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Research Article

Molecular characterization of *Arabidopsis* PHO80-like proteins, a novel class of CDKA;1-interacting cyclins

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Abstract. Cyclins are regulatory proteins that interact with cyclin-dependent kinases (CDKs) to control progression through the cell cycle. In *Arabidopsis thaliana,* 34 cyclin genes have been described, grouped into five different types (A, B, D, H, and T). A novel class of seven cyclins was isolated and characterized in *Arabidopsis*, designated P-type cyclins (CYCPs). They all share a conserved central region of 100 amino acids ("cyclin box") displaying homology to the corresponding region of the PHO80 cyclin from *Saccharomyces cerevisiae* and the related G1 cyclins from *Trypanosoma cruzi* and *T. brucei*. The *CYCP4;2* gene was able to partially re-establish the phosphate-dependent expression of the *PHO5* gene in a pho80 mutant strain of yeast. The CYCPs interact preferentially with CDKA;1 in vivo and in vitro as shown by yeast two-hybrid analysis and co-immunoprecipitation experiments. P-type cyclins were mostly expressed in proliferating cells, albeit also in differentiating and mature tissues. The possible role of CYCPs in linking cell division, cell differentiation, and the nutritional status of the cell is discussed.

Key words. *Arabidopsis thaliana*; cyclin-dependent kinase; CDKA;1; cyclin; PHO80-like protein; yeast two-hybrid.

Over the past decades many of the main molecular components that regulate cell division in eukaryotic cells have been identified. A central role is played by the cyclin-dependent kinases (CDKs), which regulate cell cycle progression through the phosphorylation of key substrates, such as the retinoblastoma protein, E2F/DP transcription factors, nuclear laminar proteins, and histones [1]. CDK activity is controlled by multiple mechanisms, including transcriptional regulation, phosphorylation, protein degradation, and the interaction with regulatory proteins. These regulatory mechanisms allow the CDKs to integrate a multitude of signaling pathways with cell cycle progression.

Cyclins are the largest class of regulatory proteins that interact with CDKs. Their binding induces a conformational change in the CDK subunit, triggering CDK activity [2]. Originally, cyclins were identified as proteins with cyclical accumulation and destruction patterns during the cell cycle [1], thereby determining the timing of CDK activity. In addition, by activating the CDK subunits, the cyclins largely establish the substrate specificity of the

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CDK/cyclin complex. Although cyclins have no strict molecular size, many of them are characterized by the presence of a highly conserved sequence domain of approximately 100 amino acids (the so-called "cyclin box") that usually contains distinct motifs necessary for the interaction with CDKs [3].

In the complete *Arabidopsis thaliana* genome, 12 CDKs, subdivided into six distinct classes (CDKA, CDKB, CDKC, CDKD, CDKE, and CDKF), and 34 cyclins (subdivided into the five major types A, B, D, H, and T) have been identified [4, 5]. A- and B-type cyclins are mitosis-specific cyclins, whereas the D-type cyclins are also referred to as G1-specific cyclins. H-type cyclins are the regulatory subunits of CDK-activating kinases [6], and the T-type cyclins are putatively involved in the control of gene transcription [4]. In addition to this classification, a highly divergent *Arabidopsis* cyclin, CYCj18, has been identified through complementation of G1-specific cyclin-deficient budding yeast [7]. The role of this cyclin remains to be elucidated.

Besides their involvement in the regulation of the cell cycle, CDK/cyclin complexes in yeast have been associated with other cellular processes [8–10], such as the regulation of gene expression in response to phosphate starvation (fig. 1). The phosphate auxotrophy gene *PHO5* of budding yeast encodes a repressible acid phosphatase (rAPase) that is transcriptionally regulated by phosphate availability in the medium via a network of intracellularly regulatory factors, comprising at least the genes *PHO2*, *PHO4*, *PHO81*, *PHO80*, and *PHO85* [11]. *PHO2* and *PHO4* encode the activators necessary for transcription of *PHO5*. When extracellular levels of phosphate are high, the PHO4 protein becomes hyperphosphorylated. This phosphorylation facilitates the nuclear exclusion of PHO4 and prevents its further association with PHO2, which is located in the nucleus. The phosphorylation of PHO4 is mediated by the PHO80/PHO85 cyclin/CDK complex that acts as a negative regulatory mechanism for *PHO5* expression [12]. In contrast, when extracellular levels of phosphate are low, the PHO81 CDK inhibitor inactivates the PHO80/PHO85 complex, and *PHO5* expression is activated [13].

Homologs of the yeast PHO80 cyclin have been characterized in other organisms, such as CYC2 and CYC6 from *Trypanosoma cruzi* [14] and *T. brucei* [15], respectively, which are involved in cell cycle and cell differentiation. Here, we report the isolation and characterization of seven PHO80-like proteins (P-type cyclins or CYCPs) from *Arabidopsis* that constitute a new class of cyclins in plants. All CYCPs share significant similarity to the PHO80 protein of yeast and the CYC2 and CYC6 cyclins of *Trypanosoma* and associate with the *Arabidopsis* cyclin-dependent kinase CDKA;1 protein. CYCP4;2 was able to reconstitute the phosphate signaling pathway of the yeast pho80 mutant. Furthermore, we demonstrate that *CDKA;1* and *CYCP* genes are expressed in a similar spatial manner. The existence of PHO80-like cyclins in plants suggests that CDKA;1 may play an even more versatile role than previously assumed.

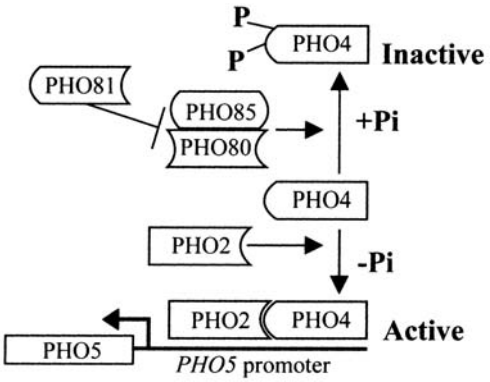

Figure 1. The 'PHO-regulatory system' of yeast. When phosphate levels are high (+Pi), *PHO5* (which encodes a secretory alkaline phosphatase) is not transcribed because the factor PHO4 is phosphorylated (inactive) by the PHO85/PHO80 complex, resulting in the translocation of PHO4 from the nucleus to the cytoplasm. Upon phosphate starvation (-Pi), the PHO85/PHO80 complex is inhibited by PHO81 and unphosphorylated PHO4 (active) remains in the nucleus. Interaction between PHO4 and PHO2 turns on *PHO5* transcription.

Materials and methods

Isolation of *CYCP* genes and yeast two-hybrid interactions

A partial sequence of a *PHO80-like protein* (*CYCP1;1*) gene (annotated with the accession clone CAB87757/At3g63120) that only lacked the start codon, had been identified in a previously described two-hybrid screen using the CDKA;1 protein of *A. thaliana* (L.) Heynh. as bait [16]. The full-length *CYCP1;1* gene was obtained by polymerase chain reaction (PCR) with a forward primer that included the missing start codon, 5′-ATGGACTCTCTCGCAACC-3′ and the reverse primer, 5′-TTGCCGATCAGCGTGC-3′. By screening of the *Arabidopsis* databases (both genomic and predicted sequence data sets), six additional *CYCP* genes were found:

accession numbers NM_113083/At3g21870 (*CYCP2;1*), AAD32828/At2g45080 (*CYCP3;1*), CAB81841/At3g60550 (*CYCP3;2*), AAC27476/At2g44740 (*CYCP4;1*), BAB09009/At5g61650 (*CYCP4;2*), and CAB87934/At5g07450 (*CYCP4;3*). Expressed sequence tags provided by the databases confirmed correct sequence annotation for all *CYCP* genes. The corresponding cDNAs were isolated by reverse transcription (RT)-PCR with RNA prepared from *Arabidopsis* cell suspensions (ecotype Columbia 0) and the Superscript RT II kit (Invitro-

gen, Carlsbad, Calif.) according to the manufacturer's protocol. Gene-specific sets of primers were designed for each P-type cyclin gene with an overhanging extension to incorporate the *Eco*RI and *Bam*HI restriction sites flanking the 5′ and 3′ ends of each cDNA. The primer set sequences used were:

5′-GGGAATTCATGGACTCTCTCGCAACC-3′ and 5′-GGGGATCCTTGCCGATCAGCGTGC-3′ (*CYCP1;1*); 5′-GGGAATTCATGTTAACCGCAGCCGGAGACG-3′ and 5′-GGGGATCCATCAAACATATAAAGATG-3′ (*CYCP2;1*); 5′-CCGAATTCATGGATTCCCTAGCGATTTCTCC-3′ and 5′-GGGGATCCCTACAACATGATTCGAGAAAATTGATGG-3′ (*CYCP3;1*); 5′-CCGAATTCATGGCCGTTTCGAATTCTTTAACAATC-3′ and 5′-GGGGATCCCTACAACAAGATTCGAGC-3′ (*CYCP3;2*); 5′-GGGAATTCATGGCGGAACTTGAGAATCC-3′ and 5′-GGGGATCCAAGACAAGATAAGAGTCCCTGCCG-3′ (*CYCP4;1*); 5′-GGGAATTCATGGCTGATCAGATTGAGATCC-3′ and 5′-GGGGATCC, GCATAAATATAATCAAGCAGCAGCG-3′ (*CYCP4;2*); and 5′-CCGAATTCATGGCTTATCAGATTGATCAG-3′ and 5′-GGGGATCCCTCAAACAGCACTGGTGAC-3′ (*CYCP4;3*).

The resulting PCR fragment of each gene was purified and digested with the restriction enzymes *Eco*RI and *Bam*HI and cloned into the corresponding restriction sites of pGAD424 (Clontech, Palo Alto, Calif.). The pGADCYCP vectors were co-transformed into yeast HF7c (*MAT*$_a$ *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 URA3::GAL4*$_{17mers(3x)}$-*CyC1*$_{TATA}$-*LacZ*) with pGBTCDKA;1, pGBTCDKB1;1 [17], or pGBTCDKC;1 [4] as described elsewhere [18]. Prey plasmids co-transformed with the empty pGBT9 vector were used as controls. Transformed yeast colonies were grown in medium lacking leucine and tryptophan for 2 days at 30 °C; the resulting colonies were plated on selective medium without leucine, tryptophan, and histidine. The protein-protein interaction was measured by the ability to grow in the absence of histidine.

Protein motifs, alignments, and phylogenetic analysis

Homologs of the *Arabidopsis CYCP* genes were identified from the non-redundant database (nrdb95) using WU-BLAST2 (http://blast.wustl.edu) and aligned using Clustal W [19]. The phylogenetic tree was constructed on the basis of more conserved residues by using the maximum likelihood (quartet puzzling, 25,000 puzzling steps) and neighbor-joining approach as implemented in the TREE-PUZZLE 5.0 [20] and TREECON packages [21], respectively. In the latter approach, evolutionary distances were calculated with Poisson correction and bootstrap analysis with 500 replications was performed to test the significance of nodes. For alignment editing and reformatting, the BioEdit [22] and ForCon [23] programs were used, respectively.

Duplicated blocks (i.e., large regions of colinearity) in the *Arabidopsis* genome were detected and dated as described [24, 25]. Genes were localized on the chromosomes with the MATDB Genome Viewer option on the *Arabidopsis* database of the Munich Institute for Protein Sequences (MATDB; http://mips.gsf.de/proj/thal/db/search/search_frame.html). With the programs SMART [26] (http://smart.embl-heidelberg.de/) and ScanProsite [27] (http://hits.isb-sib.ch/cgi-bin/PFSCAN), already characterized domains were detected, whereas the MEME program as implemented in the Wisconsin package version 10.1 (Accelrys, San Diego, Calif.) was used to identify new sequence motifs [28] and the PEST finder program [29] (http://www.at.embnet.org/embnet/tools/bio/PESTfind/) to predict PEST regions. Accession numbers for the sequences used in this analysis were: for *Arabidopsis thaliana*: *Arath*;CDKA;1 (X57839), *Arath*;CDKB1;1 (X57840), *Arath*;H4 (M17132), *Arath*;CYCP1;1 (AV441245, AV542791, AV555805, H36237, N37922, AV546320, N65480, AV440357, AV807667, AV552044, T75596, AV442812, and BE527697), *Arath*;CYCP2;1 (AA394924, N65100, AA585716, and T22252), *Arath*;CYCP3;1 (AAD32828), *Arath*;CYCP3;2 (CAB81841), *Arath*;CYCP4;1 (AU237869, AV562484, AU228970, and AV559246), *Arath*;CYCP4;2 (T46758), *Arath*;CYCP4;3 (AU239995 and AU231363), *Arath*;PSR3.2 (U72155), and *Arath*;IPS1 (AF236376); for *Beta vulgaris*: *Betvu*;CYCP1 (BQ060499); for *Glycine max*: *Glyma*;CYCP1;1 (BE346942, AW597795, BI471322, BE474970, BG405185, AW705323, and BQ273814), *Glyma*;CYCP1;2 (BE021762, AW781862, BI471322, and BE608984), *Glyma*;CYCP2;1 (AW132892), *Glyma*;CYCP3;1 (AI899864 and BQ629698), *Glyma*;CYCP4;1 (AW570625, BM885143, and AW278071), and *Glyma*;CYCP4;2 (BE057269); for *Gossypium arboreum*: *Gosar*;CYCP3;1 (BF278366 and BQ410074), *Gosar*;CYCP3;2 (BQ410110), *Gosar*;CYCP4;1 (BG440121 and AW666787), and *Gosar*;CYCP4;2 (BM358191); for *Gossypium hirsutum*: *Goshi*;CYCP1;1 (AJ513592 and AJ513344), *Goshi*; CYCP3;1 (AI725997), *Goshi*;CYCP4;1 (AI727370), and *Goshi*;CYCP4;2 (AI728307, AI727322, and AI727377); for *Hordeum vulgare*: *Horvu*;CYCP1;1 (AJ432832, AV918828, BI949176, BQ469876, BI778758, BG343785, AV913529, BF625799, BU986449, and BU986449), *Horvu*;CYCP4;1 (BF255343, BQ470176, BQ470734, BF267566, BJ475495, AV833001, and BJ470684), *Horvu*;CYCP4;2 (AL503619 and BE412786), *Horvu*;CYCP4;3 (BF259570), *Horvu*; CYCP4;4 (AL505791), and *Horvu*;CYCP4;5 (BG368263 and BG299276); for *Lotus japonicus*:

Lotja;CYCP1;1 (AW428917) and *Lotja*;CYCP3;1 (AV420231); for *Lycopersicon esculentum*: *Lyces*; CYCP2;1 (AI899677), *Lyces*;CYCP3;1 (BI933687, AW648358, AW625047, and AW648357), and *Lyces*; CYCP4;1 (BG134870); for *Medicago truncatula*: *Medtr*;CYCP2;1 (BI271124 and BI265192), and *Medtr*;CYCP4;1 (BQ148870 and BG581477); for *Mesembryanthemum crystallinum*: *Mescr*;CYCP1;1 (BE037286); for *Neurospora crassa*: *Neucr*;PREG (Q06712); for *Oryza sativa*: *Orysa*;CYCP1;1 (AU108869), *Orysa*;CYCP2;1 (AL662988), *Orysa*; CYCP3;1 (AL606633), *Orysa*;CYCP4;1 (Q9AY48), *Orysa*;CYCP4;2 (Q949F8), *Orysa*;CYCP4;3 (AU-069937), and *Orysa*;CYCP4;4 (AL606728); for *Physcomitrella patens*: *Phypa*;CYCP1 (AW598846); for *Picea mariana*: *Picma*;CYCP1 (AF051226); for *Pinus taeda*: *Pinta*;CYCP2;1 (BE643808), and *Pinta*;CYCP3;1 (BF608997); for *Populus balsamifera*: *Popba*;CYCP1;1 (BU868815), and *Popba*;CYCP4;1 (BU869365); for *Populus tremula* x *Populus tremuloides*: *Poptr*;CYCP1;1 (BU887877 and BU885056); for *Porphyra yezoensis*: *Porye*;CYCP4;1 (AV4389021 and AV434111); for *Rosa chinensis*: *Rosch*;CYCP4;1 (BI9783981); for *Rosa hybrida*: *Roshy;*CYCP4;1 (BQ1045681); for *Saccharomyces cerevisiae*: *Sacce*;PHO80 (S61983); for *Solanum tuberosum*: *Soltu*;CYCP1;1 (BM111105, BM408029, BQ513209, and BQ509371), *Soltu*;CYCP2;1 (BG097740), *Soltu*; CYCP4;1 (BI436225 and BI436217), and *Soltu*; CYCP4;2 (BF052441 and BF052241); for *Sorghum bicolor*: *Sorbi*;CYCP1;1 (BE597830); for *Stevia rebaudiana*: *Stere*;CYCP1;1 (BG5261205), and *Stere*;CYCP2;1 (BG5248023); for *Triticum aestivum*: *Triae*;CYCP1;1 (BJ2244145, BJ249891, BJ323190, BJ244107, BQ-283518, BQ282003, CA485150, CA484415, CA-484495, and CA639405), *Triae*;CYCP3;1 (BQ244528), *Triae*;CYCP4;1 (CA704764), *Triae*;CYCP4;2 (CA-684838), and *Triae*;CYCP4;3 (CA628651 and CA-638284); for *Triticum monococcum*: *Trimo*;CYCP1;1 (BG608094); for *Trypanosoma brucei*: *Trybu*;CYC2 (CAB82894); for *Trypanosoma cruzi*: *Trycr*;CYC6 (Q9GQL7); for *Vitis vinifera*: *Vitvi*;CYCP1;1 (BQ796545); and for *Zea mays*: *Zeama*;CYCP1;1 (AW066488 and AY104758).

In vitro transcription-translation and immunoprecipitation

c-Myc-tagged versions of *CYCP2;1*, *CYCP3;1*, *CYCP4;1*, and *CYCP4;2* genes were constructed by cloning into the pSK plasmid (Stratagene, La Jolla, Calif.) containing a double *c-Myc* tag [30]. The *CYCP* fragments were obtained by PCR (using the same set of primers described above) and cloned into the *Eco*RI and *Bam*HI sites of c-*Myc*-pSK. Influenza hemagglutinin (Ha)-tagged *Arabidopsis* CDKA;1 was obtained by cloning the *Eco*RI and *Bam*HI restriction CDKA;1 fragment into the corresponding sites of the pSK vector containing the HA tag [30]. *In vitro* transcription and translation experiments were performed using the TNT T7-coupled wheat germ extraction kit (Promega, Madison, Wis.) as described before [4].

Yeast pho80 mutant complementation experiments

The full-length coding regions of the P-type cyclins were cloned in the unique *Eco*RI and *Spe*I restriction sites downstream of the GAL10-inducible promoter from the pESC-TRP vector (Stratagene), in frame with the FLAG epitope (DYKDDDDK) sequences to produce a C-terminal fusion protein. As a positive control, the yeast *PHO80* gene was cloned into the same vector by PCR amplification from yeast genomic DNA with the forward and reverse primers 5′-CCGAATTCATGGAAAGCA-CATCAGGAGAACG-3′ and 5′-GGACTAGTTTAATC-TGGCTTTGATCGC-3′, containing the *Eco*RI and *Spe*I sites, respectively. The pESC-TRP empty vector was used as a negative control for the transformation. Mutant yeast strain EY0134 (*MATα pho80Δ::HIS3 K699*, *ade2-1*, *trp1-1*, *can 1-100*, *leu2-3,112*, *his3-11,15*, *ura3*, and *GAL+*) [31] was co-transformed by the lithium acetate method [25] with the diverse pESC constructs (pESC-CYCPs, pESCPHO80, and pESCTRP) and the pPHO5-GFP (EB0180) plasmid, containing a fusion between the *PHO5* promoter (nucleotides –550 to –1 upstream of the ATG) and the green fluorescent protein (GFP) [31]. After transformation, yeast cells harboring the two plasmids were selected in SD-URA, SD-HIS, and SD-TRP media. Colonies that expressed constitutively the gene coding for the GFP were grown under non-inducing (in the presence of glucose) and inducing (in the presence of galactose) conditions. After the cell density had been calibrated at OD_{600}, a spectrofluorometric *GFP* expression assay was performed by measuring the emission at 509 nm after excitation at 395 nm (Cary Eclipse Fluorescence spectrophotometer; Varian, Victoria, Australia). Co-transformed cells with the empty pESC-TRP and pPHO5-GFP (EBO180) vectors as well as non-transformed cells were used to determine background fluorescence levels. Cells from each culture were collected by centrifugation and stored at –20 °C for further analysis by Western blotting. Total proteins were extracted by boiling the cell pellet for 5 min in 1× phosphate-buffered saline (PBS); after centrifugation, 75 µg of soluble proteins of each sample were used for Western blotting with anti-FLAG antibody (Stratagene) diluted at 1:500.

Total RNA isolation and RT-PCR

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufac-

turer's recommendations using 200 mg starting material of each tissue tested. RNA quality and concentration were determined by gel electrophoresis and spectrophotometry, respectively. For the tissue-specific expression assays, 3-day-old cell suspensions, 3-week-old *Arabidopsis* ecotype Columbia 0 plants (roots, rosette leaves, stems, and flowers), and 1-week-old seedlings were used. The cell culture was routinely maintained in the laboratory in Gamborg B5 medium (Sigma-Aldrich, St. Louis, Mo.) supplemented with 20 g l^{-1} sucrose and 2 mg l^{-1} naphthalene acetic acid, pH 5.8 (with KOH) by dilution at 1:10 for each subculture.

First-strand cDNA synthesis was performed on 3 µg total RNA with the Superscript RT II (Invitrogen) and oligo $d(T)_{18}$, according to the supplier's recommendation. A 1-µl aliquot of the total RT volume was used as template in a semi-quantitative PCR amplification of 17 cycles with the same sets of primers for the *CYCP* genes as described above. Equal cDNA input was confirmed by measuring *Arabidopsis Actin2* (*ACT2;* accession number, U41998) transcript levels with the primers 5′-CTAAGCTCTCAAGATCAAAGGCTTA-3′ and 5′-TTAACATTGCAAAGAGTTTCAAGGT-3′. From the 50-µl PCR reaction, 10 µl was separated on a 1% agarose gel and transferred onto Hybond N^+ membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were hybridized at 65 °C with fluorescein-labeled probes (Gene Images random prime module; Amersham Biosciences). The hybridized bands were detected with the CDP Star detection module (Amersham Biosciences).

In situ hybridization

The mRNA in situ hybridization procedure was carried out as described by de Almeida Engler et al. [32]. Plant material was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Fixed tissue was gradually dehydrated with ethanol and embedded in paraffin. *Arabidopsis* tissue was sliced into serial 10-µm sections and attached to coated microscope slides. Probes were generated from fragments corresponding to the open reading frames of *CYCP1;1*, *CYCP2;1*, *CYCP3;1*, *CYCP3;2*, *CYCP4;1*, *CYCP4;2*, and *CYCP4;3*, cloned into the pGEM-T easy vector (Promega). Southern blot analysis demonstrated the lack of cross-hybridization between the different cyclin members. In addition, tissue sections were washed stringently in 15 mM NaCl, 1.5 mM Na_3-citrate, pH 7.0 containing 50% formamide at 47 °C to prevent potential cross-hybridization between the different CYCPs. [^{35}S]UTP-labeled sense and antisense RNA probes of the seven P-type cyclin genes were generated by run-off transcription with T7 and SP6 RNA polymerases according to the manufacturer's protocol (Roche Diagnostics, Brussels, Belgium). Labeled transcripts were reduced to an average length of 0.3 kb by alkaline hydrolysis [33]. The size of full-length and hydrolyzed transcripts was checked on a 1% formaldehyde-denaturing gel, and the concentration of synthesized RNA was calculated.

Results

Analysis of CYCP-encoding genes from *Arabidopsis*

To identify the interaction partners of the A-type CDKs, a yeast two-hybrid screen was performed with the *Arabidopsis* CDKA;1 protein as a bait [16]. One of the isolated clones encoded a protein containing a central 'cyclin box' domain that was 35% identical with the comparable domain of the PHO80 cyclin of yeast. By searching the *Arabidopsis* sequence databases, six related cDNA sequences were identified and designated *P-type cyclin* (*CYCP*) genes. The *CYCP1;1* gene corresponded to the clone originally isolated in the CDKA;1 two-hybrid screen. Searches in other sequence databases revealed that members of this novel gene family could be found throughout the plant kingdom (from algae to higher plants). P-type cyclin genes of *Arabidopsis*, as well as most of the *CYCP* genes found in other plant species, share a substantial degree of sequence identity (up to 40%) and all have a similar modular organization. Except for Sb34 of *P. mariana* (which is 284 amino acids long), CYCPs are relatively short proteins of approximately 210 amino acid residues (fig. 2A) with a predicted molecular mass ranging from 20 to 27 kDa and a pI between 5 and 8. CYCP proteins are distantly related to the plant D-type cyclins (almost 25% identity), but no significant similarity was found with the A-, B-, and H-types. All CYCPs and related proteins from other eukaryotes contain a conserved 'cyclin box' domain, mostly located in the middle of the proteins (fig. 2A). This domain shares identity scores of 35% to 60% with the corresponding domain of *T. brucei* CYC2 [15], *T. cruzi* CYC6 [14], *S. cerevisiae* PHO80 [34] and closely related PCL cyclins, and *N. crassa* PREG [35]. In the plant CYCP cyclin box, an invariable core region ([N/S]VHRLL[V/I]T) is located in the center of the domain (with the residues 95–102 in the CYCP4;1 sequence from *Arabidopsis* as a reference). No other known domains were present in the CYCP proteins. By sequence alignment, three conserved motif sequences (A, B, and C) flanking the cyclin box were found among 68 plant CYCP proteins (figs. 2A, 3). Motifs A and B are located in the N-terminal end and motif C just behind the cyclin box in the C-terminal end. According to the protein alignment, motif A is plant specific, motif B is common to all CYCPs analyzed, and motif C is specific for plants and *Trypanosoma*.

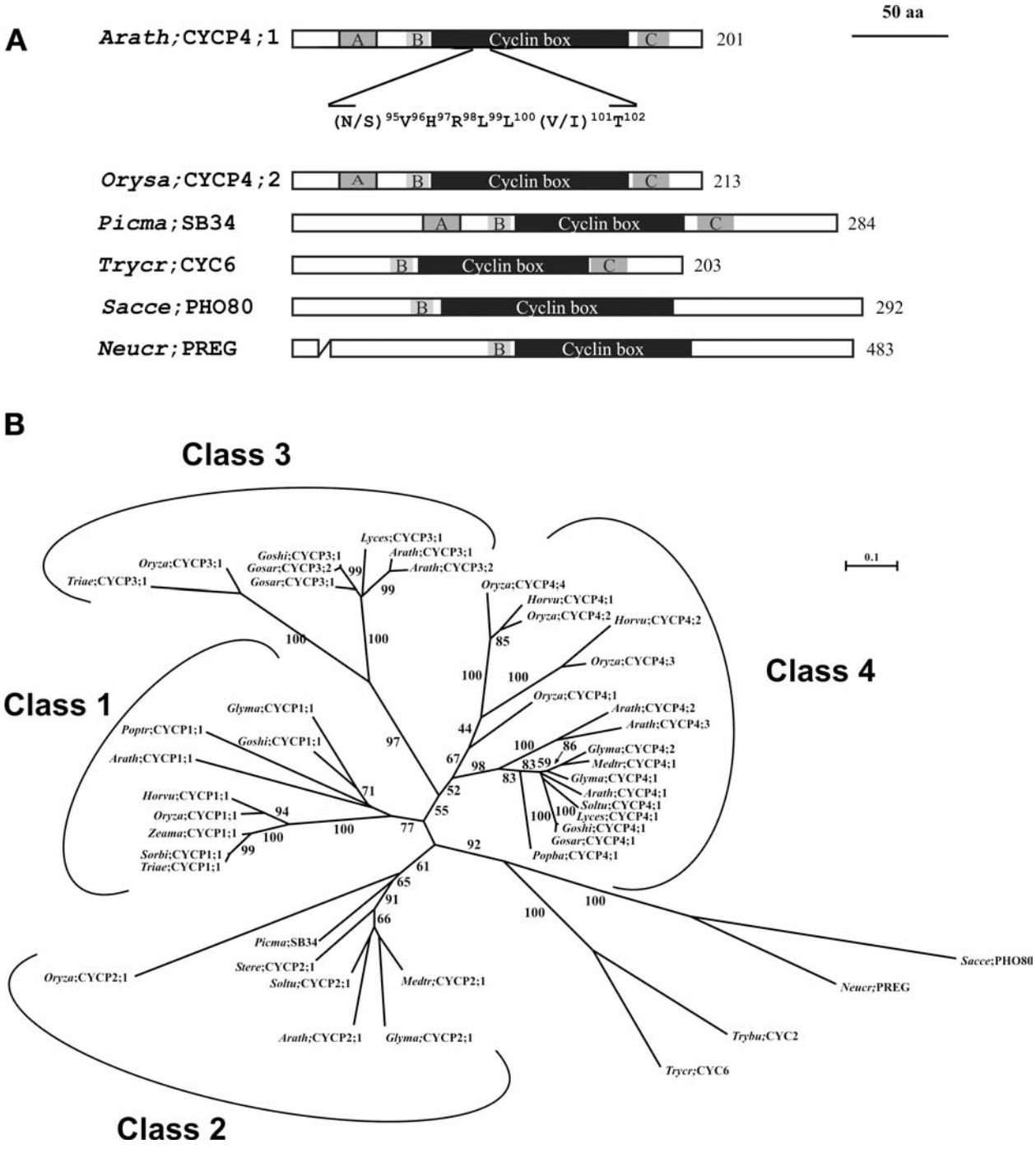

Figure 2. Sequence analyses of CYCPs. (*A*) Structural domain organization of CYCPs of *A. thaliana* (only *Arath;*CYCP4;1 is presented), *O. sativa* (*Orysa*;CYCP4;2 is given as example), *P. mariana* (*Picma*;SB34), *T. cruzi* (*Trycr;*CYC6), *S. cerevisiae* (*Sacce*;PHO80), and *N. crassa* (*Neucr*;PREG). Protein length in amino acids for each protein is indicated at the right. The scale bar above represents 50 amino acid (aa) residue length. Conserved sequence motifs (A, B, and C) and the 'cyclin box' are indicated by gray-filled and black boxes, respectively. Underneath the *Arath*;CYCP4;1, the P-type cyclin box signature is indicated. (*B*) Unrooted neighbor-joining tree of CYCPs and their counterparts in other organisms, using Poisson correction for evolutionary distance calculation. The scale indicates the evolutionary distance in number of substitutions per site. Bootstrap values give significance of nodes (values below 50% are not shown). Similar results were obtained with the maximum likelihood analysis (data not shown).

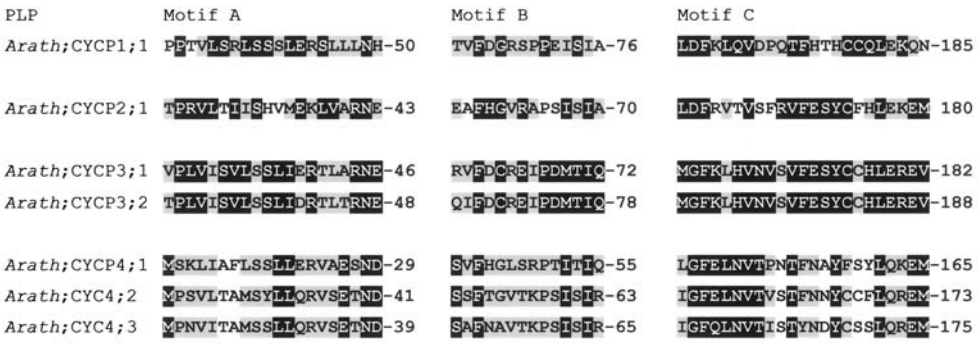

Figure 3. Conserved motif signature in CYCPs of *Arabidopsis*. Conserved amino acids, highlighted in black and grey (corresponding to <5% and <50% of sequences with a substitution at that position) were identified by sequence alignment of 68 P-type cyclins from monocotyledonous and dicotyledonous plants. The number at the right in each motif pattern defines the correct sequence position in the full-length protein.

The phylogenetic analysis revealed the presence of four classes of CYCP proteins in plants (fig. 2B). Class 2 (CYCP2;1) diverged first, followed by class 1 (CYCP1;1), class 3 (CYCP3;1 and CYCP3;2), and class 4 (CYCP4;1, CYCP4;2, and CYCP4;3), which comprises the largest number of CYCP sequences.

Two gene pairs were found in large duplicated regions in the *Arabidopsis* genome: *CYCP4;2* and *CYCP4;3* were part of a duplicated block within chromosome V, whereas *CYCP3;1* and *CYCP3;2* belong to a duplicated region between chromosome II and III. Both these regions were found to have arisen during the complete genome duplication, which occurred approximately 24–86 million years ago [24, 36–39].

Arabidopsis CYCPs interact with CDKA;1

To test the ability of CYCPs to interact with CDK proteins of *Arabidopsis*, the full-length cDNA of each *CYCP* gene was isolated and cloned into an appropriate yeast two-hybrid vector, resulting in a transcriptional fusion between the CYCPs and the GAL4 transcriptional activation domain (pGADCYCP1;1-CYCP4;3). In a yeast reporter strain, the pGADCYCP1;1-CYCP4;3 plasmids were co-transformed with vectors encoding a fusion between the CDKA;1, CDKB1;1, CDKC;1 and the GAL4 DNA-binding domain (pGBTCDKA;1; pGBTCDKB1;1 and pGBTCDKC;1), and with the empty pGBT9 vector as a control. Transformed strains were plated on medium without histidine, which allows growth of yeast only when the two proteins interact. All P-type cyclins interacted with CDKA;1, except for CYCP3;1 (fig. 4A). Only CYCP1;1 bound to CDKB1;1, and none associated with CDKC;1 (fig. 4A).

The yeast two-hybrid interaction of CYCP2;1, CYCP3;1, CYCP4;1, and CYCP4;2 with CDKA;1 was confirmed by co-immunoprecipitation. A coupled in vitro transcription-translation system was used to generate c-Myc-tagged CYCP2;1, CYCP3;1, CYCP4;1, and CYCP4;2, and HA-tagged CDKA;1. The total in vitro translation

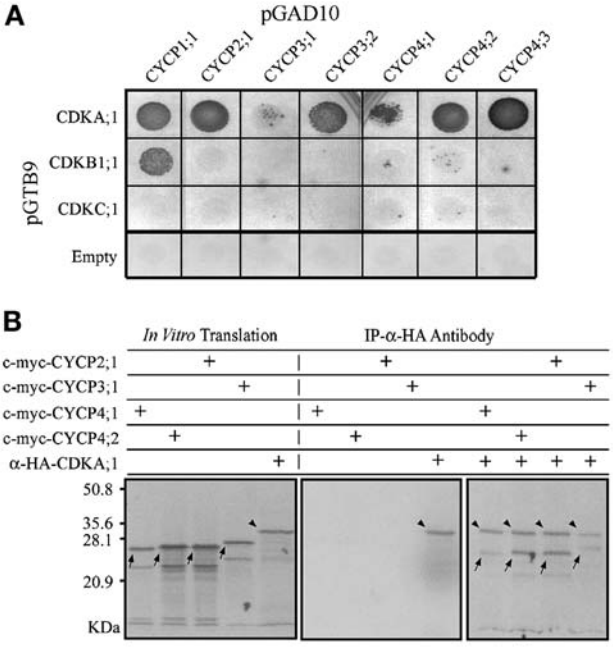

Figure 4. Interaction of *Arabidopsis* CYCPs with CDKA;1. (*A*) Two-hybrid interaction between the *Arabidopsis* CYCPs, CDKA;1, CDKB1;1 and CDKC;1. Yeast HF7c cells were transformed with plasmids containing the GAL4 DNA-binding domain fused to CDKA;1 (pGTBCDKA;1), CDKB1;1 (pGTBCDKB1;1), or CDKC;1 (pGTBCDKC;1), together with the pGADCYCPs plasmids that encode fusion proteins between the GAL4 activation domain and the CYCPs. As controls, pGADCYCPs were co-transformed with the empty pGTB9 vector. After growth on selective medium without tryptophan and leucine, co-transformants were spotted on plates with medium lacking tryptophan, leucine, and histidine. Reconstitution of GAL4 activity restores the ability to grow on histidine-deficient medium. (*B*) Co-immunoprecipitation of CYCP2;1, CYCP3;1, CYCP4;1, and CYCP4;2 with CDKA;1. A fraction of the total in vitro translation mix was analyzed directly on an SDS-PAGE gel (left panel). Another aliquot was subjected to immunoprecipitation with an anti-HA-monoclonal antibody. In the absence of CDKA;1, no CYCPs were precipitated by the anti-HA antibody (middle panel). In contrast, when CYCPs were mixed with CDKA;1, CYCPs co-immunoprecipitated with CDK1A;1 (right panel). Arrows mark the position of the CYCP proteins and arrowheads the position of the CDKA;1 protein. Molecular mass markers are given in kDa.

product of the *c-Myc-CYCP* and *HA-CDKA;1* cDNAs gave rise to a main band corresponding to the predicted molecular mass (fig. 4B, left panel). Bands of lower molecular mass were also observed, probably corresponding to truncated translation or proteolytic products. In the absence of HA-CDKA;1, no CYCPs were precipitated using the anti-HA antibody (fig. 4B, middle panel). However, when c-Myc-CYCPs were mixed with HA-CDKA;1, the CYCPs co-immunoprecipitated with CDKA;1 (fig. 4B, right panel). In contrast to what was observed in the yeast two-hybrid assay, CDKA;1 and CYCP3;1 interacted clearly (fig. 4B, far right panel). Identical results were obtained for CYCP4;3 (data not shown).

CYCP4;2 partially restores phosphate signaling in the yeast pho80 mutant

Sequence similarity between the *Arabidopsis* CYCPs and PHO80 of yeast suggests an analogous molecular function. To test whether the P-type cyclins of *Arabidopsis* function as homologs of the yeast protein, a genetic complementation experiment was performed by expressing the *CYCP* genes in a yeast *PHO80*-deficient mutant (strain EY0134) [31]. This yeast strain harbors a *GFP* gene under the control of the *PHO5* promoter. In the absence of a functional *PHO80* gene, the PHO2/PHO4 transcription factor will not be inactivated by phosphorylation under high phosphate conditions, and cells will be GFP positive. In contrast, in the presence of PHO80, the PHO2/PHO4 complex is inactivated, and GFP fluorescence will decrease.

The EY0134 strain was transformed with vectors that allowed galactose-inducible expression of the *CYCP* genes (pESC-CYCP1;1-CYCP4;3). The same strain was transformed with the empty plasmid (pESC) as a negative control, or with a plasmid allowing inducible expression of the yeast *PHO80* gene as a positive control (pESCPHO80). Non-transformed wild-type cells were included to measure background fluorescence (control). The presence of CYCPs was verified by Western blotting. Generally, protein levels increased approximately threefold in the presence of galactose in the medium compared to those under non-induced conditions (fig. 5A).

Under non-induced conditions, a lower fluorescence signal was seen for the pESCPHO80 culture than in the pESC and pESCCYCP cells (fig. 5B). This difference can be explained by the leaky expression of the galactose-inducible promoter. After induction, a decrease in fluorescence signal was only observed for pESCCYCP4;2, albeit to a lesser extent than for the native PHO80. For none of the other *CYCPs* was a decrease in GFP fluorescence observed, meaning that only *CYCP4;2* is able to partially restore the phosphate signaling pathway of the mutant yeast strain.

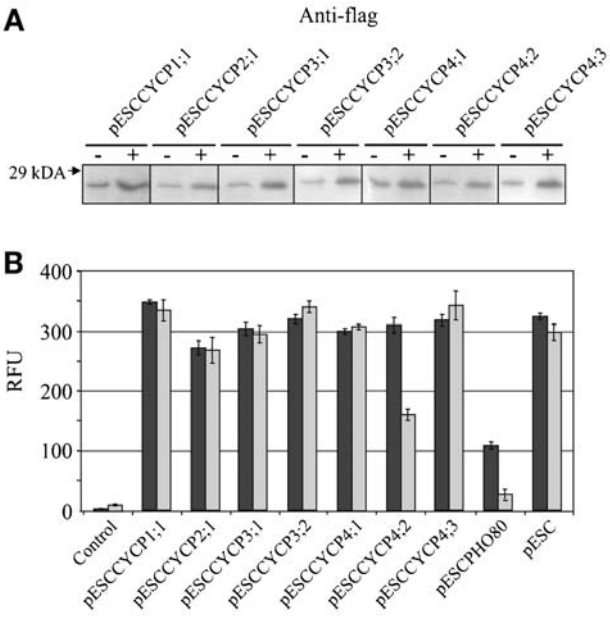

Figure 5. Yeast pho80 mutant complementation. (*A*) Expression of the different CYCPs was confirmed by Western blotting with the anti-FLAG antibody. Proteins were extracted from cells transformed with corresponding pESCCYCP vectors grown under non-inducing (–) or inducing (+) conditions. (*B*) GFP fluorescence levels in pho80 mutant yeast cells harboring the pPHO5-GFP reporter construct, transformed with the pESCCYCP vectors grown under non-inducing (black) or inducing (gray) conditions. Cells transformed with pESCPHO80 or the empty pESC vector were used as positive and negative control, respectively. Wild-type cells were included in the analysis (as control). GFP fluorescence was measured in relative fluorescence units (RFU) for emission at 510 nm from an equal amount of liquid culture. Data are the mean values of three independent experiments with error bars for the standard deviation.

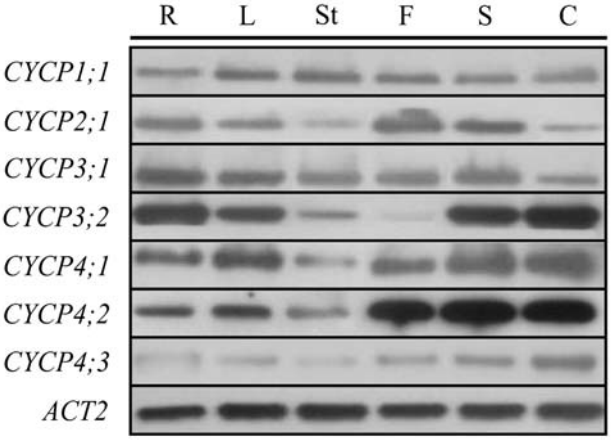

Figure 6. Transcriptional analysis of *CYCP* genes of *Arabidopsis*. Transcript levels of *CYCP* genes in 3-week-old *Arabidopsis* tissues, 1-week-old seedlings, and 3-day-old cell suspension. RNA was extracted from roots (R), leaves (L), stems (St), flowers (F), 1-week-old seedlings (S), and cell suspensions (C), analyzed by semi-quantitative RT-PCR with specific primers, and hybridized with *CYCP* probes. *Actin 2* (*ACT2*) was included as loading control.

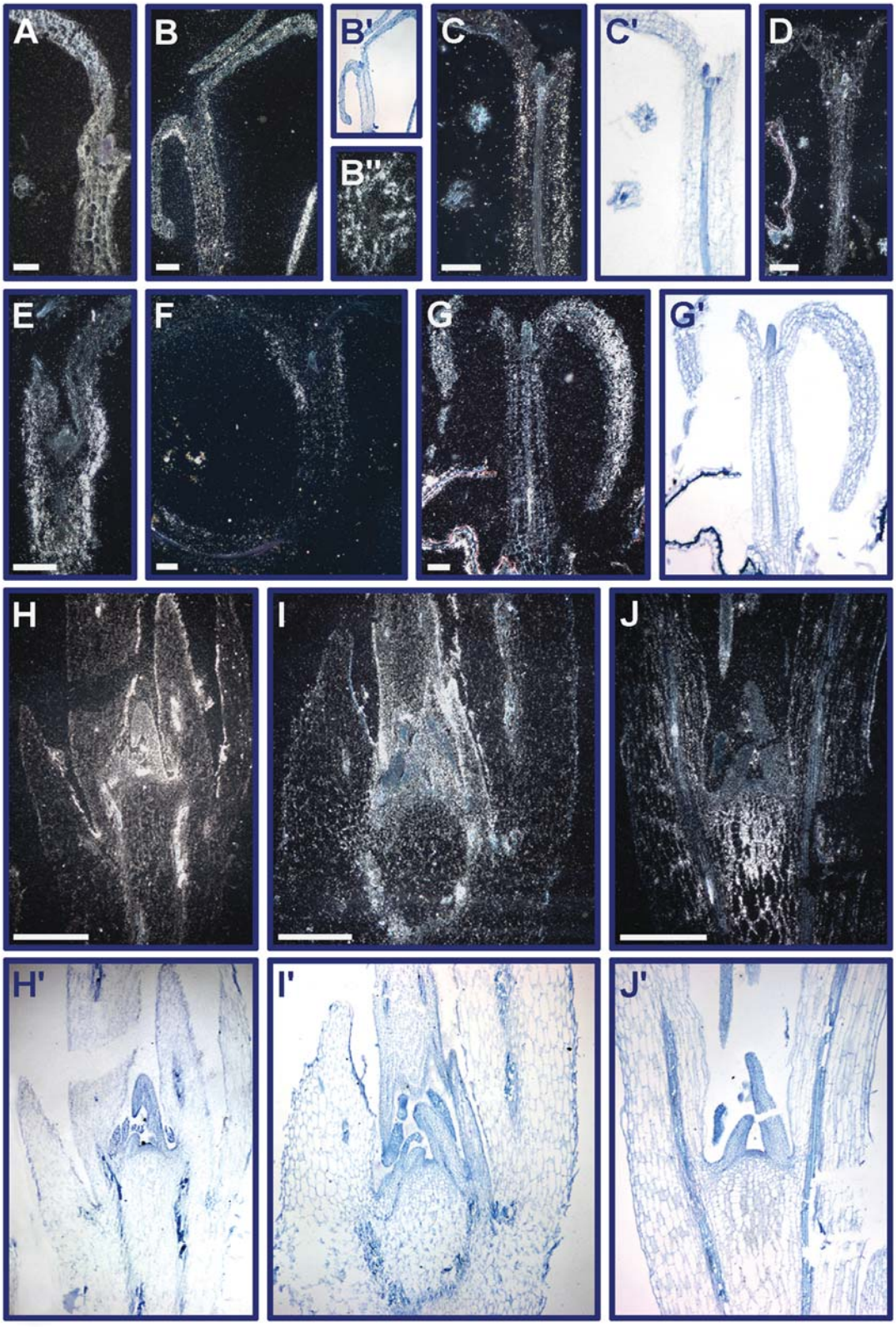

Figure 7. In situ localization of mRNA of the *CYCP* genes in *Arabidopsis* and radish seedlings. Hybridization signals are seen as white grains under dark-field optics. Some bright-field images are included to help visualize the plant morphology (*B′*, *C′*, *G′*, *H′*, *I′*, *J′*). (*A–G*) Longitudinal sections through the shoot apex of *Arabidopsis* seedlings. (B″) Cross-section through an *Arabidopsis* hypocotyl. (*H–J*) Longitudinal sections through the shoot apex of radish showing the shoot apical meristem and young leaves. Antisense probes of the seven *CYCP* genes correspond to *CYCP1;1* (*A*), *CYCP2;1* (*B*, *B′*, *B″*), *CYCP3;1* (*C*, *C′*, *H*, *H′*), *CYCP3;2* (*D*), *CYCP4;1* (*E*, *I*, *I′*), *CYCP4;2* (*F*), and *CYCP4;3* (*G*, *G′*, *J*, *J′*). Scale bars, 100 µm (*A–F*), 200 µm (*G*), and 500 µm (*H–J*).

CYCP genes are differentially expressed in plant tissues

To find out where *CYCP* genes are expressed in planta, we analyzed their expression levels in different *Arabidopsis* tissues by semi-quantitative RT-PCR. *CYCP* transcripts were detected in all tissues tested (roots, leaves, inflorescence stems, flowers, seedlings, and actively dividing cell suspensions), but at different expression levels (fig. 6). Transcripts of *CYCP1;1*, *CYCP3;1*, and *CYCP4;3* were equally detected in all tissues. *CYCP2;1*, *CYCP3;2*, *CYCP4;1*, and *CYCP4;2* mRNA levels were generally lowest in the inflorescences. In particular, *CYCP3;2* transcript levels were low in flowers.

To investigate tissue specificity of the expression patterns of the seven *CYCP* genes, mRNA of sections of shoots from *Arabidopsis* and its closely related species, radish, were hybridized *in situ* (fig. 7). Similar results were obtained for both species, but more tissue detail was seen in the larger radish cells. All *CYCP* genes were expressed in the shoot apex, leaf primordia, and young leaves, but at different intensities (fig. 7H–J). As leaves matured, expression became restricted to the developing vascular bundles and epidermal cells, with the exception of *CYCP1;1, CYCP2;1*, and *CYCP4;3* (fig. 7J) for which the expression remained detectable in the mesophyll cells. In addition, *CYCP1;1* and *CYCP4;3* (fig. 7J) were expressed in the region directly underneath the meristem. In young seedlings of *Arabidopsis* (5 days after germination), expression varied in the hypocotyl close to the apical hook. Expression was often stronger in the epidermis (*CYCP1;1, CYCP2;1*, *CYCP3;1*, *CYCP4;1*, and *CYCP4;2*) (fig. 7A–C, E, F). *CYCP1;1*, *CYCP4;1* (fig. 7A, E) and particularly *CYCP2;1* (fig. 7B″) were expressed in all hypocotyl tissues. Control hybridizations yielded no significant background (data not shown).

Discussion

Cyclins bind and activate CDKs. Previously, a total of 34 cyclins genes have been reported for *Arabidopsis* [4, 5, 7]. We described the isolation and molecular characterization of seven novel cyclins, bringing the total number of annotated cyclins in the *Arabidopsis* genome to 41. Although the CYCPs have a clear cyclin box, the previously reported in silico analysis of the whole *Arabidopsis* genome failed to detect them as cyclins because the method used to retrieve sequences was based on previously described cyclin cDNAs [5].

The CYCPs of *Arabidopsis* can be subdivided into four structural classes. Each class contains both monocotyledonous and dicotyledonous genes, indicating that the different classes diverged before the monocot-dicot split, and probably (given the presence of conifer sequences in class 2) before the gymnosperm-angiosperm divergence. Interestingly, whereas all P-type cyclins associate with CDKA;1, CYCP1;1 binds to CDKB1;1 as well. CDKB1;1 is a plant-specific CDK, thought to control plant-specific aspects of the cell cycle, whereas CDKA;1, which is most related to the mammalian CDK1 and CDK2, can complement yeast cdc2/CDC28 mutants and is, therefore, regarded as a functional homolog of the yeast CDKs [40]. The difference in binding abilities between CYCP1;1 and the other P-type cyclins suggests that CYCPs belonging to different phylogenetic classes exert distinct functions in the plant cell cycle, although this has still to be experimentally proven.

The seven cyclins described are all related to the PHO80 cyclin of budding yeast, which is known for its involvement in phosphate signaling. In plants, an inadequate supply of phosphate ultimately provokes cell cycle arrest [41]. For example, in Bright Yellow-2 cells of tobacco, cell division is inhibited when phosphate is absent from the medium [42]. At the plant level, a decrease in the phosphate concentration in the medium results in an increase of lateral root growth [43]. These data demonstrate that cell division, plant growth, and nutrient availability are interconnected. However, how this cross-talk operates is still largely unknown. Their homology with the yeast PHO80 cyclin raised the possibility that the plant P-type cyclins may coordinate cell division with phosphate availability. Accordingly, CYCP4;2 can restore the phosphate signaling pathway in the yeast pho80 mutant. However, this complementation is far less efficient than for the yeast *PHO80* gene. In addition, no clear changes in the *CYCP* transcript levels are seen when cell cultures are depleted of phosphate, or when phosphate was re-applied to phosphate-starved cells (data not shown), indicating that *CYCP* transcription is not controlled by phosphate availability, at least in cultured cells. Moreover, no clear orthologs of other components of the yeast phosphate signaling pathway can be identified in the *Arabidopsis* genome. As such, in contrast to their yeast counterpart, plant P-type cyclins are probably not involved in the regulation of expression of phosphate-scavenging enzymes. Members of the yeast PHO80 cyclin subfamily have been demonstrated to control other metabolic pathways, such as glycogen metabolism and carbon source utilization [44, 45]. Therefore, the plant P-type cyclins may control a metabolic pathway that differs from phosphate signaling. Examining the genome-wide reports on changes in the transcriptome in response to alterations of nutrient availability, such as fast nitrate [46] and sulfate [47] responses, did not allow us to assign the CYCPs to a particular nutrient signaling pathway. However, if CYCPs participated in the regulation of nutrient homeostasis, nutrient levels could affect the CYCP-containing CDK complex at the posttranscriptional level

only, as is observed for the yeast PHO80/PHO85 complex.

In contrast to most of the cyclins described, *CYCP* transcripts do not oscillate significantly during the cell cycle (data not shown; [48]). Nevertheless, transcript levels are mostly associated with dividing tissues, suggesting a clear link with cell division. Although PHO80 is not clearly involved in cell cycle regulation [10], its closer homologs PLC1 and PLC2 participate in the cell cycle progression through interaction with PHO85 [49]. The possible involvement of PHO-like proteins in the cell cycle is supported by a recent RNAi experiment on the procyclic form of *T. brucei* that demonstrated that a single CYCP cyclin homolog (CYC2) together with a single B-type cyclin homolog (CYCB2) are essential cyclins in the regulation of both G1-to-S and G2-to-M transitions [50]. In fact, protozoan cyclins are more homologous with the CYCPs than with PHO80, so that a bona fide role for the P-type cyclins in cell cycle regulation cannot be excluded.

In yeast, PHO80 and related cyclins bind exclusively to the PHO85 CDK-related protein. PHO85 is a non-essential CDK, but its absence causes a broad spectrum of phenotypes [51, 52]. By sequence comparison, no PHO85 ortholog was found in the *Arabidopsis* genomic database, and a two-hybrid screen using one of the P-type cyclins as bait did not recover a CDK other than CDKA;1 (data not shown). The binding preference of the P-type cyclins for CDKA;1 suggests that this CDK is the functional homolog of the yeast PHO85 kinase. In contrast to mammals, where the proliferating state of the tissue and the abundance of mitotic CDK mRNAs are strictly positively correlated, plant A-type CDK transcripts can also be found in non-dividing cells [53, 54]. Cells with high competence to divide retain A-type CDK transcription. The expression of CDKA in plants is regarded as the sign that cells are on 'stand-by' to initiate division, waiting for intrinsic signals or environmental conditions, a state defined as 'competence' [54]. Similar to A-type CDKs, *CYCP* transcription is found in tissues associated with active cell division, such as the stele and the meristems, but also outside dividing zones. The co-expression of *CDKA;1* and P-type cyclins, combined with the observed binding of their gene products in pull-down experiments and two-hybrid analyses, strongly suggest in planta complex formation between both proteins. As such, P-type cyclins might be a binding partner of CDKA;1 in division-competent cells. Through its differential association with the classical mitotic cyclins or P-type cyclins, CDKA;1 could participate in both cell division and the response of cells to nutrients, providing a mechanism that integrates changing nutritional signals with cell cycle activity. This hypothesis implies the potential involvement of cell cycle regulatory proteins in functions other than the regulation of the cell division process per se. To find out the exact roles played by the diverse CDK/CYCP complexes, it will be interesting to identify substrates of these complexes which might help us understanding the potential link between cell division and nutritional status of the cell.

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