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RESEARCH PAPER

Effects of cyclin-dependent kinase activity on the coordination of growth and the cell cycle in green algae at different temperatures

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Abstract

The progression of the cell cycle in green algae dividing by multiple fission is, under otherwise unlimited conditions, affected by the growth rate, set by a combination of light intensity and temperature. In this study, we compared the cell cycle characteristics of *Desmodesmus quadricauda* at 20 °C or 30 °C and upon shifts between these two temperatures. The duration of the cell cycle in cells grown under continuous illumination at 20 °C was more than double that at 30 °C, suggesting that it was set directly by the growth rate. Similarly, the amounts of DNA, RNA, and bulk protein content per cell at 20 °C were approximately double those of cells grown at the higher temperature. For the shift experiments, cells grown at either 20 °C or 30 °C were transferred to darkness to prevent further growth, and then cultivated at the same or the other temperature. Upon transfer to the lower temperature, fewer nuclei and daughter cells were produced, and not all cells were able to finish the cell cycle by division, remaining multinuclear. Correspondingly, cells placed in the dark at the higher temperature divided faster into more daughter cells than the control cells. These differences correlated with shifts in the preceding cyclin-dependent kinase activity, suggesting that cell cycle progression was not related to growth rate or cell biomass but correlated with cyclin-dependent kinase activity.

Keywords: Cyclin-dependent kinase activity, *Desmodesmus quadricauda*, DNA replication, light/dark intervals, nuclear and cellular division, protein, RNA, starch accumulation, temperature.

Introduction

Growth and cell cycle progression are interconnected: progression of the cell cycle occurs only once the initial growth phase is complete and the cell has increased in size and accumulated sufficient key macromolecules, such as RNA and protein, and/or energy reserves. It is not clear which of these or other factors are sensed by the cell as determinants of reaching the growth threshold, although reaching the threshold before cell division is essential for cell survival, otherwise cell size would decrease with each division, leading to cell death. This understanding led to the proposal of the existence of a sizer mechanism interconnecting growth and cell cycle progression, allowing progression into the cell cycle only when the growth threshold has been met.

Some chlorococcal and volvocal algae divide by multiple fission, that is, 2^n daughter cells are released at the end of the

Abbreviations: CDK, cyclin dependent kinase; CP, commitment point; μ, growth rate in terms of number of doublings per hour.

cell cycle, where *n* is the number of doublings. This is caused by the occurrence of one or more (*n*) overlapping sequences of reproductive events, such as DNA replication (S phase), G2 phase, nuclear division (M phase), G3 phase, and cytokinesis (C), within one cell cycle. Each of these reproductive sequences is preceded by a growth phase leading mostly to an approximate doubling of cell mass, bulk RNA, and bulk protein per cell (for a review, see Bišová and Zachleder, 2014; Zachleder *et al.*, 2016). The dependence of the cell cycle on growth correlates with the attainment of a cell cycle commitment point (CP), which is functionally equivalent to START in yeasts (Reed, 1980) and the restriction point in mammalian cells (Pardee, 1974). Attainment of a CP marks the beginning of each reproductive sequence and separates it from the preceding growth phase. The cell cycle of the green alga *Desmodesmus quadricauda* (formerly *Scenedesmus quadricauda*) consists of 1, 2, or 3 reproductive sequences, according to the growth conditions (Zachleder and Šetlík, 1990), each starting at CP and leading to the duplication of DNA, mitosis, and cytokinesis (Fig. 1). Individual reproductive sequences during the multiple fission cell cycle overlap and are terminated by a common cell division.

In algae that divide by multiple fission, the number of reproductive sequences and the extent to which they overlap increases with the growth rate. For autotrophically grown cultures, the main factors affecting growth rate are light intensity (John, 1984; Zachleder and Šetlík, 1990; Vítová *et al.*, 2011b) and temperature (Šetlík *et al.*, 1975; Donnan *et al.*, 1985; Vítová *et al.*, 2011a). Increasing light intensity will speed up photosynthesis, growth processes, and the accumulation of bulk RNA and protein (Vítová and Zachleder, 2005), leading to the attainment of more CPs and an increase in the number of reproductive sequences occurring within one cell cycle. The cell size at CP—the critical cell size for division—remains the same, independent of the light intensity (John, 1984; Zachleder and Šetlík, 1990). Increasing temperature within the normal physiological range will have a similar effect, leading to increased growth and faster attainment of CP (Zachleder and van den Ende, 1992; Vítová *et al.*, 2011a). However, there is one crucial difference between the two factors. Changes in temperature will affect not just growth and the number of CPs attained but also the duration of the sequences of reproductive events, an effect that is independent of light intensity.

Cell cycle progression is governed by the activity of cyclin-dependent kinases (CDKs) in all eukaryotes, including green algae. CDK activity promotes the attainment of CP and mitosis (John *et al.*, 1989; Zachleder *et al.*, 1997; Bisova *et al.*, 2000, 2005). In the plant kingdom, cell cycle progression is regulated by two related CDKs, CDKA and plant-specific CDKB. CDKA is responsible for regulating the transitions from G1 to S phase and from G2 to M phase. CDKB is responsible specifically for regulating S phase and M phase (Francis, 2007). In

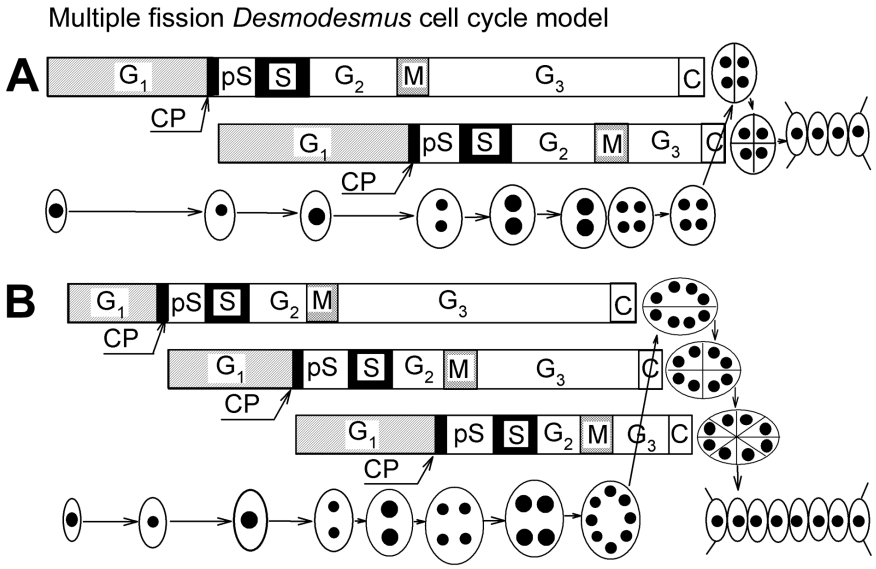

Fig. 1. Diagrams showing the multiple-fission cell cycle in the green alga *Desmodesmus quadricauda*. Two or three partially overlapping sequences of growth and reproductive events occur within a single cycle in cells dividing into four daughter cells (A) or eight daughter cells (B). Individual bars show the sequence of cell cycle phases. Schematic pictures of the cells represent their size during the cell cycle, and the black circles inside them illustrate the size and number of nuclei. Large black circles indicate a doubling of DNA. The diagonal lines extending from the terminal cells of *D. quadricauda* coenobia represent spines, typical for this species. G1, the phase during which the threshold size of the cell is attained. It can be termed a pre-commitment period because it is terminated when the commitment point is reached. CP, the commitment point. This is the stage in the cell cycle at which the cell becomes committed to triggering and terminating the sequence of reproductive events (e.g. DNA replication, nuclear and cellular divisions). pS, the pre-replication phase between the CP and the beginning of DNA replication. The processes required for the initiation of DNA replication are assumed to happen during this phase. S, the phase during which DNA replication takes place. G2, the phase between the termination of DNA replication and the start of mitosis. Processes leading to the initiation of mitosis are assumed to take place during this phase. M, the phase during which nuclear division occurs. G3, the phase between nuclear division and cell division. The processes leading to cell division are assumed to take place during this phase. C, the phase during which cell cleavage and formation of daughter cells occurs. (Modified after Zachleder *et al.*, 1997, Growth-controlled oscillation in activity of histone H1 kinase during the cell cycle of *Chlamydomonas reinhardtii* (Chlorophyta). Journal of Phycology 33, 673–681, with permission; and adapted by permission from Springer: The cell cycle of microalgae by Zachleder *et al.*, 2016, In: Borowitzka MA, Beardell J, Raven JA, eds. The physiology of microalgae. Cham: Springer International Publishing Switzerland, 3–46, Copyright 2016).

Chlamydomonas reinhardtii, another green alga that divides by multiple fission, CDKA promotes entry into the cell cycle, the attainment of CP, and initiation of the first DNA replication. CDKB is an essential CDK responsible for spindle formation and nuclear division, and subsequent rounds of DNA replication (Tulin and Cross, 2014; Atkins and Cross, 2018).

The role of light (light intensity, the illumination regimen, and light composition) in regulating the algal cell cycle is well established. The effect of temperature has been less studied because of the more complicated experimental design that is required, and investigations into the effect of changing temperature on cell cycle progression are lacking. Here, we studied the effect of temperature shifts on cell cycle progression in *D. quadricauda*. To separate the effects of temperature on growth processes and the cell cycle, we prevented further growth upon a shift in temperature by applying a dark treatment, which did not affect the cell cycle events that were already committed. This setup allowed us to independently compare the effects of temperature on growth (the synthesis of RNA, protein, and starch) and the cell cycle (DNA replication and nuclear and cellular divisions). We sought to determine whether relationships set up at one temperature would be retained after a temperature change, for example, whether there is a direct correlation between growth and cell cycle progression, as implied by the sizer hypothesis.

Materials and methods

Organism and culture methods

The chlorococcal alga *Desmodesmus quadricauda* (Turp.) Bréb., strain Greifswald/15, was obtained from the Culture Collection of Autotrophic Microorganisms kept at the Institute of Botany, Třeboň, Czech Republic. The cultures were maintained on mineral ½ SS medium as described elsewhere (Hlavová *et al.*, 2016).

Synchronization procedure and cultivation conditions

To obtain synchronous populations of daughter cells, optimal growth conditions were used: incident light intensity 420 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (400–720 nm), temperature 30 °C, and aeration with 2% added CO_2 (v/v). The use of a low concentration of cells (1×10^6 cells ml^{-1}) prevented a substantial decrease in the mean light intensity (264 µmol photons m^{-2} s^{-1}) due to a shadowing effect from growing cells. Synchronization was carried out by using 15/9 h alternating light/dark periods (Hlavová *et al.*, 2016).

Suspensions of synchronous cells were cultivated in rectangular plate-parallel vessels (44 × 24.5 × 2.3 cm, volume 2200 ml) or glass cylinders (inner diameter 3 cm, volume 300 ml) illuminated from one side by a panel of dimmable incandescent lamps (OSRAM DULUX L55W/950 Daylight) with incident irradiance at the surface of the culture vessel ~420 µmol photons m^{-2} s^{-1}. Culture vessels were immersed in water baths kept at a constant temperature of either 20 °C or 30 °C. The cultures were aerated with a mixture of air and carbon dioxide (2% v/v) at a flow rate of 15 l h^{-1} (for the tubes) or 60 l h^{-1} (for the plate-parallel vessels). Cells were counted using a Bürker counting chamber.

For the experiments, only cultures with at least 98% of eight-celled coenobia were used. Each experiment was repeated at least five times. Individual processes of the cell cycle were performed in the same time window, with the midpoints varying by a maximum of 1 h in the control cultures. In the samples placed in darkness, the progression of nuclear and cellular division depended on the actual state of the particular culture. Each of the experiments represented a time series of culture behavior in the light and dark, so the processes could be readily followed even if they were slightly delayed or advanced in the actual culture. The general rules governing culture behavior were the same and did not affect the experimental outcome.

Assessment of commitment, nuclear division, and cell division

To determine whether and how many cells attained CP and the number of CPs, cells were sampled at hourly intervals and incubated in the dark at the defined experimental temperature. Cells that had passed their CP for cell division formed daughter coenobia; the number of daughter cells per coenobium indicated the number of CPs passed by the mother cell (Vítová and Zachleder, 2005). The proportion of mother cells that formed four- and/or eight-celled daughter coenobia was determined by examination with a light microscope. The number of nuclei per cell was calculated after staining with 4,6′-diamidino-2-phenylindol (DAPI) according to Zachleder and Cepák (1987). Commitment, nuclear division, and cell division curves were obtained by plotting the cumulative percentages as a function of sampling time.

Temperature coefficient

The optimal temperature for *D. quadricauda* is 30 °C (Šetlík *et al.*, 1972) and 20 °C is within the physiological range for the alga. The temperature coefficient (Q_{10}) was calculated as the ratio of the growth rate in terms of number of doublings per hour (µ) or as the ratio of the duration of cell cycles or their phases (in h) for a given 10 °C change in temperature.

Microscopy

Observations by transmitted light and fluorescence microscopy were made using an Olympus BX51 microscope equipped with a charge-coupled device camera (F-ViewII). A U-MWIBA2 filter block (excitation/emission: 460–490/510–550 nm) was used for DAPI fluorescence.

Determination of total DNA, RNA, and protein

Total nucleic acids were extracted according to Lukavský *et al.* (1973). The DNA assay was carried out as described by Zachleder (1984). The sediment remaining after nucleic acid extraction was quantified for protein according to the procedure described by Lowry *et al.* (1951).

Determination of starch

A modification of the method of McCready *et al.* (1950) was used, as described previously (Brányiková *et al.*, 2011).

Inhibition of cyclin-dependent kinase activity

Roscovitine, a specific inhibitor of CDK (Planchais *et al.*, 1997), was added to synchronized cultures transferred into darkness at 30 °C after 8 hours of growth at 30 °C in light. Roscovitine was prepared from a 20 mM stock solution dissolved in DMSO to a final concentration of 50, 100, 200, or 400 µM.

Kinase assay

For each assay, the same number of cells from the same volume of culture was used; the cultures were not diluted during experiments. Cell pellets containing 2×10^7 cells were harvested during the cell cycle, washed with SCE buffer [100 mM sodium citrate, 2.7 mM sodium EDTA, adjusted to pH 7 with citric acid], snap frozen in liquid nitrogen, and stored at −70 °C. Protein lysates were prepared as described by Bisova *et al.* (2005). The cleared lysates were directly used for the assay or were affinity purified with CrCKS1 beads as described by Bisova *et al.* (2005), with the modification of a 2 h incubation at 4 °C as described by Hlavová *et al.* (2011). Histone H1 kinase activity was assayed as described by Langan *et al.* (1989) in a final volume of 10 µl with either 7 µl of cleared

whole-cell lysate or the CrCKS1 bead-purified fraction, corresponding to 20 µl of whole-cell lysate. The reactions were started by adding the master mix to a final composition of 20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.1 mM ATP, 0.2% (w/v) histone (Sigma H5505), and 0.370 MBq [γ ^{32}P] ATP. For the kinase inhibitor experiments, roscovitine was added directly to the master mix from a 20 mM stock in DMSO to a final concentration of 200 µM (Bisova *et al.*, 2005). All reactions were incubated for 30 min at room temperature and then stopped by adding 5 µl of 5×SDS sample buffer [250 mM Tris–HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 100 mM DTT, 0.5% (w/v) bromophenol blue], incubated for 2 min at 98 °C, and immediately cooled. Proteins were separated by SDS-PAGE in 15% gels (Laemmli, 1970). Phosphorylated histone bands were visualized by autoradiography and analyzed using a phosphoimager (Storm 860, Molecular Dynamics). The extent of phosphorylation was quantified using Image Studio Lite software (LI-COR Biosciences). To compare between samples and experiments, the sum of pixel intensity within the same area was normalized to the background pixel intensity to yield pixel intensity values of the signal. These were further normalized to the pixel intensity of histone bands in the gels stained with Coomassie Brilliant Blue. Resulting values are presented as the sum of pixel intensity and are comparable across experiments. Each experiment was repeated at least three times and representative experimental results are shown.

Results

In continuous light, growth is slower at 20 °C but cell cycle progression is similar at 20 °C and 30 °C

Before the experiment, cultures were grown for three consecutive cell cycles at 30 °C or 20 °C under a light/dark illumination regime and then left to grow in continuous light. At both temperatures, three consecutive CPs were attained by all cells (Fig. 2). Once committed, cells consecutively performed three nuclear divisions, becoming progressively binuclear, tetranuclear, and finally octanuclear (Fig. 2). Their cell cycle was terminated by the formation of eight-celled (octuplet) daughter coenobia (Fig. 2).

All pre-commitment and post-commitment phases, as well as the whole cell cycle (as measured at midpoints of corresponding curves; Fig. 2), were markedly longer at the lower temperature (Fig. 2B). The temperature coefficients (Q_{10}), calculated as the ratios of (i) growth rates (µ), (ii) the duration of pre- and post-commitment phases, and (iii) the duration of the whole cell cycle at the two temperatures, ranged from 1.5 to 4 (Table 1).

The Q_{10} values of the growth-related processes (RNA and protein accumulation) fluctuated around 2, suggesting that these processes were temperature controlled (Table 2). The Q_{10} of DNA replication varied from 1.56 to 2 (Table 2).

Daughter cells from cultures grown for several cell cycles at 20 °C (Fig. 3) contained two-fold higher levels of RNA, protein, and DNA at the beginning of the cell cycle than those grown at 30 °C (Fig. 3A, B, D, Table 2). Consequently, the relative content of these macromolecules at the end of the cell cycle was higher than in cells grown at 30 °C, and resulted in the release of larger daughter cells, although the number of daughter cells remained at eight. Cells grown at 20 °C contained two-fold higher amounts of DNA per nucleus (compare Figs 2B and 3D) and thus entered the cell cycle with duplicated genomic DNA.

The proportion of cells progressing through the cycle decreases upon a shift to a lower temperature

Cultures grown at 30 °C in the light were transferred after 4, 6, 8, and 14 hours to darkness and kept at the same temperature (Fig. 4A–D) or at 20 °C (Fig. 4E–H). Prolongation of light exposure at 30 °C caused a gradual increase in the fraction of the population that attained the first, second, and third CPs. In the dark at 30 °C, each attainment of CP was, in the corresponding fraction of the population, followed by the first, second, and third nuclear divisions (Fig. 4A–D) and then the release of the corresponding proportion of four- or eight-celled daughter coenobia.

Cells of the same culture transferred to the dark at 20 °C showed nuclear division later and in a lower proportion of cells than those committed in the light at 30 °C (compare Fig. 4A–D and E–H). The most remarkable effect of the transfer to a lower temperature in the dark was that tetranuclear cells were unable to finish their cell cycles by cellular division. Consequently, no four-celled daughter coenobia were formed

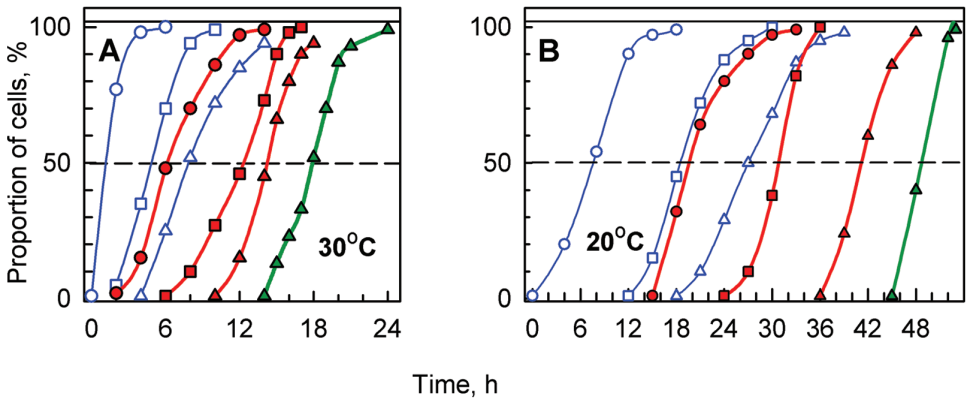

Fig. 2. Synchronized cultures of the green alga *Desmodesmus quadricauda* grown under continuous light. The synchronized cultures were grown at the same mean light intensity of 420 µmol photons m^{-2} s^{-1} and at 30 °C (A) or 20 °C (B). Blue lines: percentage of cells that attained the commitment point for the first (open circles), second (open squares), and third (open triangles) sequence of reproductive events. Red lines: percentage of cells that passed the first (closed circles), second (closed squares), and third (closed triangles) nuclear divisions. Green lines: percentage of cells that released eight daughter cells.

Table 1. *Growth rate, duration of the cell cycle and its phases, number of daughter cells, and temperature coefficients in synchronized populations of the alga* Desmodesmus quadricauda *grown at temperatures of 30 °C and 20 °C*

	μ	*Pre-CP1*	Pre-CP2	Pre-CP3	Post-CP1	Post-CP2	Post-CP3	CC	DC
20 °C	0.064	8	11	8	12	12	15	47	8
30 °C	0.166	2	4	3	6	8	8	18	8
Q_{10}	2.6	4	2.8	2.7	2	1.5	1.8	2.6	

μ, Growth rate in term of doublings per hour; CC, duration (h) of the cell cycle; pre-CP1, pre-CP2, pre-CP3, post-CP1, post-CP2, post-CP3, phases of the cell cycle (duration in h); DC, number of daughter cells; Q_{10}, temperature coefficient.

Table 2. *DNA, RNA, and protein content, and temperature coefficients at the start and end of the cell cycle and at the commitment points in synchronized populations of the alga* Desmodesmus quadricauda *grown at 20 °C and 30 °C*

	Start	End	CP1	CP2	CP3
RNA (pg cell^{-1})					
20 °C	8	55	15	30	45
30 °C	4	22	8	15	23
Q_{10}	2	2.5	1.88	2	1.96
Protein (pg cell^{-1})					
20 °C	50	400	70	150	300
30 °C	25	175	30	60	140
Q_{10}	2	2.28	2.33	2.5	2.14
DNA (pg cell^{-1})					
20 °C	0.4	3.5	0.8	1.5	2.5
30 °C	0.2	1.6	0.4	0.8	1.6
Q_{10}	2	1.56	2	1.88	1.56

CP1, CP2, CP3, First, second, and third commitment points (at midpoints of commitment curves; Q_{10}, temperature coefficient for a given 10 °C change in temperature as the ratio of compound content in pg cell^{-1} for the two temperatures.

in the dark, and only cells whose nuclei divided into eight completed their cell cycle by division into eight-celled daughter coenobia (Fig. 4E–G; see also Fig. 5B).

The proportion of cells progressing through the cycle increases upon a shift to a higher temperature

Cultures grown at 20 °C in the light were transferred after 15, 21, 27, or 39 h of growth to darkness and kept at the same temperature (Fig. 5A–D) or at 30 °C (Fig. 5E–H). Growth processes at 20 °C were slower than at 30 °C (Table 2), which led to a slower and more temporally spread attainment of CPs (compare the time distances between the curves of attaining individual CPs and their steepness in Figs 4 and 5). Similarly, the rate of nuclear division was slower both in the light and dark, due to the cells' slower metabolism at the lower temperature (compare the time distances between the curves of individual nuclear divisions in Figs 4 and 5).

After transfer to the dark at 21 h, similar to the situation for transfers from 30 °C to 20 °C, only some of the cells that underwent nuclear division into four nuclei also divided into four-celled daughter cell coenobia (Fig. 5B).

For cultures grown in light for 27 and 39 h (Fig. 5C, D), the number of cells able to undergo nuclear division increased, reaching 100% of the cell population (Fig. 5D). In contrast to the cultures transferred to darkness after shorter light intervals, all cells that were committed to division of their nuclei into four or eight completed their cell cycles, dividing into four- or eight-celled daughter coenobia (Fig. 5C, D).

When the cells grown in light at 20 °C were transferred to darkness at 30 °C, there was a significant increase in the rate and proportion of nuclear and cellular divisions within the population relative to cultures transferred to darkness at 20 °C (compare Fig. 5A–C and 5E–G). The cells underwent more nuclear and cellular divisions than were committed to in the light at 20 °C, and this took place over a shorter time interval. All cells that completed at least two nuclear divisions into four nuclei completed their cell cycles by cell division (compare Fig. 5A–D and 5E–H or Figs 7 and 8). The cells transferred into the dark at 30 °C after 39 h in the light showed nuclear division into eight nuclei and released eight-celled daughter coenobia, similar to cells transferred into the dark at 20 °C (Fig. 5H). However, 30% of the newly born daughter cells underwent a nuclear division, becoming binuclear (Fig. 5H). This nuclear division was part of the next cell cycle. No such additional nuclear division occurred in the cells kept in the dark at 20 °C (Fig. 5D).

Rates of macromolecular synthesis vary with temperature

In separate experiments, the synthesis of macromolecules related to cell growth (total RNA and protein), the accumulation of energy reserves (starch), and cell reproduction (DNA replication) were followed at 30 °C in cells grown in the light and then transferred after 9 or 13 h to darkness, where they were kept at 30 °C or 20 °C (Fig. 6A–D). In another set of experiments, cultures grown at 20 °C were transferred to the dark after 10 or 20 h and kept at 20 °C or 30 °C (Fig. 6E–H).

RNA and protein accumulated during the continuous light period until values corresponding to the number of daughter cells formed at the end of the cell cycle were attained (Table 2). For cultures grown at 30 °C or 20 °C in the light, there was no significant difference in the accumulation of RNA (Fig. 6A, E) or protein (Fig. 6B, F), or the degradation of starch (Fig. 6C, G) when transferred to either 20 °C or 30 °C in the dark. Starch degradation started immediately after transfer to the dark regardless of the temperature at which cells had been maintained in the light, but was slightly faster in cells transferred to 30 °C than 20 °C (Fig. 6C, G).

DNA replication was faster in the dark at 30 °C than 20 °C (Fig. 6D, H). The final DNA content in cultures transferred to the dark later in the cell cycle was comparable between the two treatments (Fig 6D). In contrast, in cultures transferred to the dark earlier in the cell cycle, the final DNA content per cell

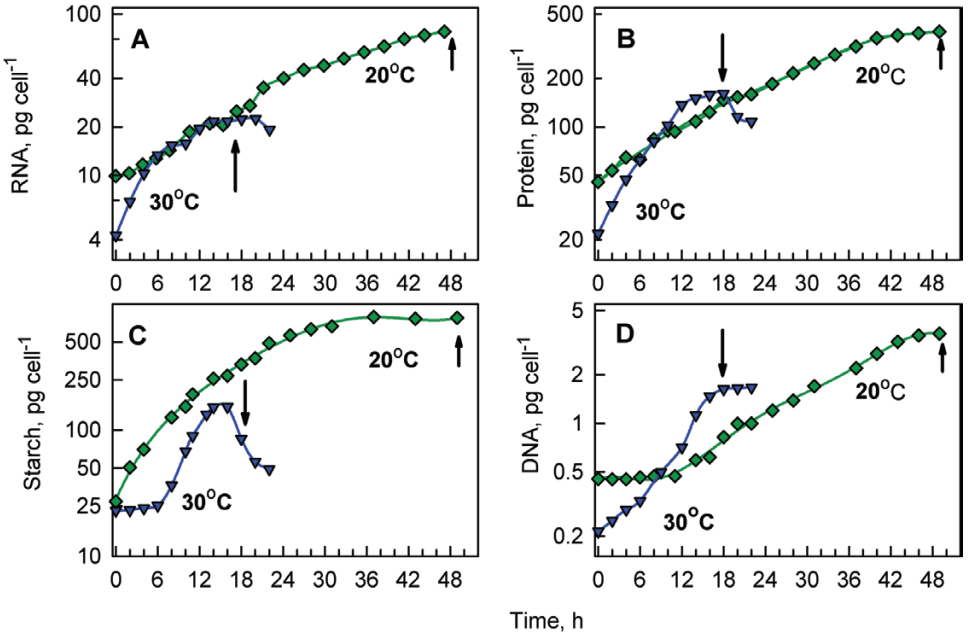

Fig. 3. Changes in the contents of RNA (A), protein (B), starch (C), and DNA (D) in synchronized populations of the alga *Desmodesmus quadricauda* grown at different temperatures. The cultures were grown in continuous light with a mean light intensity of 420 µmol photons m^{-2} s^{-1} at either 30 °C (triangles) or 20 °C (diamonds). Arrows indicate the time midpoints of cellular division.

was about 1.5 times higher at 30 °C than at 20 °C (Fig 6D). Faster and more pronounced DNA replication was a prerequisite and correlated with the faster and more numerous nuclear divisions observed in cultures transferred to 30 °C in the dark (Figs 4, 5, 7, 8).

Cell cycle progression correlates with CDK activity

Differences in cell cycle progression upon transfer from one temperature to the other could not be explained by changes in any macromolecule measured, suggesting that the levels of these macromolecules levels were not critical. We therefore determined whether the onset of activity of the main cell cycle regulator, CDK, was correlated with total RNA or bulk proteins as determinants of critical cell biomass, or with cell cycle processes being performed. We analyzed two types of kinase activities present in whole-cell lysates, activity relating to the attainment of CP and to nuclear division, and activity purified by affinity to CrCKS1 protein, relating to nuclear division (Bišová *et al*., 2000). Comparison of the two activities revealed that, in most cases, they overlapped, so that CKS1-bound kinase activity was 'hidden' within the whole-cell lysate activity, although it was about 10-fold lower (compare Supplementary Fig. S1 at *JXB* online with Figs 7 and 8). The only difference was a more prominent peak of CKS1-bound activity preceding cellular division(s) (Supplementary Fig. S1), which was very low or absent in the whole-cell lysates. In both the whole-cell lysate and CKS1-bound fraction, kinase activities were inhibited by roscovitine (Supplementary Fig. S2), a specific inhibitor of CDK (Planchais *et al*., 1997), confirming that they represent CDK-like activities.

The activities of whole-cell lysates were assayed in aliquots of synchronized populations grown in light at 30 °C (Fig. 7) or 20 °C (Fig. 8), and subsequently transferred to darkness at either the same temperature or to the lower or higher temperature (i.e. 20 °C or 30 °C, respectively). The activities of CDK before and after transfer into darkness were compared with changes in nuclear and cellular division(s). Individual synchronized cultures differed slightly (see Materials and methods), which led to slight variations in the timing of CDK activity between individual experiments, but the correlations described below were maintained.

The rate of change and level of CDK activity decreases upon a shift to darkness at a lower temperature

For cultures grown at 30 °C, subpopulations of synchronized cultures were transferred into darkness at 30 °C or 20 °C after 4, 6, 8, 10, or 14 h of illumination (Fig. 7).

As described above, with prolonged light exposure at 30 °C, the number of committed cells as well as the number of attained CPs increased, followed by an increase in the proportion of cells undergoing nuclear division (Fig. 7A–E). In all experimental variants, nuclear divisions were tightly coupled with and related to variations in CDK activity (Fig. 7; Supplementary Fig. S3). In the dark at 30 °C, individual nuclear divisions followed each other within a short time interval (Fig. 7B, C), concomitant with a sharp peak of CDK activity. These peaks related to the start of individual nuclear divisions and overlapped in time (Fig. 7B, C, D).

In the cultures grown in 30 °C and then transferred to darkness at 20 °C, the numbers of cells undergoing nuclear division into two, four, or eight nuclei during the dark period were lower than those committed in the light at 30 °C and then undergoing nuclear division in the dark at 30 °C (Fig. 7F–J). The level of CDK activity after transfer from 30 °C in the light to 20 °C in darkness (Fig. 8F–I) was lower, and the peaks were broader than those measured in cells maintained in darkness at 30 °C. This was in line with the slower rate and lower proportion of

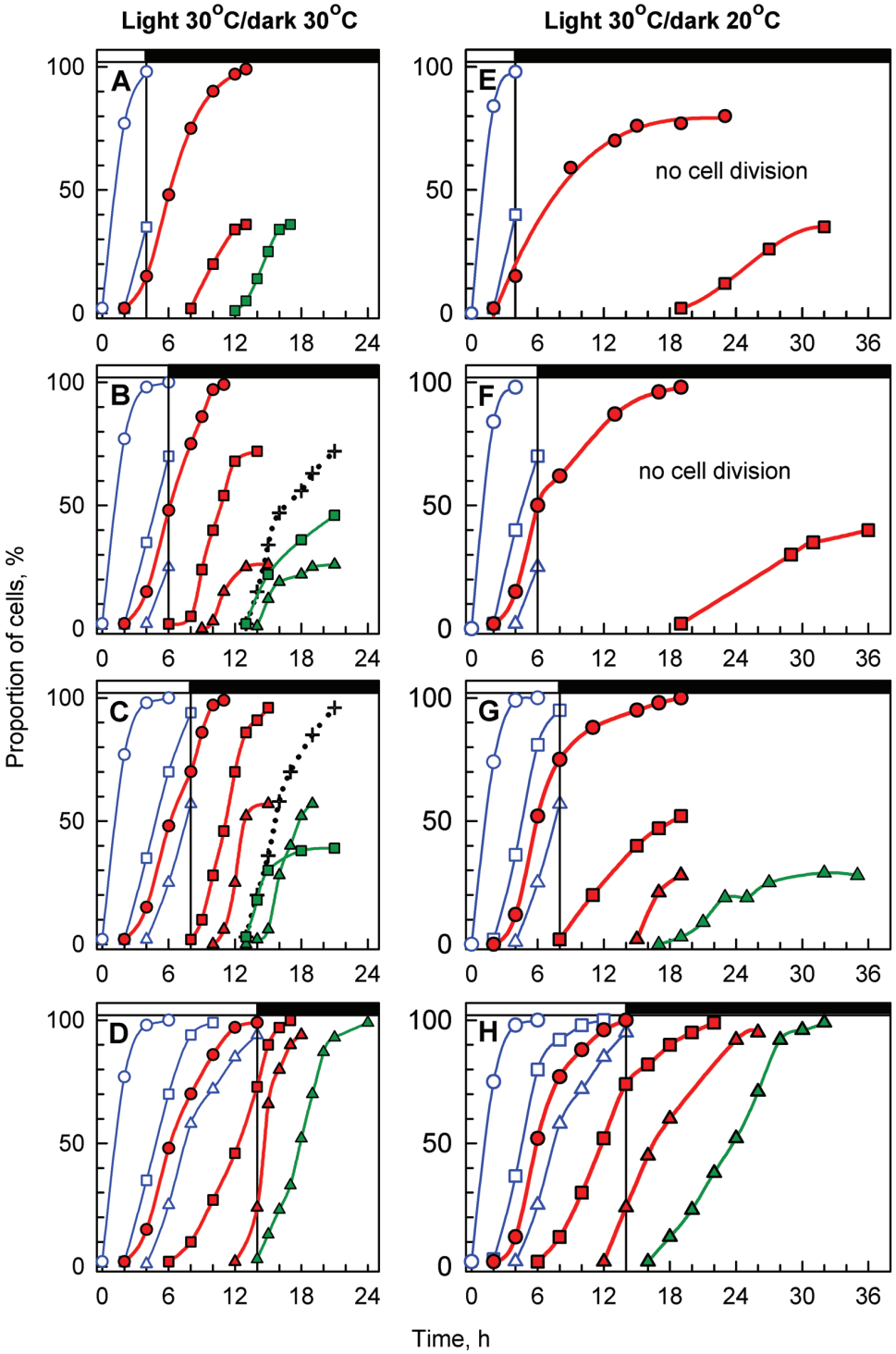

Fig. 4. Time courses of individual commitment points, nuclear divisions, and cellular divisions in synchronized populations of *Desmodesmus quadricauda* grown in light at 30 °C and then placed into the dark after 4, 6, 8, or 14 h and kept at 30 °C (A–D) or 20 °C (E–H). Blue lines: percentage of cells that attained the commitment point for the first (open circles), second (open squares), and third (open triangles) sequence of reproductive events. Red lines: percentage of cells that passed the first (closed circles), second (closed squares), and third (closed triangles) nuclear divisions. Green lines: percentage of cells that completed the second (closed squares) and third (closed triangles) cellular divisions and released four or eight daughter cells. Dotted black lines: percentage of all cells that produced daughter cells (crosses). Periods of dark are marked with black bars at the top of each graph.

nuclear divisions in populations at 20 °C (compare Fig. 8A–D and 8F–J; see also Figs 4, 5, 7, 8). As at 30 °C, the peaks of CDK activity were concomitant with nuclear divisions. Thus, at both 30 °C and 20 °C, the timing of the increase in CDK activity correlated with the timing and proportion of nuclear divisions in the populations, even though there were significant differences between the cultures placed into darkness at the two temperatures. To visualize the correlation better, we used cumulative time-course values of kinase activities and compared them with the cumulative number of nuclei per cell over time

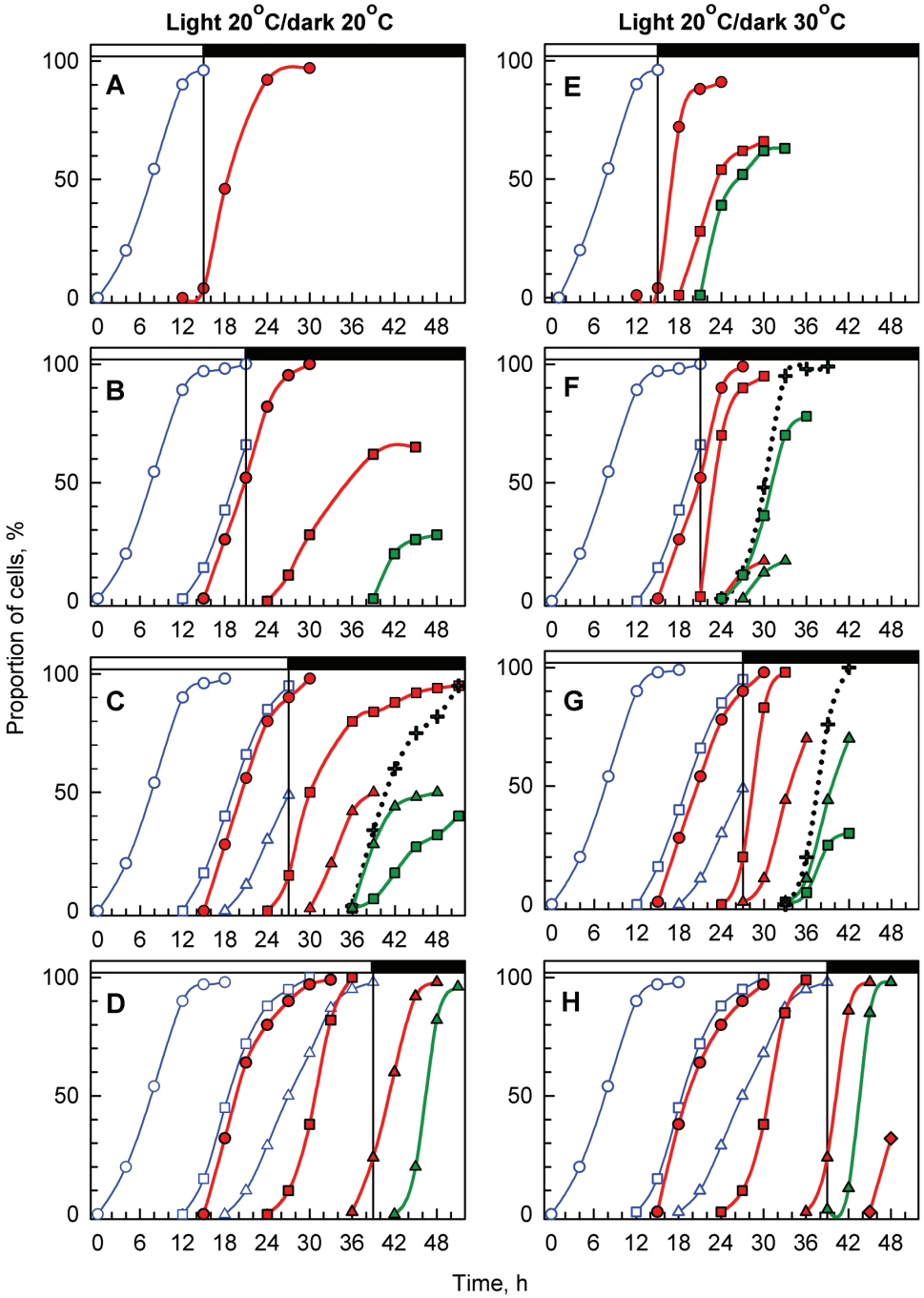

Fig. 5. Time courses of individual commitment points, nuclear divisions, and cellular divisions in synchronized populations of *Desmodesmus quadricauda* grown in light at 20 °C and then placed into the dark after 15, 21, 27, or 39 h and kept at 20 °C (A–D) or 30 °C (E–H). Blue lines: percentage of cells that attained the commitment point for the first (open circles), second (open squares), and third (open triangles) sequence of reproductive events. Red lines: percentage of cells that passed the first (closed circles), second (closed squares), and third (closed triangles) nuclear divisions; daughter cells that underwent additional nuclear division are indicated with diamonds. Green lines: percentage of cells that completed the second (closed squares) and third (closed triangles) cellular divisions and released four or eight daughter cells. Dotted black lines: percentage of all cells that produced daughter cells (crosses). Periods of dark are marked with black bars at the top of each graph.

(Supplementary Fig. S3). This type of plot clearly showed that CDK activity preceded nuclear division, which was proportional to the levels of CDK activity (Supplementary Fig. S3).

The rate of change and level of CDK activity increases upon a shift to darkness at a higher temperature

Due to the slower cell metabolism at 20 °C, the cell cycle was more than twice as long as at 30 °C (Fig. 2). Because of this, to transfer cells grown at 20 °C in light to the dark at a similar cell cycle time, the light period had to be prolonged. Cultures grown at 20 °C were transferred into the dark (at 30 °C or 20 °C) after 15, 21, 27, or 39 h (Fig. 8). The first division of nuclei occurred later in the culture grown at 20 °C than at 30 °C (20 h versus 9 h) and the following nuclear divisions were further apart at 20 °C (12 h versus 4 h) (compare Figs 7E and 8E).

As for the cells grown at 30 °C, the activity of CDK increased before nuclear division (Fig. 8; Supplementary Fig. S3), and

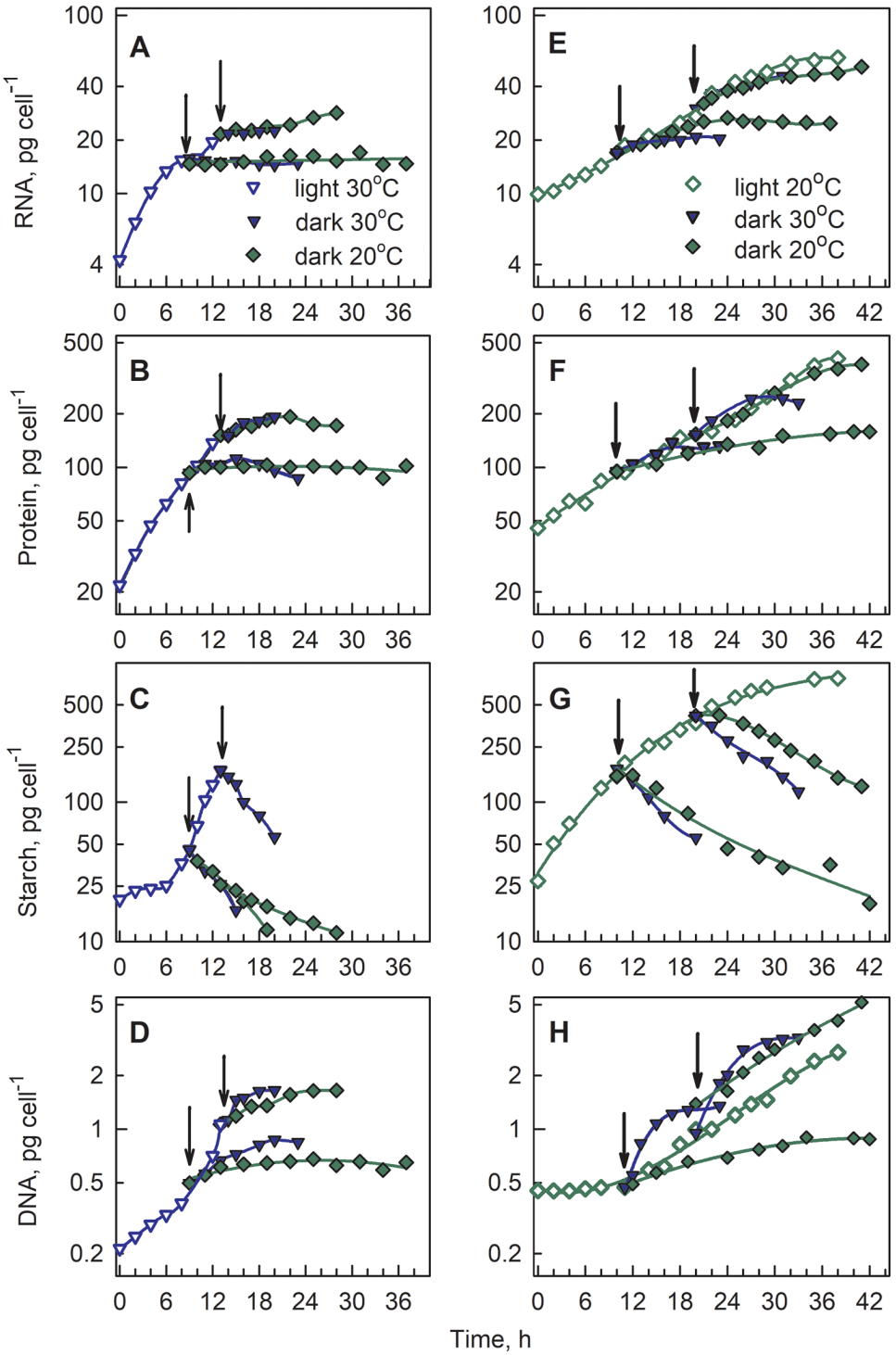

Fig. 6. Changes in the content of RNA (A, E), protein (B, F), starch (C, G), and DNA (D, H) during the cell cycle in synchronized populations of *Desmodesmus quadricauda*. The cultures were grown at 30 °C (A–D) or 20 °C (E–H). (A–D) Macromolecular synthesis in cells permanently illuminated at 30 °C (blue line, open triangles) or placed in darkness after 9 or 13 h (arrows) and kept at 30 °C (blue line, closed triangles) or 20 °C (green line, closed diamonds). (E–H) Macromolecular synthesis in cells permanently illuminated at 20 °C (green line, open diamonds) or placed in darkness after 10 or 20 h (arrows) and kept at 20 °C (green line, closed diamonds) or 30 °C (blue line, closed triangles).

the level and timing of CDK activity correlated with the number of cells undergoing nuclear division and the number of nuclear divisions in individual cells (Fig. 8). In cells cultivated in the light at 20 °C, CDK activity was delayed compared with those at 30 °C, and its peaks were more separated in time, corresponding with individual nuclear divisions (Fig. 8A). With prolongation of the light period to 21 h or more, the activities of CDK preceding the first and second nuclear divisions started to overlap, resulting in a broader distribution of CDK activity with two peaks (Fig. 8B–E). In contrast to cultures grown in the light at 30 °C, division into eight nuclei in cultures grown at 20 °C was in all cases preceded by a peak of

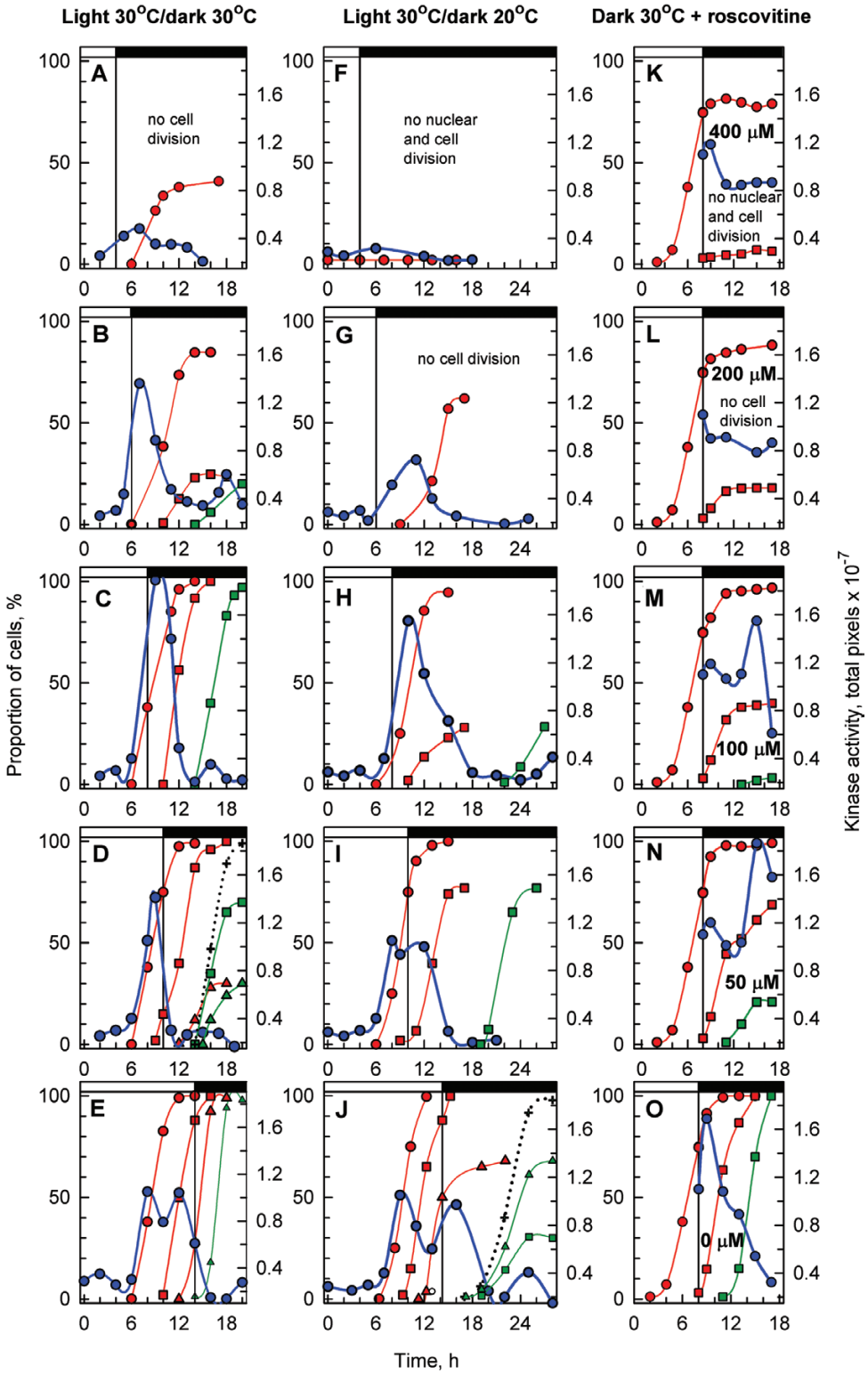

Fig. 7. Activity of CDK in whole-cell lysates and the courses of nuclear and cellular divisions in synchronized populations of *Desmodesmus quadricauda* grown in light at 30 °C and then placed into darkness after 4, 6, 8, 10, or 14 h, and kept at 30 °C (A–E) or 20 °C (F–J). The specific inhibitor of CDK activity roscovitine at different concentrations (50–400 µM) (K–N) or DMSO as a control (O) were applied to cultures transferred to darkness at 30 °C after 8 h of light. Periods of dark are marked with black bars at the top of each graph. Blue lines: activity of CDK. Red lines: percentage of cells that passed the first (closed circles), second (closed squares), and third (closed triangles) nuclear divisions. Green lines: percentage of cells that completed the second (closed squares) and third (closed triangles) cellular divisions and released four or eight daughter cells. Dotted black lines: percentage of all cells that produced daughter cells (crosses).

CDK activity that was well separated in time from preceding peaks (compare Figs 7 and 8).

Transfer of cells grown at 20 °C into darkness at 30 °C caused extensive changes in the timing of cell cycle processes, which were correlated with changes in CDK activity. As a rule, nuclear divisions occurred shortly after transfer to 30 °C, at a higher rate and in more cells than in cells kept 20 °C (compare Fig. 8A–D and 8F–I). CDK activity reached a sharp peak

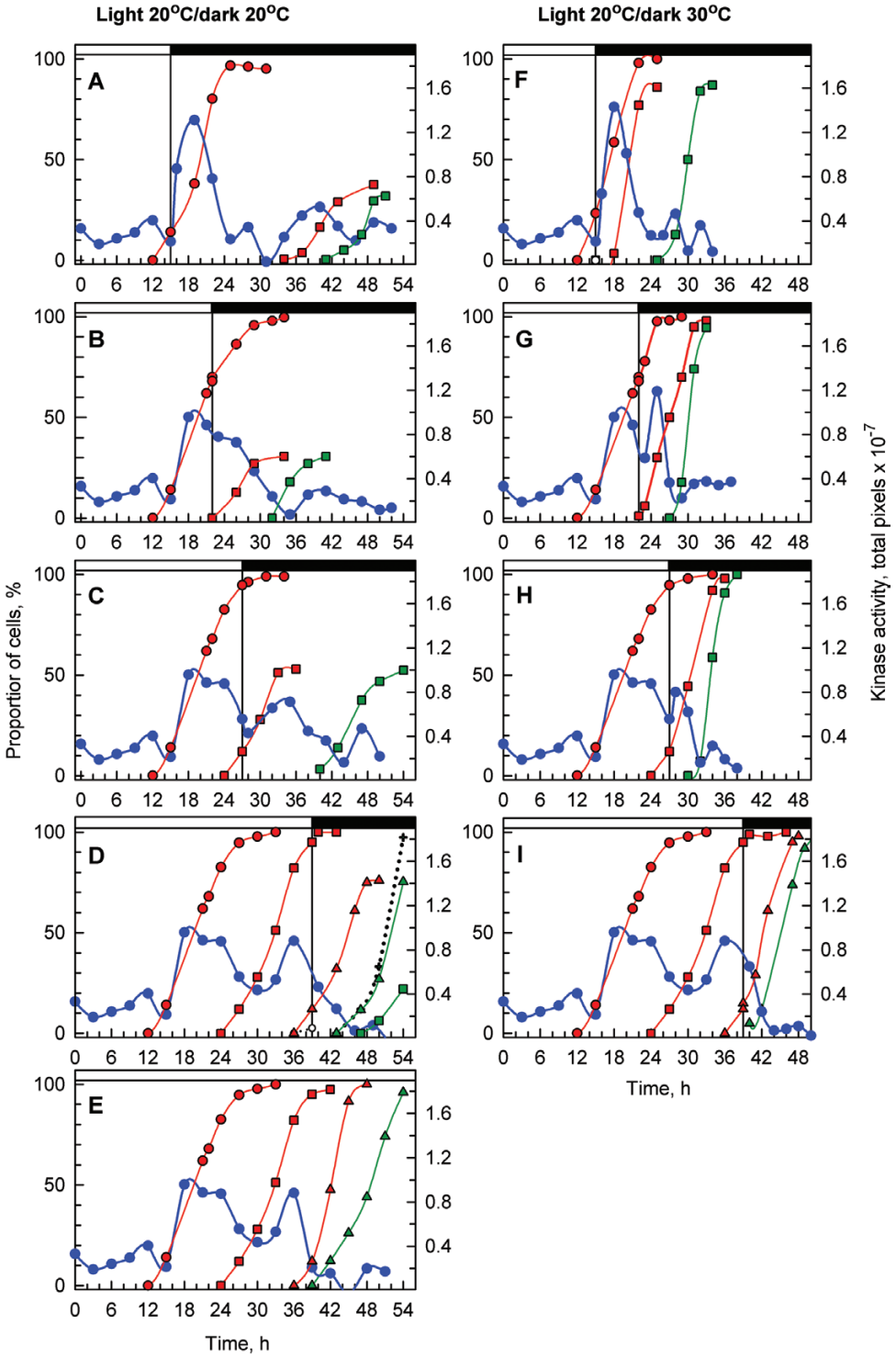

Fig. 8. Activity of CDK in whole-cell lysates and the courses of nuclear and cellular division in synchronized populations of *Desmodesmus quadricauda* grown in light at 20 °C and then placed into darkness after 15, 22, 27, or 39 h and kept at 20 °C (A–D) or 30 °C (F–I). One culture was grown at 20 °C in continuous light (E). Blue lines: activity of CDK. Red lines: percentage of cells that passed the first (closed circles), second (closed squares), and third (closed triangles) nuclear divisions. Green lines: percentage of cells that completed the second (closed squares) and third (closed triangles) cellular divisions and released four or eight daughter cells. Dotted black lines: percentage of all cells that produced daughter cells (crosses). Periods of dark are marked with black bars at the top of each graph.

within 1 or 2 h after transfer to darkness at 30 °C (Fig. 8G, H) while such sharp peaks were absent in cultures transferred to darkness at 20 °C (compare Fig. 8B, C, G and H). Kinase activity maxima were higher at 30 °C than at 20 °C, and the peaks were sharper, in line with faster completion of nuclear division. The lower kinase activities at 20 °C correlated with the slower completion of nuclear and/or cell divisions and the lower proportion of cells in the population undergoing nuclear and/or cell division. For another illustration of the correlation, see Supplementary Fig. S3.

To determine whether there were temperature-dependent differences in total kinase activity, we compared the cumulative CDK activity at the two temperatures with the accumulated number of nuclei per cell (Supplementary Fig. S3). The results clearly demonstrate that although the rates of change of CDK activity were slower at 20 °C, total CDK activity correlated only with the number of completed nuclear and/or cellular divisions within the population, and not with temperature (Supplementary Fig. S3). In all cases, CDK activity correlated with nuclear divisions, confirming a tight connection between CDK activity and cell cycle progression.

Inhibition of CDK activity by roscovitine simulates its decrease through transfer to lower temperature

To assess whether the correlation between CDK activity and changes in cell cycle progression was causative or not, we transferred subcultures of synchronized cultures grown in light at 30 °C for 8 h into darkness with increasing concentrations of the CDK inhibitor roscovitine (Fig. 7K–O). In control cultures without roscovitine, all the cells underwent nuclear division into four nuclei and completed cell division into four-celled coenobia (Fig. 7O, compare also with Fig. 7C). Increasing concentrations of roscovitine progressively inhibited nuclear and cellular division as well as kinase activities in whole-cell lysates (Fig. 7K–N). With the highest concentration of roscovitine (400 μM), CDK activity was completely blocked, which was reflected by a complete block of nuclear division and cell division (Fig. 7K). The effect of the inhibitor mimicked the effect of shorter light exposure (compare Fig. 7M with 7B and 7C). This proved a causal relationship between changes in CDK activity at different temperatures and cell cycle progression, as was suggested by the correlations illustrated in Figs 7 and 8 and Supplementary Fig. S3.

Discussion

In green algae dividing by multiple fission, the critical cell size at which the cells attain CP remains constant at different growth rates set by different light intensities. The rate of attaining the critical cell size and the number of CPs vary, but the duration of the reproductive sequences is set by the metabolic rate and remains constant (Vítová *et al*., 2011*b*). This is true not only in algae but also in yeasts (Beach *et al*., 1982), mammalian cells (Pardee, 1989), and plant cells (Jones *et al*., 2017). In contrast, temperature affects both growth-related processes before CP attainment and the duration of the sequence of reproductive events, even at the same light intensity (Fig. 2). This stems from a temperature effect on general metabolism and fits with established knowledge about temperature responses in other algae (Zachleder *et al*., 2002; Vítová and Zachleder, 2005).

Results of experiments performed at 20 °C and 30 °C suggested that threshold levels of the macromolecules assumed to be involved in establishing a constant critical size (RNA, protein, and starch) were doubled at 20 °C compared with 30 °C. The question was whether this threshold requirement for entry into CP would be retained after transfer between two different but physiological temperatures (either from 20 °C to 30 °C or from 30 °C to 20 °C) when the cells are placed into darkness to block any direct involvement of light on growth. Should there be a direct relationship between critical cell size (and/or its determinants) and attainment of CP, we expected that progression of the cell cycle would continue after transfer, similar to the control culture maintained at the original temperature, although with different kinetics dictated by different metabolic rates at the two temperatures. In contrast, if critical cell size and CP attainment were not simply correlated, then upon transfer the progression of the cell cycle should differ from that of the control at the original temperature. The results of the transfer experiments rather unexpectedly proved the second of these options. The role of temperature in the timing of cell cycle events thus goes beyond its effect on growth rate, in contrast to changing the light intensity at a constant temperature, which has a simple trophic effect (Zachleder and Šetlík, 1990).

Upon transfer to the dark, at either 30 °C or 20 °C, the accumulation of RNA and proteins ceased, and their levels stayed constant (Fig. 6A, B, E, F). Starch was extensively degraded in the dark at rates that were faster at 30 °C, but the final levels of starch were similar at both temperatures (Fig. 6C, G). In general, all growth processes that depend directly on a carbon and energy supply stopped or substantially slowed upon transfer to the dark. In contrast, processes essential for cell survival and reproduction, particularly DNA replication and nuclear division, are not directly dependent on energy from photosynthesis; even in the light they depend on starch (and other) reserves, and thus they can be performed in the dark (Wanka, 1968, 1975). Although general protein synthesis slows or ceases in the dark, this does not affect gene expression and protein synthesis related to cell cycle events, which still run in the dark. If protein synthesis is completely inhibited by the addition of cycloheximide, DNA replication and nuclear division are completely suppressed (Zachleder *et al*., 2002).

The accumulation of DNA was comparable for cultures grown at 30 °C and then moved to the dark at either 30 °C or 20 °C (Fig. 6D). In contrast, there was a striking difference in DNA accumulation in cultures grown at 20 °C and then placed into darkness at either 30 °C or 20 °C. In the cultures placed into darkness at 30 °C, DNA accumulation started earlier, was faster, and could have reached higher values (Fig. 6H). This result suggested that there might be different effects of a temperature shift on the progression of the cell cycle. Generally, cell cycle progression was accelerated in cultures transferred from the light at 20 °C to darkness at 30 °C. The cells behaved as if they attained more CPs; they underwent more nuclear divisions and completed more cell divisions. Some of these cells completed cell divisions even under conditions where no cell division was detectable in the control cultures kept at 20 °C (Figs 5, 7, 8). The opposite behavior was observed in cultures grown in the light at 30 °C and transferred to the dark at 20 °C. Cells in these cultures were unable to complete nuclear or cell divisions committed at 30 °C (Fig. 4). The increased rate of individual cell cycle processes at 30 °C and the slower rate at 20 °C were expected and were most probably caused simply by different metabolic rates at the two temperatures, as reflected by the temperature coefficient, Q_{10} (Table 1). In contrast, the fact that cells seemingly attained more or fewer

CPs than implied by their critical cell biomass at the culture temperature suggested that the cells are at different stages in the cycle, independent of cell size. This also implies that neither critical cell size (biomass) nor any other macromolecule we analyzed is a determinant of CP. Thus, the two processes—that is, growth up to critical cell biomass and attainment of CP—are simply correlated under stable growth conditions but are not causally related.

CDK activity changed distinctly with temperature shifts, preceding both temporally (Figs 7, 8; Supplementary Fig. S3) and functionally (Fig. 7K–O) nuclear and to some extent cellular divisions in the cultures and determining the progression of division. The completion of each nuclear division correlated with cells reaching certain threshold total CDK activity levels, which were similar at 30 °C and 20 °C (Figs 7, 8; Supplementary Fig. S3), although reaching the threshold activity was delayed at 20 °C. Furthermore, the application of increasing concentrations of roscovitine, a specific CDK inhibitor (Planchais *et al.*, 1997), altered the extent of cell cycle progression, confirming that alternating CDK activity levels control cell cycle progression. In some organisms, CDK activity seems to be related to cell size. In plants, a model incorporating an increase in active CDK with cell size fits the behavior of dividing cells in the apical meristem (Jones *et al.*, 2017). In *C. reinhardtii*, the abundance, and possibly the activity, of a specific CDK, CDKG1, regulates the number of mother cell divisions and thus the daughter cell size (Li *et al.*, 2016). In other organisms, alternating CDK activity can be a regulatory variable on its own. In the simple system of fission yeast encoding a single cell cycle-regulating CDK, the progression of the cell cycle can be simplified so that a single monomolecular module of CDK linked with cyclin is able to drive the cell cycle in a manner similar to wild-type cells (Coudreuse and Nurse, 2010). This is achieved simply by alternating two CDK activity thresholds at entry to the S and M phases, and functions even in the absence of most forms of canonical regulation. It is not clear whether such a simple model is operating in *D. quadricauda*, where, as in other plants, cell cycle progression is regulated by two CDKs, CDKA and CDKB, which have different roles in cell cycle regulation (Tulin and Cross, 2014; Atkins and Cross, 2018). Although we could detect a threshold CDK activity related to nuclear division (Supplementary Fig. S3), it is not clear if this represents CDKA and/or CDKB. Moreover, we were unable to detect a threshold CDK level related to DNA replication. In plant meristems, altering either CDKA or CDKB complex kinase activity will affect the cell size and duration of the individual cell cycle phases but not the total length of the cell cycle (Jones *et al.*, 2017). It would be interesting to see how this relates to cell cycle progression in *D. quadricauda* that is not directly connected to attaining a critical cell size. Unfortunately, given the lack of genetic tools, we were unable to separate the CDKA and CDKB kinase activities, as antibodies to CDKA and CDKB recognized and immunoprecipitated both proteins (Hlavová *et al.*, 2011). Moreover, CDK activity in whole-cell lysates generally overlapped with the kinase activity specifically bound to CKS1 beads (Supplementary Fig. S1), suggesting that the CKS1-bound protein fraction also contains both CDKA and CDKB. In some cases, CKS1-bound kinase activity differed from that in whole-cell lysates (Supplementary Fig. S1), hinting at the possibility that one of the complexes is preferentially affinity purified. This was true particularly for the CKS1-bound activity peak at cellular division (compare Figs 7, 8, and Supplementary Fig. S1). In *C. reinhardtii*, CDKB is an essential CDK responsible for spindle formation and nuclear division and subsequent DNA replication, once CDKA-dependent initiation has occurred (Tulin and Cross, 2014; Atkins and Cross, 2018). We assume that cell division-related kinase activity might be the action of CDKB. Clearly, mutants in CDKA and/or CDKB would be very useful in analyzing the roles of the two kinases in regulating cell cycle progression in *D. quadricauda*, the relationship to temperature shifts, and the nature of threshold CDK activities.

In the temperature shift experiments, the critical cell size (and/or the amount of different macromolecules), or the state of the cell more generally, seems to be 'reinterpreted' based on the current cultivation temperature. This reinterpretation was manifested as changes in CDK activity followed by changes in the number of nuclear divisions (and other cell cycle-related processes) per cell, and/or their proportion within the population. The activity of the CDK complex is regulated on multiple levels: (i) transcription of CDK or the cyclin subunit, (ii) post-translational modification, (iii) protein–protein interaction, and (iv) subunit degradation (Francis, 2007; Inagaki and Umeda, 2011; Van Leene *et al.*, 2011). Changes in kinase activity occurred within 1–2 h of a temperature shift (Fig. 8G, H), suggesting that they might have occurred at the level of changes in post-translational modification and/or protein–protein interactions of existing CDK complexes. Alternatively, they could be caused simply by temperature sensitivity of the CDK complexes themselves. The protein levels of both CDKA and CDKB are quite steady during the cell cycle of *D. quadricauda* (Hlavová *et al.*, 2011). Similarly, the level of the CDK inhibitor Wee1 kinase was steady. Moreover, the levels of Wee1 kinase do not seem to interfere with CDK activity (Hlavová *et al.*, 2011). Thus, it is feasible that the activity of CDK is affected by changes in protein–protein interactions. Candidates for such regulators are CDK inhibitors that have not so far been identified in green algae owing to their high sequence divergence (Bisova *et al.*, 2005).

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. The activity of CDK in the CKS1-bound fraction and the courses of nuclear and cellular division in synchronized populations of *Desmodesmus quadricauda*.

Fig. S2. Inhibition of CDK activity by roscovitine in whole-cell lysate and in the CKS1-bound fraction.

Fig. S3. Cumulative time-course values of kinase activities in whole-cell lysates, cumulated number of nuclei, and completed cell divisions in synchronized populations of *Desmodesmus quadricauda*.

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