 1). Among the up-regulated genes, two cell cycle-related genes were found. T43227 encoded a putative protein showing similarity to a cell proliferation related protein from Mus musculus. The cDNA under the accession number N95936 encoded a cell cycle-related protein EXO showing sequence similarity with the tobacco PHI-1 gene (Farrar et al., 2003). These two genes were further examined by RNA gel-blot analysis to evaluate the validity of the expression profile obtained from cDNA microarray analysis. As shown in Fig. 6, when expression of AtCPR was high in the overexpressing transgenic lines S2 and S16, expression of both T43227 and EXO were high. However, in a line (S3) that did not show higher expression of AtCPR, both T43227 and EXO genes were expressed at a low level. This result showed that the expression data obtained by RNA gel-blot analysis were consistent with those obtained by microarray analysis, and also indicated that T43227 and EXO genes may be the downstream genes of AtCPR.

**Discussion**

Cell division is a fundamental biological process sharing conserved features and controls in all eukaryotes. Regulated gene expression is an important mechanism for controlling cell cycle progression and genes involved in cell-division-related processes often show transcriptional regulation dependent on cell cycle position (Menges et al., 2002). In this study, a previously undescribed cell cycle-related gene, AtCPR, was cloned and characterized. Blast analysis showed that its deduced amino acid sequence displayed 53.2% identity to a M. musculus protein p38-2G4 (Fig. 1). p38-2G4 is a cell cycle-specifically modulated and proliferation-associated nuclear protein of 38 kDa containing a number of putative phosphorylation sites, a nuclear localization signal composed of six lysine residues, and an amphipathic helical domain (Radomski and Jost, 1995).
Table 1. Number of clones involved in different functional groups up-regulated or down-regulated at least 2-fold in AtCPR transgenic line S2

<table>
<thead>
<tr>
<th>Functional category</th>
<th>Gene number</th>
<th>Representative gene names</th>
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<tbody>
<tr>
<td><strong>Up-regulated</strong></td>
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<td></td>
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<tr>
<td>Protein kinases</td>
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<td>One GTP pyrophosphokinase, one receptor-like protein kinase, one putative protein kinase, one protein inositol 1,3,4-trisphosphate 5/6-kinase</td>
</tr>
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<td>Photosynthesis</td>
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<td>One photosystem II type I chlorophyll a/b binding protein</td>
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<td>Amino acid</td>
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<td>One glutamine-dependent asparagine synthetase</td>
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<td>Vacular-type H+-translocating inorganic pyrophosphatase</td>
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<td>Serine/threonine protein kinase</td>
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<td>Two zinc finger transcription factor</td>
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<td>Stress-associated</td>
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<td>One superoxidase dismutase, one water stress-induced protein, two peroxidase, one pinoresinol-lariciresinol reductase, one endochitinase</td>
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<tr>
<td>Ionic homeostasis</td>
<td>9</td>
<td>Nine metallothionein-like protein</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>1</td>
<td>Lipase</td>
</tr>
<tr>
<td>metabolism</td>
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<tr>
<td>Lipid-transfer</td>
<td>1</td>
<td>Lipid-transfer protein</td>
</tr>
<tr>
<td>protein</td>
<td></td>
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</tr>
<tr>
<td>Osmotic precursor</td>
<td>2</td>
<td>Two osmotin precursor</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>2</td>
<td>60S ribosomal protein I21, pr-4</td>
</tr>
<tr>
<td>WD repeat protein</td>
<td>1</td>
<td>WD-40 repeat protein</td>
</tr>
<tr>
<td>No hits</td>
<td>5</td>
<td>Five unknown protein</td>
</tr>
</tbody>
</table>

Computational analysis of the present AtCPR sequence also predicted a number of putative phosphorylation sites and a putative nuclear localization signal enriched in lysine between amino acids 357 and 367. The subcellular location analysis revealed that the AtCPR-GFP fusion protein was present in the guard cells, especially in the nuclear region (Fig. 3), suggesting that AtCPR might be a nuclear protein. Yeast one-hybrid assay revealed that AtCPR did not possess transcriptional activation activity (Fig. 4). However, a potential winged-helix DNA-binding domain was found in AtCPR. Therefore AtCPR might function as a transcription suppressor or other regulatory protein. Alternatively, AtCPR might function as a peptidase or a fibronectin-like protein since homologies to these proteins have been observed in AtCPR.

Dispersed plant cell suspension cultures allow the study of cell division in the absence of developmental processes, by providing a homogeneous population of near-identical cells (Gould, 1984). Synchronization of such cultures can provide material representative of specific cell-cycle phases. Usually, this may be achieved either by removal and subsequent resupply of a nutrient source, or by applying reversible blocks at different stages of the cell cycle using specific inhibitors (Menges and Murray, 2002). In Arabidopsis, Fuerst et al. (1996) blocked exponentially growing cells in the G1 phase with a low concentration of cycloheximide. A combined phosphate starvation followed by an aphidicolin block and release produced 50–60% of cells in the S and G2 phases (Callard and Mazzolini, 1997). Riou-Khamlichi et al. (1999) achieved partial synchrony from G1 into S phase using sucrose removal and resupply. Menges and Murray (2002) reported the best synchrony obtained to date with a maximum of 80% of cells in the S phase and 92% in the G2 phase a few hours later, after applying the toxin aphidicolin to cell suspension MM2d. Here, the removal and resupply of sucrose combined with cycloheximide treatment was used to obtain partially synchronous Arabidopsis suspension cultures. Molecular cell-cycle marker genes expressing at different phases were used to give information about the cell-cycle stages. CDKA;1 is constantly expressed and shows activity at both G1-to-S and G2-to-M transition points (Mironov et al., 1999). By contrast, CDKBI;1 expression is activated at the start of the S phase and its mRNA reaches a peak during late G2/early mitosis. The mRNA level corresponding to the D-type cyclin CYCD3;1 was reported to increase during the G1 phase (Riou-Khamlichi et al., 1999, 2000). In the present study, expression of CDKA;1 was relatively stable, expression of CDKBI;1 increased gradually and reached a peak at 14 h after treatment and then declined after 16 h, and CYCD3;1 mRNA level increased after 6 h. The transcript of AtCPR increased steadily between 2 h and 6 h, reached a peak at 8 h, and then decreased (Fig. 5). Although further estimation of the particular steps of the cell cycle using techniques such as flow cytometric analysis, [3H] thymidine labelling, and cytology is needed, from the expression data of the molecular markers used it is suggested that transcript of AtCPR accumulated at the G1-to-S phase. The present AtCPR gene has not been identified in the set of cell cycle-modulated genes reported by

![RNA gel-blot analysis](https://academic.oup.com/jxb/article-abstract/56/413/807/550019/807.png)
Menges et al. (2002). The reason for this may be due to the different synchronization procedure used. Alternatively, the relatively low abundance of the AtCPR mRNA may block the identification of this gene in the previous study (Menges et al., 2002). It is also possible that other unknown reasons may be involved in the prevention of the identification of this gene.

cDNA microarray has become a useful tool for direct and large-scale comparative analysis of gene expression in a wide range of organisms. In plants, this chip-based technology has been adopted to investigate genome expression profiles in different plant biological processes including defence responses, mechanical wounding responses, far-red light regulation of gene expression, and drought and cold stress responses (Petersen et al., 2000; Reymond et al., 2000; Ma et al., 2001; Seki et al., 2001; Tepperman et al., 2001). Since efforts to overexpress AtCPR gene in Arabidopsis wild-type plants did not reveal any phenotypic change in planta, cDNA microarray was further performed to examine the putative downstream genes regulated by AtCPR. Among the up-regulated genes, there was an identified cell cycle-related gene EXO. Previous studies showed that EXO mRNA is preferentially abundant during the M phase of the cell cycle by using semi-synchronized cells; plants homozygous for the T-DNA insertion mutation of the EXO gene and a series of independent sense and antisense transgenic lines did not show abnormal phenotype and double mutant studies revealed that the exo mutation can suppress the defective root meristem phenotype of the hydra2 mutant, suggesting that EXO may be a component of a negative regulatory system for cell division (Farrar et al., 2003). Because EXO was up-regulated by AtCPR, it is possible that AtCPR also functions as a negative regulator of cell division. Further studies using T-DNA insertion lines of AtCPR and double mutant analysis with the EXO mutant may disclose more roles of AtCPR in plant cell division.

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