Journal of Plant Physiology 208 (2017) 84–93

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Changes in nitric oxide/hydrogen peroxide content and cell cycle progression: Study with synchronized cultures of green alga *Chlamydomonas reinhardtii*

Wojciech Pokora[a,*], Anna Aksmann[a], Agnieszka Baścik-Remisiewicz[a], Agnieszka Dettlaff-Pokora[b], Max Rykaczewski[a], Magdalena Gappa[a], Zbigniew Tukaj[a]

[a] Department of Plant Physiology and Biotechnology, University of Gdańsk, ul. Wita Stwosza 59, 80-308 Gdańsk, Poland
[b] Department of Biochemistry, Medical University of Gdańsk, ul. Dębinki 1, 80-211 Gdańsk, Poland

ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form 7 October 2016
Accepted 9 October 2016
Available online 15 November 2016

Keywords:
Chlamydomonas
Nitric oxide
Hydrogen peroxide
Cell cycle

ABSTRACT

The present study aimed to evaluate the possible relationship between the changes in hydrogen peroxide (H_2O_2) and nitric oxide (NO) content and the course of growth and reproductive processes of the cell cycle of *Chlamydomonas reinhardtii*. The peak of H_2O_2 observed at the beginning of the cell cycle was found to originate from Fe-SOD and Mn-$SOD_{chl.}$ activity and result from the alternation in the photosynthetic processes caused by the dark-to-light transition of daughter cells. A rapid increase in NO concentration, observed before the light-to-dark cell transition, originated from NR and NIR activity and was followed by a photosynthesis-independent, Mn-$SOD_{chl.}$-mediated increases in H_2O_2 production. This H_2O_2 peak overlapped the beginning of *Chlamydomonas* cell division, which was indicated by a profile of *CYCs* and *CDKs* characteristic of cells' passage through the G1/S and S/M checkpoints. Taken together, our results show that there is a clear relationship between the course of the *Chlamydomonas* cell cycle and typical changes in the H_2O_2/NO ratio, as well as changes in expression and activity of enzymes involved in generation and scavenging of these signaling molecules.

1. Introduction

The progress and regulation of the eukaryotic cell cycle undergo the universal model in which cyclins (CYCs) and cyclin-dependent kinases (CDKs) synthesis and degradation play a central role (Morgan, 2007). Conserved homologs (orthologs) of CYCs and CDKs have been identified in yeast, animal and plant cells (Morgan, 2007). However, some cell cycle regulatory protein/protein complexes, such as Rb (suppressor protein involved in G1 checkpoint control and cell cycle S phase entry), E2F (transcription factor involved in cell cycle regulation and nucleic acids synthesis) and cyclins: A, B and D are present in animals, plants and green algae but not in yeasts, suggesting that cell cycle control in the eukaryotes was an early evolutionary development with this set of regulatory proteins (Tulin and Cross, 2014). Research in plants or their green algal relatives like *Chlamydomonas* may provide direct information on the cell cycle control network of all eukaryotic cells. In the cultures of green alga *Chlamydomonas reinhardtii*, the optimal growth conditions and the alternating light/dark period lead to synchronization of cell growth. This allows us to analyze the population of cells in the same moment of the cell cycle, and synchronized cultures of *Chlamydomonas* were, for many years, a convenient tool for the study of different physiological and developmental processes during a cell cycle (For review see Cross and Umen, 2015; Cross et al., 2011).

Cells of *Chlamydomonas reinhardtii* divide by multiple fission into "2^n" daughter cells, where "n" DNA replication rounds and "n" nuclear divisions occur during a single cell cycle (Lien and Knutsen, 1979). The *Chlamydomonas* cell cycle has a long G1 phase,

Abbreviations: ABS/RC, the amount of energy absorbed by single reaction center of photosystem II; APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; CYC, cyclin; CDK, cyclin-dependent kinase; Cu/Zn-SOD, copper-zinc superoxide dismutase; DI_0/RC, nonphotochemical energy dissipation by single reaction center of photosystem II; DMSO, dimethylsulfoxide; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ET_0/RC, the amount of energy used for electron transport by single reaction center of photosystem II; Fe-SOD, iron superoxide dismutase; H_2O_2, hydrogen peroxide; Mn-SOD, manganese superoxide dismutase; NBT, nitrobluetetrazolium, chloride; NiR, nitrite reductase; NR, nitrate reductase; NO, nitric oxide; PS II, photosystem II; PVP, polyvinylpyrrolidone; Rb, retinoblastoma; RC, reaction center of PS II; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TR_0/RC, the amount of energy trapped in single reaction center of photosystem II.

* Corresponding author.
E-mail address: pokora@ug.edu.pl (W. Pokora).

http://dx.doi.org/10.1016/j.jplph.2016.10.008
0176-1617/

occupying up to 80% of the cell cycle, when the cell growth and multiplication of cell structures take place. It is followed by a short division phase in which mother cells alternate rapidly between S and M phases, and daughter cells are finally released (Coleman, 1982; Umen and Goodenough, 2001). Matsumura et al. (2010) showed that *Chlamydomonas* cell division progresses through three critical points and that its cell cycle is regulated by an 'interdivision timer,' 'commitment sizer' and 'mitotic sizer.' As in other plant cells, the progress of the *Chlamydomonas* cell cycle undergoes regulation involving cyclins (CYCs) and cyclin-dependent kinases (CDKs) synthesis and degradation. *Chlamydomonas* encodes orthologs of all major CDK, CYC and E2F in higher eukaryotes involved in the regulation of cell cycle progress (G1/S transition and DNA replication) genes, identified in higher plants' genome (Bišová et al., 2005). The expression of a subset of *Chlamydomonas* genes (e.g. *CYCA1*, *CYCB1*, *CDKB1*, *RNR1*, *POLA4*), most of which are likely to be Rb-E2F pathway targets, is up-regulated prior to commitment and down-regulated just after passage through commitment (Umen and Goodenough, 2001). The same genes are up-regulated a second time during the S/M checkpoint, when they are likely to function (Bišová et al., 2005; Cross and Umen, 2015). mRNA for *CDKA1* is present constitutively during the cell cycle, with its expression increasing as cells enter the growth phase at the beginning of the light period and increasing markedly further during the S/M phase. mRNA for *CDKB1* shows two peaks of expression, one corresponding to the passage through commitment G1/S and the second, very strong peak, during the S/M phase (Bišová et al., 2005). The current status of the studies on the cell cycle regulation in green alga dividing by multiple fission, particularly the *Chlamydomonas* cell cycle, can be found in review papers of Bišová and Zachleder (2014) and Cross and Umen (2015).

The set of enzymes that is involved in the generation and scavenging of H_2O_2 in *C. reinhardtii* cells (superoxide dismutases (SODs), peroxidases (POXs), catalase (CAT)) and NO (nitrate (NR) and nitrite (NiR) reductase), undergo up- or down-regulation in response to the cellular level of H_2O_2 and/or NO (Zago et al., 2006). The available experimental data indicate three equally probable ways of such regulation: direct interaction with receptor proteins (Mittler, 2002), interaction with redox-sensitive transcriptional factors (e.g. NPR1, HSFs) (Neill et al., 2002) or direct inhibition of phosphatase activity leading to a modification of signal transduction cascades (e.g. OXI1 protein expression) (Rentel et al., 2004). With respect to SODs and APX regulation on both the transcriptional and translational levels has been demonstrated (Sung et al., 2009). It is also known that the activity of SODs can be regulated by the H_2O_2 concentration by direct enzyme activity inhibition or its mRNA degradation (Miyake et al., 2006). H_2O_2 was found to induce the generation of NO, another signaling molecule in plant cells (She et al., 2004; He et al., 2005). The removal of H_2O_2 using antioxidants or via inhibition of its synthesis resulted in the prevention of NO production (Bright et al., 2006). NO is known to be involved in many developmental processes in plants, such as leaf expansion, root growth or phytoalexin production (Leshem and Haramaty, 1996; Noritake et al., 1996). In addition, there are a few reports indicating the involvement of NO in cell cycle regulation. For instance, Correa-Aragunde et al. (2006) reported that NO modulates the expression of cell cycle regulatory genes involved in the G1-to-S phase transition during the lateral root initiation in tomato. Experiments reported by Ötvös et al. (2005) indicate that NO may influence the entry into the cell cycle (G0-to-G1 transition) but not cell cycle progression in alfalfa cell cultures. With respect to green microalgae, only a study of Lehner et al. (2009) demonstrating the NO-mediated suppression of growth and development of *Micrasterias denticulata* is available. The role of NO in developmental and growth processes still remains to be investigated. Hypotheses vary from NO action as a nonspecific antioxidant protecting IAA against oxidation (Fernández-Marcos et al., 2011; Correa-Aragunde et al., 2016) through the existence of NO-specific molecular targets such as cell-cycle genes or enzymes involved in signal transduction (Correa-Aragunde et al., 2004), to maintaining the redox balance in plant cells (Correa-Aragunde et al., 2015).

Circadian oscillations of the activity of antioxidative enzymes, determining the amount of formed and scavenged H_2O_2, have been reported in algal cells (Carvalho et al., 2004). Further, circadian fluctuation in synthesis and degradation of nitrate reductase – the enzyme primarily responsible for the formation of NO, has been observed (Lehner et al., 2009). Both H_2O_2 and NO are molecules involved in signal transduction cascades of MAP kinases, leading to the activation of transcription factors regulating the expression of CYCs and CDKs. Thus, changes in intracellular concentrations of H_2O_2 and NO may be important factors involved in the modulation of expression of genes required for cell cycle progression. To determine the possible relationship(s) between changes in NO and H_2O_2 content and cell cycle progress in a synchronized culture of *Chlamydomonas* we examined: i) cells' growth and photosynthetic activity, ii) NO/H_2O_2 level and expression of main enzymes involved in their metabolism, iii) nucleic acid content and the level of main CYCs and CDKs, as well as the transcription factor (E2F).

2. Methods

2.1. Cultures

Chlamydomonas reinhardtii strain cc-1690 was purchased from the Chlamydomonas Resource Center (University of Minnesota, USA). Cultures were grown on mineral, liquid high salt medium (HSM; Sueoka, 1960) of pH 6.9 ± 0.1, in 200 mL glass test tubes at 30 °C and illuminated by cool white light with irradiance of 300 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR, 380–690 nm). The cultures were aerated with the atmospheric air containing 2.5% (v/v) CO_2, which was passed through a bacteriological filter.

The algal cells' growth was synchronized by alternating light and dark periods (L/D −10/14 h). At the beginning of each light period, the algal culture was diluted to a constant density of 1.5×10^6 cells mL^{-1}. The lengths of the light periods of the cell cycle were selected so that three replication rounds (n = 3) during one 24 h L/D period occurred. A dark period of 14 h was sufficient to liberate all of the daughter cells. At least 5 L/D cycles were needed for cells' growth synchronization.

The number and size of cells were determined by an electronic particle counter (Beckman Coulter Z2), the observations of cell morphology were performed under the light microscopy.

2.2. RNA/DNA content

The total DNA/RNA content was detected using SYTO16® fluorescent nucleic acid stain (S7578, molecular probes) (Frey, 1995). For staining, 1 mL of the cell suspension withdrawn from the culture was centrifuged (3000*g*, 5 min) and suspended in 0.2 mL 25 mM HEPES buffer pH 7.5 (RNA staining) or 10 mM TRIS, 1 mM EDTA, 50 nM NaCl, pH 7.4 (DNA staining). The solution of SYTO16® dye in DMSO was added to the final concentration of 50 μM. Then the cells were incubated for 60 min in darkness at room temperature. The fluorescence signal was detected using a Varioskan® Flash Spectral Scanning Multimode Microplate Reader (ThermoScientific) run by a Varioskan Flash SkanIt Software. Ex./Em. wavelengths: 488/518 nm for DNA detection and 494/525 nm for RNA were applied. Samples containing algal cells without fluorochrome, fluorochrome solution in buffer, as well as samples treated with DNAse or RNAse (15 min, RT) prior to incubation with SYTO16® dye were used as controls