We previously showed that nuclear DNA replication (NDR) is regulated by a checkpoint monitoring the occurrence of organelle DNA replication (ODR) in a unicellular red alga *Cyanidioschyzon merolae*. These analyses depended on the use of chemical CDK inhibitors such as CDK2 inhibitor II and roscovitine, but subsequent analyses yielded conflicting results depending on the experimental conditions. In the present study, we identified significantly short half-lives of the used chemicals in the sulfur acidic cultivation medium, which reconciles the discrepancy among these results.

Key Words: CDK; cell cycle; *Cyanidioschyzon merolae*; inhibitor; red algae

*Cyanidioschyzon merolae* is a unicellular red alga living in acidic hot springs, and was introduced as a novel model eukaryotic cell to elucidate various fundamental cell processes because it contains the simplest structure among eukaryotic cells. The complete genome sequences of the nucleus, mitochondria, and chloroplast were determined, and various tools for molecular genetics analyses have been developed (Fujiwara et al., 2013; Imamura et al., 2009; Kobayashi et al., 2010; Kuroiwa, 1998; Matsuzaki et al., 2004; Nozaki et al., 2007; Taki et al., 2015). *C. merolae* has been extensively used for studying organelle division processes, cell cycle regulation, and structural biology.

In a previous study, we investigated a mechanism underlying the coordination of organelle DNA replication (ODR) with nuclear DNA replication (NDR) in *C. merolae* (Kobayashi et al., 2009). Under periodic light-dark cultivation conditions, the algal cell cycle is arrested at the G1 phase in the dark, and initiated by illumination. Detailed analyses revealed that both mitochondria and chloroplast genomes replicate upon the onset of illumination, and then NDR occurs afterward. As the underlying mechanism, we revealed that an increase of intracellular Mg-ProtoIX, an intermediate of chlorophyll biosynthesis produced in the chloroplast, induced by the ODR occurrence was recognized as a retrograde signal from the chloroplast to the nucleus to activate CDKA, responsible for activating NDR.

During these experiments, specific inhibitors for cyclin-dependent kinases (CDK) were conveniently used to analyze the underlying mechanism. CDK2 inhibitor II is a commercially available inhibitor for animal CDK2 and plant CDKA (Merck, Darmstadt, Germany) (Davis et al., 2001). As expected, biochemical and physiological analyses revealed that this compound inhibited the relevant CDK (CDKA) activity and NDR in *C. merolae* (Kobayashi et al., 2009). Another CDK inhibitor used was roscovitine (Merck, Darmstadt, Germany), that is known as a general inhibitor for various CDK types, as revealed by its inhibition of cdc2/cyclin B, cdk2/cyclin A, cdk2/cyclin E, and cdk5/p53 in mammalian cells (Xie et al., 2016). Our results indicated that CDK inhibitor II inhibited only NDR, while roscovitine inhibited both ODR and NDR, and, thus, it was suggested that ODR is under the control of a CDK other than CDKA in *C. merolae*. Because both CDK inhibitors inhibit NDR at the cell cycle S phase, their addition to the medium was expected to result in the growth arrest. However, little inhibitory effect on OD750 increases was observed during 3 days after adding the drugs to the medium at the concentration used in the previous DNA replication analyses, 600 nM or 5 μM of CDK2 inhibitor.
II or roscovitine, respectively (Figs. 1A and B). To explain this, we wondered if these inhibitors might be unstable and rapidly lose their activity in the sulfur acidic cultivation medium, MA2, since we previously found that acid lability in MA2 medium masks the physiological effects of exogenously added abscisic acid (ABA) (Kobayashi et al., 2016).

Because of the instability of the compounds in the acidic medium, periodic addition of the compounds to the medium may accomplish the growth inhibitory effect. To examine this, we repeatedly added the same dose of inhibitors to the medium at 12-hour intervals and observed cell growth. With the new protocol, we found that both CDK2 inhibitor II and roscovitine markedly inhibited cell growth (Lines-R in Figs. 1A and B). Accumulation of the inhibitor was not the reason for growth inhibition, because adding 8-fold higher doses of the inhibitors (4.8 mM CDK2 inhibitor II; A and C, 40 μM Roscovitine; B and D) at the initial time point only slightly affected the growth (Lines-Ex in Figs. 1A and B). Increases of cell numbers during the cultivation were also measured during the time course, shown in Figs. 1C and D. While severe inhibition of cell number increase was observed by repeated addition of both drugs, it was also observed during the first 24 hours by the addition of CDK2

![Fig. 1. Effect of CDK2 inhibitor II and roscovitine on cell growth.](image-url)
Lability of CDK inhibitors in sulfur acidic cultivation medium

Inhibitor II (Ex 4.8 μM, Fig. 1C) or roscovitine (5 μM or
Ex 40 μM, Fig. 1D) at the initial time point. Thus, in-
ccrease in cell number, which is relevant to the function of
CDK inhibitors, is more sensitive to these drugs than the
increase of the cell mass.

The half-lives of the two compounds in the C. merolae
cultivation medium were analyzed by HPLC analysis.

Table 1. Drug stability under high acidity or high temperature conditions.

| Incubation condition | 0 h | pH 7.4, 25°C, 24 h | pH 7.4, 42°C, 24 h | pH 2, 25°C, 24 h |
|---------------------|-----|-------------------|-------------------|------------------|
| CDK2 inhibitor II   | 19.79 ± 0.77 μM | 15.93 ± 2.43 μM | 3.25 ± 0.92 μM | 9.05 ± 4.83 μM |
| Roscovitine         | 51.7 ± 6.27 μM  | 52.71 ± 13.9 μM | 41.56 ± 7.67 μM | 0 ± 0 μM       |

In this study, we found that CDK2 inhibitor II and
roscovitine are rather unstable under C. merolae cul-
tivation conditions. From a structural point of view, CDK2
inhibitor II contains an acid-labile hydrazone structure,
whereas roscovitine is highly basic and protonated under
acidic conditions. As mentioned above, ABA also pos-
sesses acid-labile hydroxy groups (Supplementary Fig.
S1). These molecular characteristics are well-consistent
with our experimental observation. Thus, we should be
especially cautious when using inhibitory drugs to analyze
cellular mechanism of organisms cultivated in extremely
acidic conditions.
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Supplementary Materials

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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