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Natural synchronisation for the study of cell division in the green unicellular alga *Ostreococcus tauri*

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Abstract

Ostreococcus tauri (Prasinophyceae) is a marine unicellular green alga which diverged early in the green lineage. The interest of *O. tauri* as a potential model to study plant cell division is based on its key phylogenetic position, its simple binary division, a very simple cellular organisation and now the availability of the full genome sequence. In addition *O. tauri* has a minimal yet complete set of cell cycle control genes. Here we show that division can be naturally synchronised by light/dark cycles and that organelles divide before the nucleus. This natural synchronisation, although being only partial, enables the study of the expression of CDKs throughout the cell cycle. The expression patterns of *Ot*CDKA and *Ot*CDKB were determined both at the mRNA and protein levels. The single *Ot*CDKA gene is constantly expressed throughout the cell cycle, whereas *Ot*CDKB is highly regulated and expressed only in S/G2/M phases. More surprisingly, *Ot*CDKA is not phosphorylated at the tyrosine residue, in contrast to *Ot*CDKB which is strongly phosphorylated during cell division. *Ot*CDKA kinase activity appears before the S phase, indicating a possible role of this protein in the G1/S transition. *Ot*CDKB kinase activity occurs later than *Ot*CDKA, and its tyrosine phosphorylation is correlated to G2/M, suggesting a possible control of the mitotic activity. To our knowledge this is the first organism in the green lineage which showed CDKB tyrosine phosphorylation during cell cycle progression.

Introduction

Cell division is a basic property of all living organisms and studies on the molecular control of cell division began about three decades ago on unicellular yeasts or on animal models, such as sea urchin eggs, which are naturally highly synchronised. These studies showed that the regulation of the eukaryotic cell division occurs at multiple points and is highly conserved throughout evolution (Dewitte and Murray, 2003). The regulation of the cell division cycle is basically ensured by the activities of CDK-complexes which are heterodimers composed of a cyclin dependent kinase (CDK) subunit that binds a regulatory cyclin subunit (Mironov *et al.*, 1999). CDK-cyclin complexes are present in all evolutionary lineages hitherto studied including plants (Joubès *et al.*, 2000). However, despite this globally conserved regulation of the cell cycle in plants, there are many adaptations specific to this phylum due to the immobility of plants, making their growth and

development much more dependent on the environment than compared to animals (Rossi and Varotto, 2002).

However, the precise role of these conserved genes in the green lineage is often hard to grasp because of the high complexity of plant model genomes, namely, the presence of several copies of key genes such as CDK and cyclins (Vandepoele *et al*., 2002). The function of each copy is difficult to study since its independent role is blurred: silencing one copy does not necessarily yield the complete phenotype associated with the gene as part or all of the function of the silenced copy can be rescued by other copies. Therefore there is a need for a simpler model organism for the green lineage that can be used to unravel aspects of the cell cycle that are unique to this phylum.

Chlamydomonas reinhardtii is a well-known model organism belonging to the green lineage and could represent such a model. Many studies on the regulation of the cell division of this unicellular alga have already been published but the complexity of its cell division process by multiple fission is a limiting factor for such studies (Umen and Goodenough, 2001; Bisova *et al*., 2005). The red unicellular alga *Cyanidyoschyzon merolae*, whose complete genome has been sequenced recently (Matsuzaki *et al*., 2004), also represents a potentially interesting model. However, although the red lineage is related to the green lineage, phylogenetic extrapolations to higher plants remain difficult. Furthermore, *C. merolae* is found in very unusual extreme environments such as high temperature and low pH. Several other unicellular photosynthetic organisms have also been used to study the cell division, such as *Euglena*, *Chlorella* or other picoeukaryotes (Donnan *et al*., 1985; Vaulot, 1995; Jacquet, 2001), but these studies often remain incomplete because very few molecular data are available.

Ostreococcus tauri is an unicellular alga and is the smallest free-living eukaryotic cell described to date (diameter around 1 μm). It possesses a minimal cellular organisation consisting of a naked, non-flagellated cell with one nucleus, one chloroplast and one mitochondrion (Courties *et al*., 1994; Chrétiennot-Dinet *et al*., 1995). Its 12.6 Mbp genome is now entirely sequenced (E. Derelle, personal communication). The interest of *O. tauri* as a potential model to study plant cell division is based on its key phylogenetic position at the base of the green lineage, its simple binary division, a very simple cellular organisation and now the availability of the full genome sequence. In addition, *O. tauri* has a minimal, yet complete, set of cell cycle control genes, with only one copy of every gene, including one copy of *Ot*CDKB which is known as a plant-specific CDK (Fobert *et al*., 1996; Magyar *et al*., 1997; Corellou *et al*., 2005; Robbens *et al*., 2005).

Furthermore, marine phytoplankton represents until 40–50% of our planet's annual net primary biomass production and the understanding of its growth control is a priority to better understand the control of this biomass production and, consequently, the control of the carbon cycle in oceans (Field *et al*., 1998; Falkowski *et al*., 2004).

In this article, we characterise the growth of *O. tauri* by the development of a natural synchronisation of cell cultures by simple light/dark cycles. This synchronisation enabled us to investigate the basic cellular and molecular features of the *O. tauri* cell division cycle. The interest of *O. tauri* as a simple model organism to study cell cycle regulation in the green lineage is discussed.

Materials and methods

Algal strains and culture conditions for natural synchronisation

The *Ostreococcus tauri* strain OTTH0595 was used for all experiments (Courties *et al*., 1998) and was grown in Keller-medium (Sigma-Aldrich, Saint-Quentin Fallavier, France), prepared in 0.22 μm filtered sea water (salinity: 36‰). Cultures were maintained in presence of an antibiotic cocktail (Penicillin: 25 μg ml^{-1}, Neomycin: 20 μg ml^{-1} and Kanamycin: 25 μg ml^{-1}). Axenisation of cultures was checked with flow cytometry after staining with SYBR®green I (Molecular Probes Inc., Eugene, Oregon, USA). Cultures were grown under a light/dark cycle (6, 10, 12 and 16 h of light in a 24 h cycle) at 20 ± 1 °C and under mild agitation. For the commitment assay, cultures were submitted to 2, 4, 6, 8, 10, 12, 14 and 16 h of light in a 24 h cycle after one week in a continuous light condition and a last day in darkness. A light intensity of 100 μmol photon m^{-2} s^{-1} with a blue filter was used in all experiments. Cells were cultured at least one week under different light conditions before analysis.

Synchronisation of the Ostreococcus *cultures by blocking agents*

Drugs as hydroxyurea (HU: 1 mM, Sigma), aphidicolin (20 μM, Sigma) or propyzamide (6 μM for biochemical and molecular analyses or 10 μM for microscopy analyses, Sigma) have been used as control to block cells in particular stages of the cell cycle. Aphidicolin and HU were used to inhibit DNA replication (inhibition after G1/S transition, cells are in S/G2 phase). Propyzamide is known to depolymerise the microtubule cytoskeleton by preventing the addition of tubulin dimers to dynamic microtubules and was used to obtain cells blocked in M phase. High percentages of cells in S/G2 or in mitosis were obtained.

When cultures entered the light phase, HU was added. Treatment was performed for 12 h and then cells were harvested by centrifugation using 0.1% (w/v) pluronic acid at 8000 *g* for 10 min at 4 °C. Pellets were resuspended and transferred to microcentrifuge tubes and then centrifuged at 8000 *g* for 4 min at 4 °C. Supernatants were removed and the pellets were frozen quickly in liquid nitrogen before storing at −80 °C.

Cultures that was prepared for electron microscopy was treated with 10 μM propyzamide or 10 μM aphidicolin at the beginning of the light phase for 12 h. For biochemical and molecular analyses, HU inhibition was performed at the beginning of the light phase. Cultures were subjected to continuous light. After 12 h, cells were either harvested immediately (HU blockages) or released from HU blockage (propyzamide blockage) by washing the cells twice using 0.1% (w/v) pluronic acid solution and once without pluronic acid. Afterwards, 6 μM propyzamide was added and cells were harvested 3 h later (maximum of cells in G2/M). HU blockage followed by a washing step allowed the cells to complete their cycle progression with a high synchrony (Corellou *et al.*, 2005). Furthermore, a new blockage with propyzamide after HU release even gave a better M phase (70–80% of the cells in this phase).

Flow cytometry analysis

Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser providing 15 mW at 488 nm. *O. tauri* detection and cell number evaluation were performed according to their forward angle light scatter (related to cell size) and fluorescence response, which depends on the pigment contents (red for chlorophylls). Parameters of light scatter and fluorescence emissions were normalised using fluorescent beads (diameter, 1.002 μm, Polysciences, Warrington, PA, USA) as described elsewhere (Derelle *et al.*, 2002). All samples were fixed for 30 min with 0.25% (v/v) glutaraldehyde before analysis, or quickly frozen in liquid nitrogen, and stored at −80 °C until analysis. For cell cycle analysis, SYBR®green I (Molecular Probes Inc., Oregon, USA) was added at a dilution of 1/10 000 of the commercial solution (Marie *et al.*, 1997). Samples were incubated for 30 min at room temperature in the dark before analysis. Values obtained with or without RNase treatments were similar and no signal was detected without SYBR®green I and that signal disappeared after DNase treatment. Comparisons of the data on the genome size (12.6 Mb) and the DNA content showed that the nuclear genome represents at least 95% of the total cellular DNA in *O. tauri*.

Results were treated with the CellQuest v3.1 software (Becton-Dickinson, San Jose, California) and a modelisation was proposed with the ModFit v2 software (Verity Software House, Inc, USA).

Transmission electron microscopy (TEM)

Samples were prefixed in culture medium with 1% (v/v) glutaraldehyde for 30 min at room temperature. Then, cells were centrifuged at 2600 *g* for 15 min at 20 °C. Pellets were resuspended in 2 ml fixative solution (3% (v/v) glutaraldehyde, 0.1 M cacodylate and 30 mM NaCl) and then centrifuged at 2600 *g* for 15 min. Pellets were rapidly resuspended in 20 μl of agar-agar using a micropipette. The tubes obtained were incubated in fixative solution for 1 h at room temperature. Agar tubes were washed 3 times for 20 min each in 0.4 M sucrose, 1% (v/v) osmic acid, 0.1 M cacodylate and 30 mM NaCl. Post-fixation was achieved using 1% (v/v) osmic acid, 0.1 M cacodylate and 49 mM NaCl for 1 h at room temperature. Samples were washed a further 3 times for 20 min each in 0.4 M sucrose, 1% (v/v) osmic acid, 0.1 M cacodylate and 30 mM NaCl before dehydrating by successive ethanol bathes containing increased ethanol concentrations. Samples

were then embedded in Epon. Ultrathin sections (500 Å) were prepared by using a microtome and contrasted with uranyl acetate and lead citrate. Sections were observed under a Hitachi H-7500 Transmission Electron Microscope.

Northern blot and semi-quantitative RT-PCR

The northern blot protocol was performed as described previously (de O Manes *et al*., 2001). Total RNAs were extracted with the RNAeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol and 30 μg of total RNA were used for the blot. Signals were quantified using a STORM phosphorimager with the Image QuanT software (Molecular Dynamics, Sunnyvale, CA). Probes were labelled with the Prime-a-Gene® Labeling System (Promega) and purified with ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech).

For semi-quantitative RT-PCRs, cDNAs were obtained using the RETROscript™ kit (Ambion) according to manufacturer's protocol. Then, PCR was performed in 50 μl using 20 cycles to obtain linear amplification products. A 315 bp internal 18S standard from QuantumRNA™ Universal 18S Internal Standard kit (Ambion) was used in PCR experiments and primers were added after 10 cycles. Products (10 μl) were separated in a 1.5% (w/v) agarose gel and transferred onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech), and subsequently fixed with 50 mM NaOH. Fluorescein-labeled probes were prepared using the Gene Images Random Prime Labelling Module (Amersham Pharmacia Biotech) and signal detection was performed with The Gene Images CDP-STAR Detection Module (Amersham Pharmacia Biotech). Bands were quantified with the Phoretix *Total*Lab software v2.01 (ALPHELYS SARL, France). For both techniques, the 18S probe was used as control of normalisation.

Primers used for Northern probes and PCR amplifications were *Ot*CDKA 5′-TCAAA ACTTT GGGTACTCCG-3′ and 5′-GG TTGCTCGAAA ATAGATCTC-3′ ; *Ot*CDKB 5′-TTGGATCAAG ATTTGAAGCAG-3′ and 5′-TG GAAACCGCT CGTATTTCA-3′ ; *Ot*CYCA 5′-C TAAATTCAA ACTTCCCGGTG-3′ and 5′-G GTTGCTCGAA AATAGATCTC-3′ ; *Ot*CYCB 5′-ACGCGACG-CACGTATCTAT-3′ and 5′-CGGCGACCGTCA GTTTCA-3′; *Ot*CYCD 5′-ATCGTGGACATCG AACATGTA-3′ and 5′-TTCAAGCTCACTCCT-CATCCC-3′; *Ot* Histone H4 5′-GTCCCATCAT-CAAGCGTTTA-3′ and 5′-AGCGCATCTCTGG TCTCATCT-3′.

Extraction and purification of CDK-like proteins

Cells were harvested as above (see Synchronisation of the *Ostreococcus* cultures by blocking agents), and pellets frozen in liquid nitrogen before storing at −80 °C. Protein was extracted from cells in 500 μl of extraction buffer containing 0.1% nonidet P-40 and an anti-protease cocktail (P9599, Sigma) as described previously (Corellou *et al*., 2000). Cells were broken by grinding in liquid nitrogen twice and sonicating briefly 3 times for 5 s on ice, then centrifuged at 10 000 *g* for 10 min at 4 °C.

CDK-like proteins were purified on $p9^{CKS1Hs}$ or $p10^{CKS1At}$ Sepharose beads. For each sort of beads, protein extracts were incubated with 10 μl of beads on a rotary shaker for 45 min at 4 °C. Beads were spun at 2000 *g* for 2 min and washed 3 times in bead buffer (Azzi *et al*., 1994).

Polyacrylamide gel electrophoresis and immunolabelling experiments

$p9^{CKS1Hs}$ or $p10^{CKS1At}$ beads were resuspended in 30 μl of 4 × Laemmli buffer and proteins were eluted by boiling for 5 min. Proteins purified by affinity were separated in denaturing polyacrylamide gels (10% separating gel, 4% stacking gel) using the Mini-Protean II system (BioRad Laboratories, California, USA). Proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech) in a wet blotting system (BioRad Laboratories, California, USA) at 90 V for 1 h. The membrane was subsequently treated according to the ECL+Plus System protocol (Amersham Pharmacia Biotech). Detection of *Ot*CDKA was achieved with a anti-PSTAIR monoclonal antibody (Sigma) diluted 1/5000, and the secondary antibody was a goat anti-mouse IgG peroxidase-conjugated antibody (Sigma), diluted 1/10 000. PSTAIR antibody can recognise both *Ot*CDKA and *Ot*CDKB (PSTALRE motif). For *Ot*CDKB, the primary antibody has been raised against *O. tauri* peptide (-YFDSLDKSQF-), and was used at a 1/1000 dilution. The antibody did not detect *Ot*CDKA and its immuno-affinity was assayed by competition with the antigenic peptide

(Corellou *et al*., 2005). The secondary antibody was a goat anti-rabbit IgG peroxidase-conjugated antibody (Sigma) used at a 1/5000 dilution. Phosphorylation on the tyrosine residue was detected with the p-Tyr horseradish peroxidase-conjugated monoclonal antibody (PY99-HRP, Santa Cruz Biotechnology, Inc., CA) at a 1/5000 dilution. Alkaline phosphatase (CIP: Calf Intestinal Phosphatase, BioLabs) treatment was applied on $p10^{CKS1At}$ beads to ensure the specificity of the phosphorylation detected. Ten units of CIP were applied for 60 min at 37 °C in a 50 μl reaction. Detection of the targeted proteins was performed by chemoluminescence using the ECL + Plus or ECL System (Amersham Pharmacia Biotech).

Histone H1 kinase activity assay

The ability of *O. tauri* CDK A and B to phosphorylate histone H1 was determined as described previously (Azzi *et al*., 1994; Corellou *et al*., 2000). The activity was measured at 30 °C for 30 min using [γ^{32}P] ATP. Roscovitine (200 μM) was applied to *Ot*CDKA and *Ot*CDKB to inhibit specifically CDK activities just prior to [γ^{32}P] ATP addition. The signal of labelled histone H1 was quantified using a STORM phosphorimager with the Image QuanT software (Molecular Dynamics, Sunnyvale, CA) considering the highest activity level of each protein as the 100% reference.

Results

Synchronisation of O. tauri *cultures by light/dark cycles*

O. tauri cultures were subjected to various light/dark conditions and the synchronisation rates of cell divisions were followed by measuring the cellular DNA content by flow cytometry. Cultures grown in continuous light showed 1.8 daily division (doubling time of 13 h 20) when cells are in exponential phase (cell concentration inferior to 10 million cells per millilitre), and no synchronisation of cell division could be observed. In contrast, no division occurred when cells were maintained in constant dark whereas a partial synchronisation was obtained when cells were entrained under a light/dark cycle (Figure 1). A progressive increase of the light period showed that no cell division occurred before 6 h of light (Figure 2). In our experimental conditions, this time corresponded to the minimal light requirement necessary for cell to commit to a mitotic cycle.

One division per day was seen under a 12:12 light/dark (12:12 L/D) cycle, and under this regime, cell division occurred at the light/dark transition (Figure 1C). Half of the cells divided before the end of the light phase and half divided at the beginning of dark phase. The cell division rate was more or less similar under a 10:14 L/D cycle (global daily 0.9 division) but percentage of synchronisation was lower (Figure 1B).

An increase of the light period to 16 h slightly increased the percentage of S cells from 35 to 40–45% (Figure 1D). However, in this 16:8 L/D cycle, a first round of cell division occurred 10 h after the beginning of the light phase (as in 12:12 L/D condition), followed by a second overlapping cell division (global daily 1.5 division). When the daily light period was shortened to 6 h, only 70% of the cells divided, divisions began slightly sooner and cells divided almost entirely in dark (Figure 1A). However, in that case, S and G2/M peaks overlapped which did not rend a good precision for cell cycle study.

Histone H4 mRNA expression, which is a known molecular marker of S phase entry (Bilgin *et al*., 1999; Meshi *et al*., 2000), was followed for the 12:12 L/D culture (Figure 3A,C). As described in other organisms, *O. tauri* histone H4 mRNA was also strongly induced in early S in agreement with the flow cytometry results. A good correlation between the S phase and the H4 mRNA expression was observed.

Synchronisation of the O. tauri *cultures by blocking agents*

Light/dark cycles mimicked natural light variations but the synchronisations obtained were partial and, for example, only 35% of the cells were in S-phase when the 12:12 L/D cycle was applied. Furthermore, the S, G2 and M phases partially overlapped. To increase the level of synchronisation, drugs known to block cells at different stages were also tested. When aphidicolin or HU were added to 12:12 L/D acclimated cultures at the beginning of the light phase in mid G1, no DNA replication or cell division occurred (Figure 3B, C). Propyzamide was also

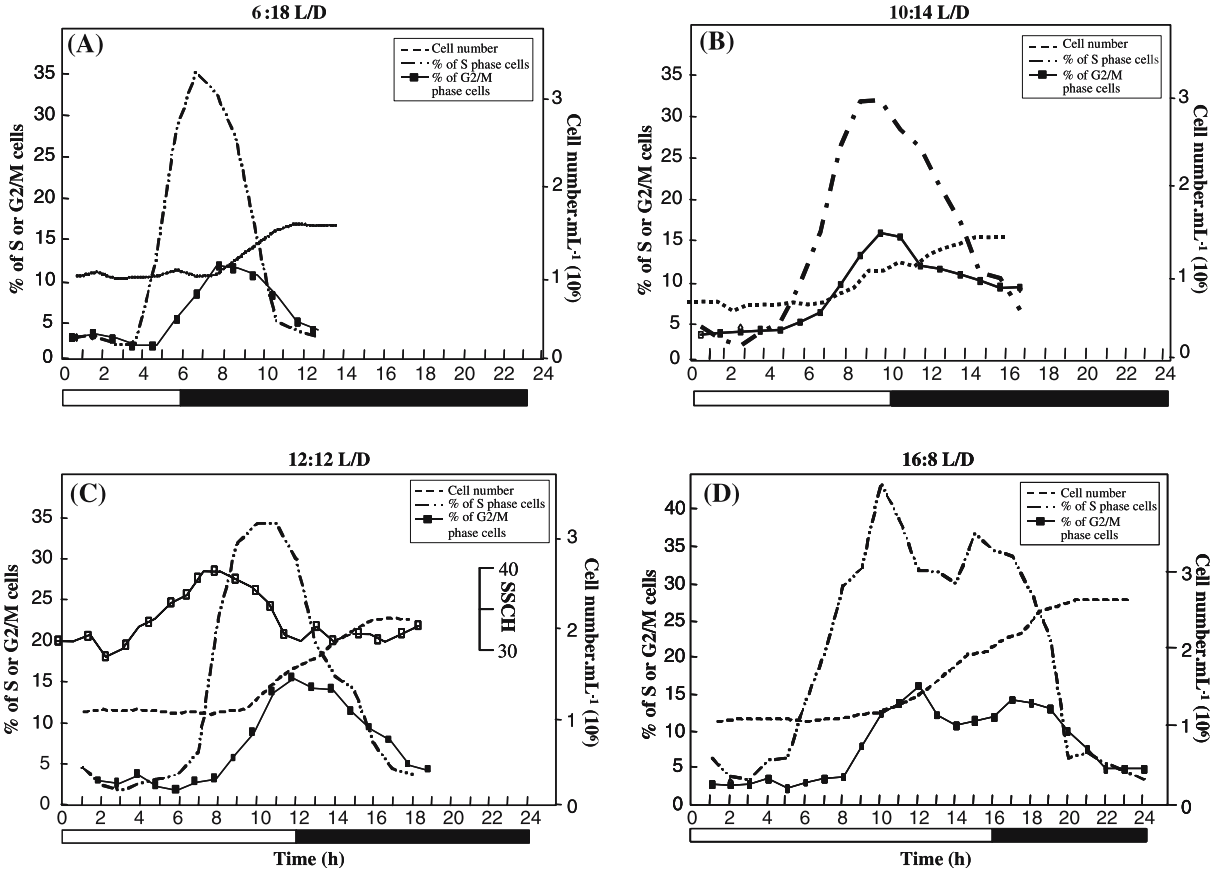

Figure 1. Growth of *O. tauri* cells and percentage of cells in the S phase and G2/M phases for four different light regimes. Bars below are a schematic representation of the light/dark period. (A) 6:18 L/D cycle; (B) 10:14 L/D cycle; (C) 12:12 L/D cycle; (D) 16:8 L/D cycle.

introduced after HU release and about 70–80% of *O. tauri* cells were blocked in G2/M, i.e. after the duplication of their DNA and when no further histone H4 mRNA was detected (Figure 3B, C). The H4 mRNA expression was followed by RT-PCR after addition of block conditions. We used aphidicolin (cells in S/G2 phase), HU-treated cells harvested just after washing step (beginning of DNA synthesis), HU-treated cells three hours after the release (S/G2/M cells accumulation) and finally propyzamide-treated cells during two hours (double blockage, S/G2/M phase cells accumulation). G1 cells 2 h after the beginning of the light period was used as negative control (Figure 4). After 2 h of light, no H4 mRNA was observed, suggesting that its expression is not light dependent. H4 mRNA was not expressed prior to DNA synthesis (Figure 3C) but when the DNA polymerase was inactivated (aphidicolin) as previously

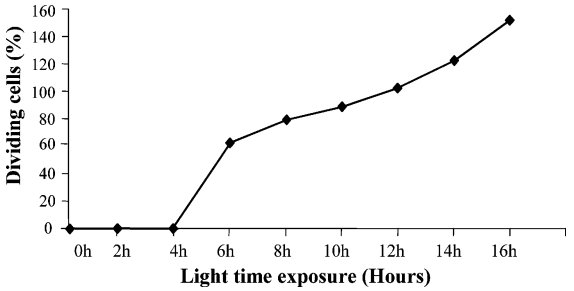

Figure 2. Commitment assay. Dividing cells percentage evaluated according to light time exposure in a 24 h cycle. Culture were harvested 24 h after the end of the light.

described by Reichheld and co-workers who showed that H4 expression was independent to DNA synthesis (Reichheld *et al.*, 1995). Immediately after the HU release, S phase initiation coincided with H4 mRNA expression (Figure 4).

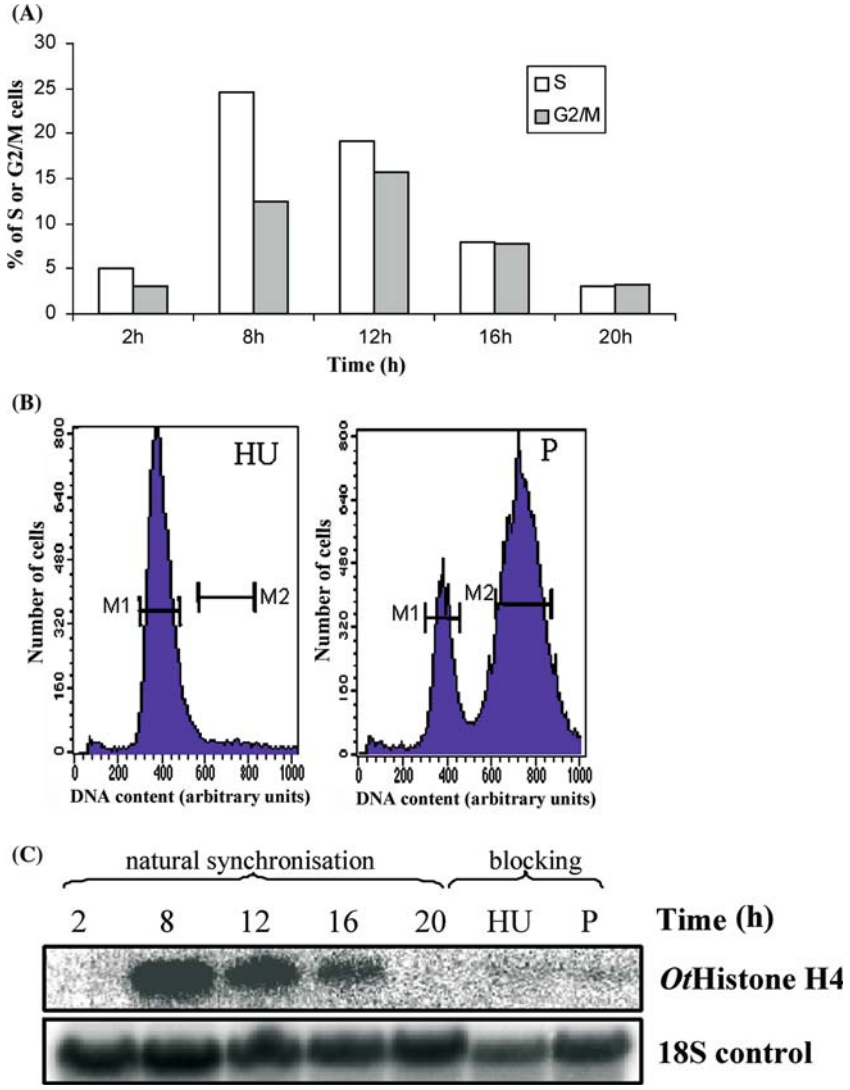

Figure 3. Synchronisation rates after natural synchronisation and after blocking with drugs. (A) Flow cytometry analysis of synchronised cultures under a 12:12 L/D cycle. (B) Flow cytometry analysis for HU or propyzamide treated cells showing cells in the phases G1 (M1) or S/G2/M (M2). (C) Expression of H4 histone mRNA compare to the 18S rRNA control in a 12:12 L/D synchronised culture.

Three hours after release, most of the cells were in G2/M, and H4 mRNA expression decreased. In presence of propyzamide after HU release, at two hours treatment, H4 mRNA was present (proportional to percentage of cells in S phase obtained by FACS results). In mitosis (Figure 3C), no further mRNA expression of H4 was seen, leading to the conclusion that H4 is expressed prior to M phase. These results are consistent with an expression of H4 mRNA in S phase and confirm that H4 mRNA level can be used as a control of the entry in S phase for these cultures synchronised by light/dark cycles.

Kinetics of Ostreococcus tauri *cell division*

Cultures synchronised by light (12:12 L/D) were fixed at different times and observed by TEM (Figure 5). All cells fixed at the beginning of the light phase (mid G1) were small and contained only one nucleus, one mitochondrion and one chloroplast (Figure 5A). Cells fixed at 10 h (2 h

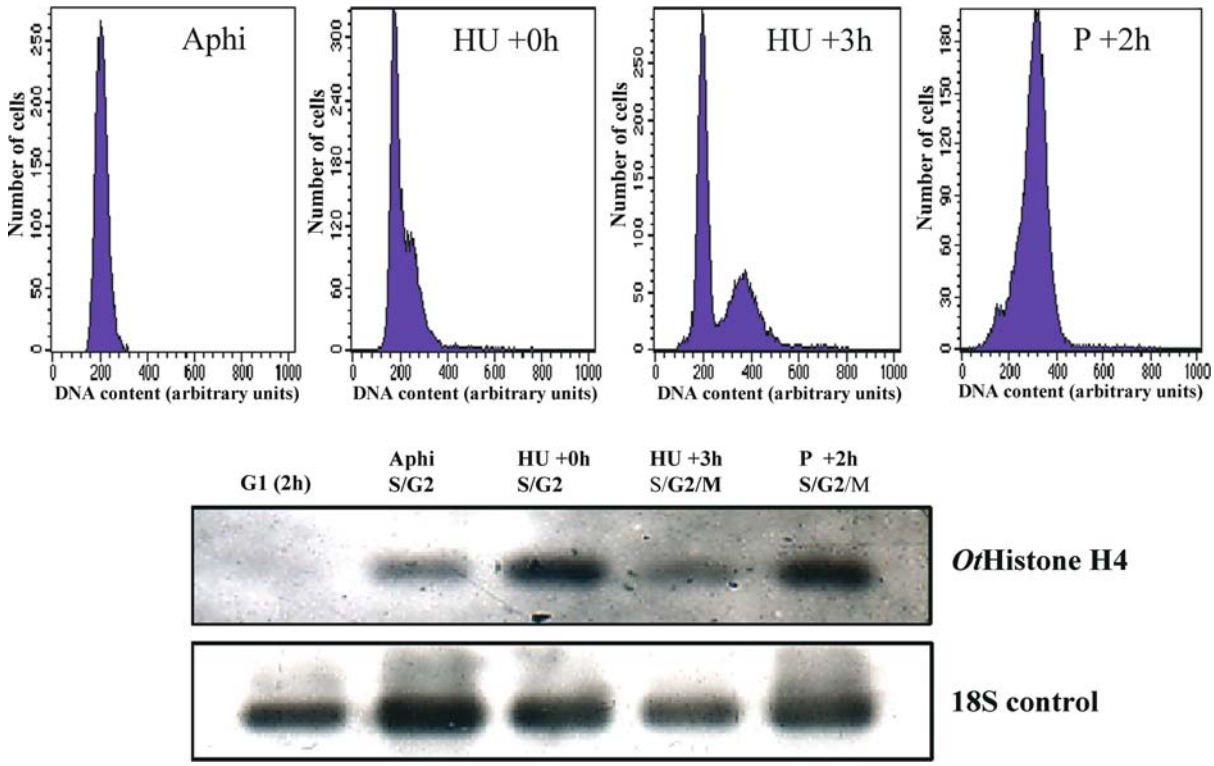

Figure 4. Histone H4 mRNA expression in particular stages of the cell cycle. cells in S/G2 phase (aphidicolin treatment), HU-treated cells harvested just after washing step (beginning of DNA synthesis), HU-treated cells three hours after the release (S/G2/M cells accumulation) and finally propyzamide-treated cells during two hours (double blockage, S/G2/M phase cells accumulation). G1 cells 2 h after the beginning of the light period was used as negative control.

before dark at the maximum of S cells), were bigger and around 30–35% of them showed either a complete or a partial division of their chloroplast whereas the nucleus and the mitochondrion were still not dividing (Figure 5B). In cultures fixed at 12 h, at the light/dark transition (maximum of G2/M), most of the cells showed a division of the nucleus and the organelles although the cells themselves were not yet dividing (Figure 5C). Furthermore, at this time, few cells showed division of the chloroplast and the mitochondrion without the division of the nucleus. Cultures blocked by propyzamide also showed a division of organelles but not of the nucleus (Figure 5D). These observations clearly revealed a sequential division of the chloroplast and the nucleus in *O. tauri*: the chloroplast divided first, during the S phase, followed by the nucleus before cytokinesis. The kinetic of mitochondrion division was less clear in these observations, although it seems dividing after the chloroplast and before the nucleus.

Transcription of core cell cycle genes in Ostreococcus tauri

Analysis of the *O. tauri* genome showed that this organism has a minimal, yet complete, set of core cell division control genes (CDK/Cyclin): 1 CDKA, 1 CDKB, 1 Cyclin A, 1 Cyclin B and 1 Cyclin D (Robbens *et al.*, 2005). The mRNA expression kinetics of these genes was followed in a 12:12 L/D acclimated culture (Figure 6A).

*Ot*CDKA mRNA was expressed at constant level throughout the cell cycle (although a slightly lower expression was seen in early G1) whereas *Ot*CDKB mRNA was highly regulated (Figure 6B). *Ot*CDKB mRNA expression was restricted to the S-phase, decreased in G2/M and disappeared in G1. Such an accumulation of *Ot*CDKB transcripts from S until late G2 has already been described in *A. thaliana* and more recently in *C. reinhardtii* (Segers *et al.*, 1996; Mironov *et al.*, 1999; Bisova *et al.*, 2005; Menges *et al.*, 2005). *O. tauri* Cyclin A and B mRNAs were expressed similarly to CDKA

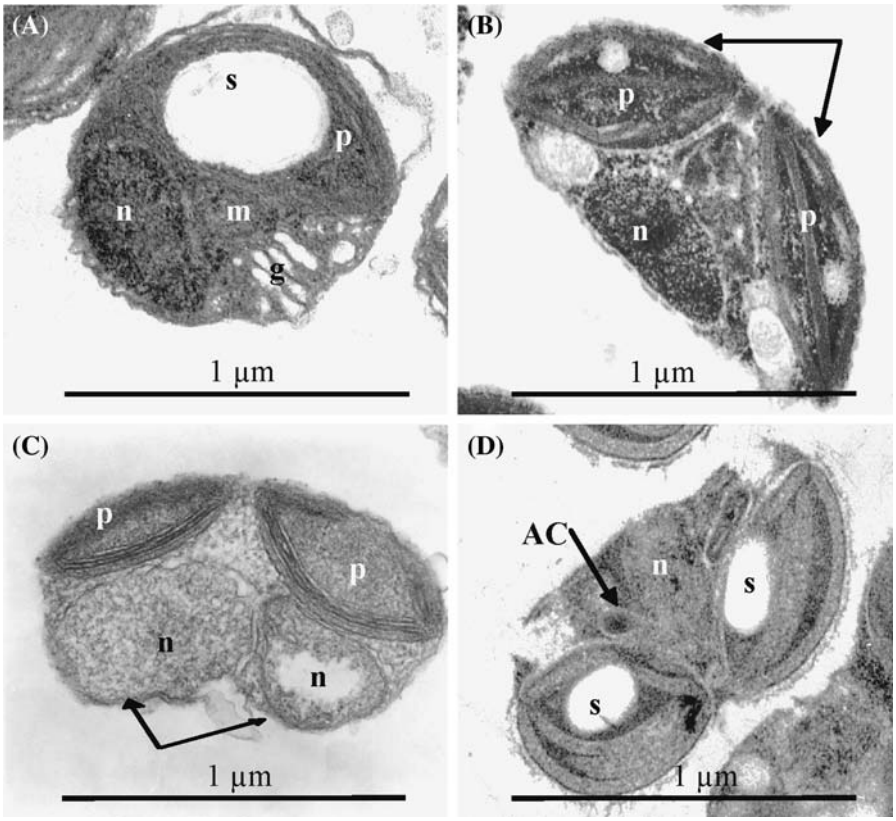

Figure 5. Observation of *O. tauri* cell division by transmission electronic microscopy. Cells from a 12:12 L/D synchronised culture fixed: (A) At the beginning of the light phase (3:00 h); (B) At 10:00 h, 2 h before dark. Arrows: division of the chloroplast; (C). At 12:00 h, at the light/dark transition. Arrows: division of the nucleus. (D) Culture blocked by propyzamide, arrow and AC: chromatin dense structures. s: starch; p: chloroplast; m: mitochondrion; n: nucleus.

and B, respectively, whereas *O. tauri* cyclin D mRNA showed a moderate expression all along the cycle with a decrease in early G1. Blocking with HU and propyzamide revealed a later expression of *Ot*CYCB compared to that of *Ot*CDKB. Histone H4 and 18S mRNAs were also detected as regulated and constant controls, respectively.

CDK accumulation, phosphorylation and activity during the division of O. tauri

Expression of CDK proteins is typically low and it is necessary to concentrate them before detection. This is usually carried out by affinity chromatography on CDK subunits (CKS) bound to sepharose beads (Azzi *et al.*, 1994). Only *Ot*CDKA binds to human CKS1 (p9), whereas both *Ot*CDKA and *Ot*CDKB bind to *A. thaliana* CKS1 (p10) (Stals *et al.*, 2000). This differential affinity towards p9 and p10 was used to separate *Ot*CDKA and B (Corellou *et al.*, 2005). Cellular extracts were first incubated with p9 to purify *Ot*CDKA until total depletion. Then, using the same extract, *Ot*CDKB was purified using p10 to obtain *Ot*CDKB alone. This protocol enabled the respective analysis of the expression, phosphorylation and kinase activity of these two proteins within the same sample. The detection of *Ot*CDKA as a doublet is probably due to isoforms. This doublet was still visible after an incubation of *Ot*CDKA with alkaline phosphatase indicating that it might be not linked to phosphorylation status of the protein (data not shown). Moreover, the use of different drugs showed distinct relative intensities of these two bands, without a clear correlation between *Ot*CDKA expression level and kinase activities throughout the cell cycle (Corellou *et al.*, 2005).

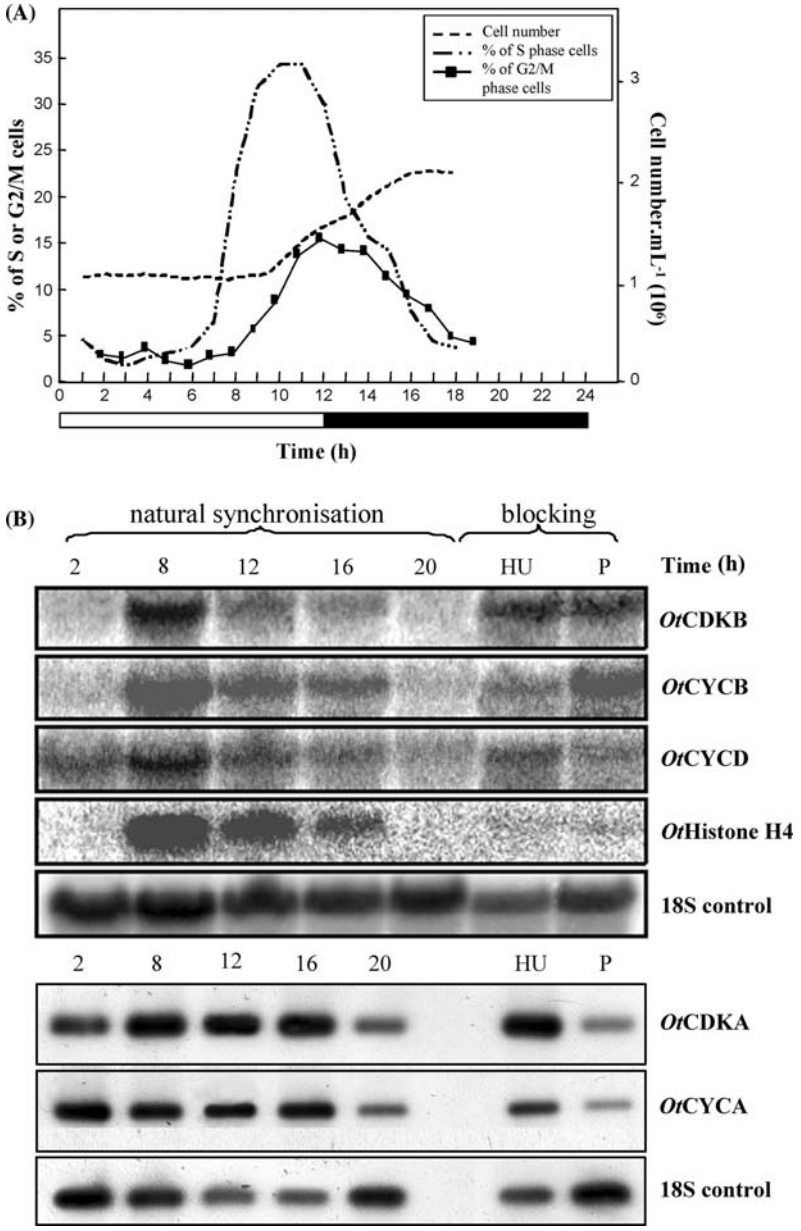

Figure 6. Expression of *O. tauri* CDK and cyclin mRNAs in a 12:12 L/D synchronised culture. (A) Growth and percentage of cells in the S or G2/M phase; (B) Northern blots of CDKB, Cyclins B and D, 18S rRNA and histone H4 genes; or RT-PCR of CDKA, CYCA and 18S rRNA.

Western blots revealed that *Ot*CDKA levels were constant throughout the cell cycle whereas *Ot*CDKB was present in S and G2/M phases and absent in G1 (Figure 7A). Using the commercial antibody PY99-HRP specific to tyrosine phosphorylation, no phosphorylation of *Ot*CDKA could be observed at any stage of the cell cycle whereas a strong signal was obtained for *Ot*CDKB, mainly in S/G2/M (Figure 7A). Cells treated with propyzamide were used to demonstrate that the epitope recognised on *Ot*CDKB is really phosphorylation dependent. The alkaline phosphatase was shown to dephosphorylate the Tyr phosphorylation revealed by PY99 antibody (Figure 7C). The different pattern of phosphorylation between *Ot*CDKA and *Ot*CDKB was confirmed in cells

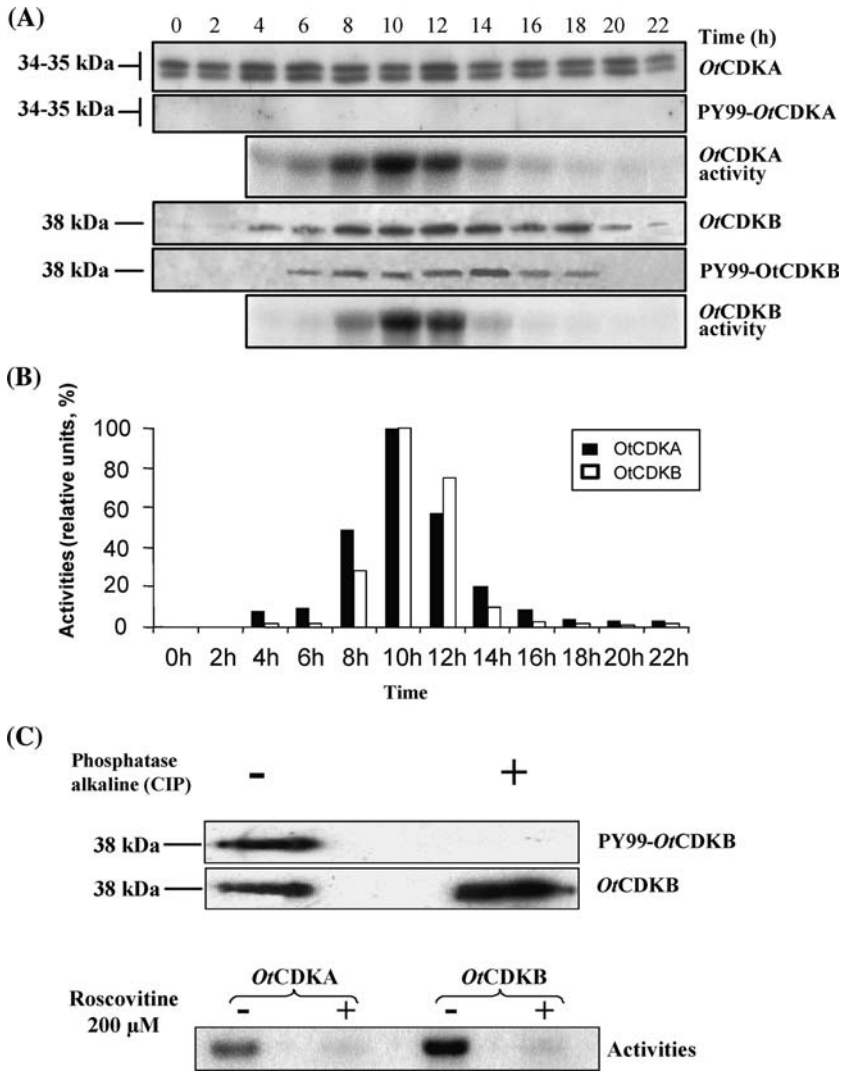

Figure 7. Protein expression, phosphorylation and kinase activity of *O. tauri* CDKA and CDKB. (A). Western blots showing *Ot*CDKA and *Ot*CDKB protein expression with time under a 12:12 L/D regime. Tyrosine phosphorylation of these two proteins is shown (lines PY99-*Ot*CDKA or B). Autoradiography of the histone H1 kinase activity of *Ot*CDKA and *Ot*CDKB; (B) Relative histone H1 activities of *O. tauri* CDKA and CDKB over a 24 h period; (C) Controls of the specificity of tyrosine phosphorylation using phosphatase alkaline treatment on *Ot*CDKB; and of *Ot*CDKs activities using a roscovitine treatment on *Ot*CDKA and B.

blocked by HU or by propyzamide (Figure 8). Non phosphorylated *Ot*CDKA accumulated at the same level in both naturally synchronised G1 cells and HU or propyzamide blocked cells. *Ot*CDKB accumulation was weak in HU blocked cells though highly phosphorylated (Figure 8). The expression of *Ot*CDKB started in early S and in HU treated samples (S/G2) a low accumulation of *Ot*CDKB with a strong phosphorylation was observed. In contrast, *Ot*CDKB greatly accumulated in propyzamide blocked cells but it was weakly phosphorylated (Figure 8). Supposing that *Ot*CDKB dephosphorylation is necessary for the completion of the G2/M transition, the decrease in tyrosine phosphorylation rate observed in propyzamide blocked cells corroborates this idea.

Histone H1 kinase activities of *Ot*CDKA and *Ot*CDKB were regulated during the cell cycle (Figure 7A,B). Kinase activities were carried out on dividing cells (after 12 h of light) and addition of roscovitine induced a strong inhibition of *Ot*CDKA and *Ot*CDKB activities (Figure 7C)

demonstrating that specific kinase activities are only due to *Ot*CDKA and B. *Ot*CDKA kinase activity, in contrast to its protein expression, was not constant, indicating a post-translational regulation of the enzymatic activity, although no tyrosine phosphorylation could be observed. Globally, both proteins showed a maximum of their kinase activity at 10–12 h, when cell division occurred. However, *Ot*CDKA activity appeared 2 h before the beginning of S phase, while *Ot*CDKB activity started 4 h later, at the G2/M phase.

Discussion

Synchronisation of eukaryote unicellular algae by light/dark cycles has been known for a long time, both in red (*Cyanidioschyzon*) and green (*Chlamydomonas*, *Chlorella*, *Euglena*, picoeukaryotes) algae, and most of them divide at the light/dark transition (Spudich and Sager, 1980; Donnan *et al*., 1985; McAteer *et al*., 1985; Yee and Bartholomew, 1988; Suzuki *et al*., 1994; Jacquet, 2001; Bisova *et al*., 2005). A similar synchronisation of cell division by light was observed in *Ostreococcus tauri*. While half of the cells divided at the end of the light phase, others divided in the beginning of the dark period when under a 12:12 L/D cycle. Our results also show that, although light was an absolute requirement to induce cell growth, the cell division itself could be light independent. For example, under a 6:18 L/D cycle, 70% of the total number of cells divided daily in the dark phase. This means that when a cell passes a commitment point, the division occurs independently of light. The progressive increase of the light period showed that this commitment point in our experimental conditions was between 4 and 6 h of light exposure. It can be estimated that in *O. tauri*, for a 12:12 L/D cycle, the G1 phase lasts around 21 h (15 h in darkness and 6 h in light), S phase about 2 h (the time observed between the beginning of S and the beginning of G2/M) and G2/M phases about 1 h (the time observed between the beginning of G2/M phases and the first cell divisions). This timing corresponds to a single cell, but as our synchronisation by light was only partial, the time frame necessary for all cells to complete the cycle was longer than the time necessary for one single cell to do the same. Interestingly, the S phase started around 7 h after light switched on, whereas the commitment point has been found between 4 to 6 h. Cells commit 1 to 3 h before S phase entry. The natural synchronisation obtained by L/D cycles was enough to distinguish cell cycle phases as demonstrated by mRNA analysis using the core cell cycle genes as markers (CDK and Cyclins). This synchrony is comparable to that reported for *A. thaliana* (Menges and Murray, 2002) which makes *O. tauri* an interesting model to study cell cycle progression.

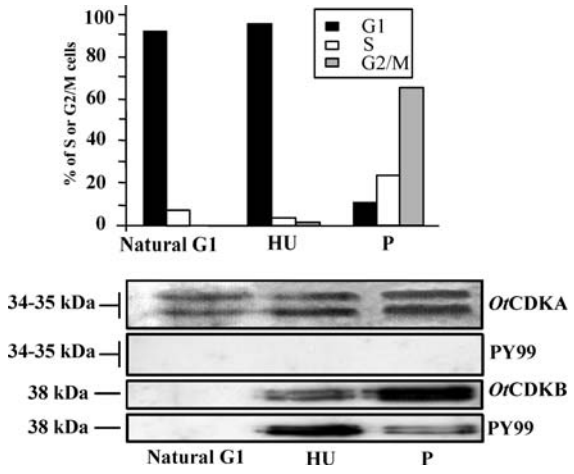

Figure 8. Protein expression and phosphorylation of *Ot*CDKA and *Ot*CDKB in *O. tauri* cultures blocked with hydroxyurea (HU) and propyzamide (P). Histogram shows the percentage of cells in the G1 phase, S phase and G2/M phases in naturally synchronised cells (natural G1) and in HU or P blocked cells. The corresponding protein expression and phosphorylation patterns are showed below for CDKA (*OtCDKA* and PY99 respectively) and CDKB (*OtCDKB* and PY99 respectively).

Few studies report on the synchronisation of the division of cell organelles compared to the nucleus. Plant cells usually possesses numerous chloroplasts and mitochondria and a clear sequence of division of the organelles remains difficult to be observed. Here we showed in *O. tauri* the division of the chloroplast followed by the nucleus. The kinetic of division of the mitochondrion remained uncertain, although our transmission electron microscopy observations suggest that it divides after the chloroplast and before the nucleus. A similar successive division of the organelles before the division of the nucleus has also been described for the red unicellular alga *Cyanidioschyzon merolae* (Suzuki *et al*., 1994).

The CDKA of plants is the ortholog of *S. pombe* cdc2, *S. cerevisiae* cdc28 or mammalian CDK1, and

contains a conserved PSTAIRE amino acid motif in the cyclin-binding domain. Only one copy of such a PSTAIRE CDK gene has been found in the *O. tauri* genome (Robbens *et al.*, 2005). As in plants, this gene shows both a constant expression throughout the cell cycle and a regulation of its kinase activity (Hemerly *et al.*, 1995; Sorrell *et al.*, 2001; Menges and Murray, 2002). The possibility of post-translational modification of *Ot*CDKA by sumoylation, or other polypeptide tag linkage can be hypothesised to explain the doublet in western analysis (Novatchkova *et al.*, 2004; Downes and Vierstra, 2005) although sulphation and glycosylation can not be excluded (Berger *et al.*, 1995; Lerouge *et al.*, 1998; Wilson, 2002). B-type CDKs are specific to plants and a CDKB ortholog is present in *O. tauri*. The four *A. thaliana* CDKBs are subdivided in two subgroups (PPTALRE motif for CDKB1 and PPTTLRE motif for CDKB2), each with two members. *O. tauri* possesses only one B-type CDK with a PSTALRE signature, which is intermediate between CDKA and CDKB. However, a careful phylogenetic analysis has shown that *Ot*CDKB is a true CDKB ortholog and not a modified CDKA (Robbens *et al.*, 2005). In contrast to CDKA, each subgroup of CDKB in *A. thaliana* possesses a specific expression pattern along the cell cycle, both at mRNA and protein levels (Segers *et al.*, 1996; Mironov *et al.*, 1999; Menges *et al.*, 2002; Menges *et al.*, 2003; Menges *et al.*, 2005). CDKB1 genes are expressed from S until G2/M phase whereas CDKB2 genes are present in G2/M and M phases. This is also true in alfalfa (cdc2MsD and cdc2MsF) and *Antirrhinum* (cdc2c and cdc2d) (Fobert *et al.*, 1996; Magyar *et al.*, 1997). *Ot*CDKB was not expressed in G1 phase, increased at the S phase entry and then decreased in M phase reaching non-significant levels in early G1 phase. This expression is very similar to that of the B1-type CDK described in plants (Segers *et al.*, 1996). Moreover, *Ot*CDKB gene sequence shows a KEN-box motif which is an APC recognition signal (-FLRE**KEN**RMAQ*) not yet described in plant CDKs (Pfleger and Kirschner, 2000; Capron *et al.*, 2003). *Ot*CDKB might activate the ubiquitin-proteasome pathway APC/C, the anaphase-promoting complex or cyclosome, which regulates the abundance of several proteins, and particularly during division for a normal progression in the cell cycle.

At the G1/S transition, the CDK activating kinase (CAK) activates CDKA by phosphorylation of the threonine-160 or a functionally equivalent residue, allowing conformational changes and the binding of a cyclin partner. Then, at the G2/M transition, an inhibitory phosphorylation of the amino-terminal residues threonine-14 and tyrosine-15 occurs under the action of the kinase WEE1 (Bell *et al.*, 1993; Zhang *et al.*, 1996; McKibbin *et al.*, 1998; Sun *et al.*, 1999; Sorrell *et al.*, 2002; De Veylder *et al.*, 2003). This inhibitory phosphorylation can be released by the dual-specific phosphatase Cdc25 or Cdc25-like as reported very recently in *A. thaliana* (Stals *et al.*, 2000; Dewitte and Murray, 2003; Landrieu *et al.*, 2004).

In plants, PSTAIRE CDK phosphorylation at the tyrosine residue in the S-G2-M phases is not well documented. Data are only available for *Fucus* zygote (brown alga) where, as in animals, tyrosine phosphorylation is a major mechanism involved in CDK regulation (Corellou *et al.*, 2001). The absence of tyrosine phosphorylation of CDKA in *O. tauri* throughout the cell cycle is surprising because, like in animals and plants, this tyrosine is conserved (Robbens *et al.*, 2005). This means that the regulation of the *Ot*CDKA kinase activity observed in our experiments is ensured either at another phosphorylation site (threonine or serine phosphorylation for example) or by another unknown post-translational modification. In contrast to *Ot*CDKA, *Ot*CDKB revealed a strong tyrosine phosphorylation/dephosphorylation throughout cell cycle. Phosphorylation occurred after the accumulation of the protein and stopped before its disappearance (Figure 6B). Although such a regulation by phosphorylation has been suspected for the plant CDKB at the G2/M transition, this has never been shown (Dewitte and Murray, 2003). This is probably due to the presence of several copies of both CDKB1 and 2 in plants which blurred the signals detected. To our knowledge this is the first organism in the green lineage which showed of CDKB tyrosine phosphorylation during cell cycle progression.

Little is also known about CDKB kinase activity in plants but it is presumed to be limited to the G2/M transition with a peak in mitosis (Boudolf *et al.*, 2004; Porceddu *et al.*, 2001; Sorrell *et al.*, 2001). We report here a similar cell cycle regulated *Ot*CDKB kinase activity, beginning later than *Ot*CDKA and occurring after the G1/S transition. However, the link between *Ot*CDKB phosphorylation and *Ot*CDKB activity remains

unclear. Actually the correlation between CDKB phosphorylation and its activity has not been studied yet in any organism. A peak of *Ot*CDKB activity can be seen at 10 h, when G2/M phase increases rapidly (a large proportion of cells are committed into M phase) with a theoretically decrease in *Ot*CDKB phosphorylation on tyrosine. This is not observed in Figure 7; time 10 h also corresponds to S phase peak, with an increase of *Ot*CDKB protein level and of tyrosine phosphorylation (Figure 6A: overlapping S-G2/M phases due to the natural synchronisation obtained), compensating global *Ot*CDKB phosphorylation decrease. Consequently, even with the observation of a rather constant level of phosphorylation, a peak of *Ot*CDKB activity can be expected. Tyrosine phosphorylation and kinase activity kinetics of CDKB in *O. tauri* suggested that, as in plants, *Ot*CDKB is supposed to control the M phase entrance via a tyrosine phosphorylation not yet described in plants. However, at 14 h, when CDKB activity decrease sharply, tyrosine phosphorylation was still observed. A similar observation has been reported by Mészáros and co-workers in alfalfa cells (2000). *Ot*CDKB possesses many potential tyrosine phosphorylation sites (27 tyrosine residues) and another tyrosine phosphorylation could not be excluded.

The role of *Ot*CDKA appears less clear, although the regulation of its kinase activity along the cycle indicates that it plays a role before *Ot*CDKB, probably at the G1/S transition. However, its participation in the G2/M transition in addition to *Ot*CDKB, cannot be excluded. In conclusion, *O. tauri* can be easily cultured and one binary division a day can be obtained under 12:12 L/D condition. This natural synchronisation, although being only partial, enables the study of the molecular control of its cell division. The natural synchronisation added to the compact nature of its genome, presenting the simplest set of genes known to regulate cell division, make *O. tauri* a very powerful model to study the regulation and the evolution of the cell division in the green lineage.

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