Light intensity and reactive oxygen species are centrally involved in photoregulatory responses during complementary chromatic adaptation in *Fremyella diplosiphon*

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Introduction

Photosynthetic organisms that have limited mobility in their environment demonstrate finely regulated abilities to sense and respond to fluctuations in the external environment. As light is required for photosynthesis, it is one of the most important external factors that impacts the growth and development of photosynthetic organisms. Cyanobacteria, which exhibit oxygenic photosynthesis comparable to higher plants, have been long studied to understand the effect of changes in external light conditions on the composition and function of the light-harvesting complexes and associated impacts on photosynthetic efficiency. The freshwater cyanobacterium *Fremyella diplosiphon* is one of the organisms that has been most extensively studied for its ability to dynamically adapt its growth to a range of environmental conditions, including different colors and intensities of light.

In the aquatic ecosystems in which organisms such as *F. diplosiphon* are found, both light quality and quantity are altered at different depths within the water column. *F. diplosiphon* is most responsive to changes in available red light (RL) and green light (GL). In a natural aquatic ecosystem context, the availability of RL decreases at greater depths as it is absorbed by water, as well as by chlorophyll and RL-absorbing phycobiliproteins [PBPs; i.e., phycocyanin (PC) or allophycocyanin (AP)] contained in organisms higher in the water column. Organisms such as *F. diplosiphon*, which possess major light-harvesting complexes called the phycobilisome (PBS), can regulate the PBP composition of the PBSs to support photosynthesis in blue- or green-light enriched benthic environments. Many cyanobacteria exhibit such an ability to alter the pigment or protein composition of their PBSs in response to external changes in the prevalent, available wavelengths of light, in a phenomenon commonly known as complementary chromatic adaptation (CCA).

In addition to the changes in pigmentation that are associated with optimizing light absorption for energy production through photosynthesis, changes in the morphology of cells and filament...
The observations that increasing the intensity of GL to which cells are exposed and a RL-associated regulation of ROS levels impact cellular morphology led us to conduct complementary studies under increasing intensities of RL to determine the impact on cellular pigmentation, ROS levels and morphological characteristics. Here, we describe an investigation that suggests that a number of cellular morphology-impacting factors are linked, i.e., an increasing light intensity is positively correlated with an increased accumulation of ROS and a change in cellular morphology to more spherical. Our results suggest a causative impact of auto increases in ROS levels on cellular morphology, as the observed light intensity-mediated impacts that are ultimately manifested as a change in cell shape are reversible when cells are transitioned from high intensity to lower intensity light.

Results

Higher intensity RL is correlated with an alteration of morphology to rounded cells in *F. diplosiphon*. Increasing the intensity of RL to which cells are exposed results in an increase in the spherical nature of cellular morphology, as well as a reduction in cell length (Fig. 1). This observation is similar to what was previously reported for cells under increasing intensities of GL. The change in cellular morphology became more apparent after reiterative dilution and growth of cells under increased fluences of RL over many generations (Fig. 1). Taken together with prior results under GL, these results provide significant evidence for an impact of light intensity, in addition to the previously recognized role for light quality (i.e., RL vs. GL), on the photoregulation of cellular morphology in *F. diplosiphon*. 

Figure 1. Confocal laser scanning microscopy analyses of cellular morphology of wild-type (WT) *Fremyella diplosiphon* UTEX481 strain under red light (RL) conditions of varying light intensity. *F. diplosiphon* WT was grown in BG-11 culture medium containing 20 mM HEPES at 10, 50, 75 or 100 μmol m⁻² s⁻¹ (numbers indicated at left) at 27°C with shaking at ~175 rpm. Representative optical slices from a Z-series of differential interference contrast (DIC) images (upper half) and maximum intensity projection of phycobiliprotein fluorescence (auto) images (lower half) of WT. Numbers of consecutive dilutions (5, 13, 21, 37 or 45) made before imaging are indicated at the top of each column. All images were acquired with a 40× oil objective with 2× zoom. Bar represents 10 μm.
Increased RL intensity results in increased carotenoid levels indicative of stress in *F. diplosiphon*. We also assessed other cellular parameters for cells growing at varying intensities of RL. We investigated the impact of increasing light intensity on the levels of distinct photosynthetic pigments in cells. We determined that PBP levels were decreased at higher RL intensities (Fig. 2A–C), similar to prior observations for growth under elevated GL.\(^2,3\) In some cases, PBP levels began to recover after many generations under high RL (Fig. 2A–C), suggesting an acclimation response to high RL over time.

Chl\(a\) levels were not as severely impacted by increasing RL intensities (Fig. 2D), suggesting that cells likely respond to the potentially damaging excess of RL by first reducing cellular PBP levels to reduce light absorption. Notably, chl\(a\) content was also less severely affected under increased intensity of GL,\(^4\) although the regulation of chlorophyll levels under these conditions may be distinct from those under RL. In this regard, the need for maintaining PBS under GL conditions to maintain light absorption by PE, the sole GL-absorbing pigment and transfer energy from the PBS to the photosystem core for the production of photosynthate and reductants, even under stress conditions, has been previously discussed for *F. diplosiphon*.\(^1,3,14\)

Carotenoids accumulate under high light or other stresses as a part of a cellular protective mechanism in cyanobacteria,\(^15\) including *F. diplosiphon*.\(^6\) Under increasing intensities of RL, carotenoid levels were in general higher than those measured for cells grown at 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\) (Fig. 2E). A similar increase in carotenoid levels under elevated GL intensity was previously noted for this organism.\(^5\)

**Figure 2.** Photosynthetic pigment content in *Fremyella diplosiphon* strain UTEX481 grown under increasing intensities of RL. Concentrations of photosynthetic pigments, including (A) phycoerythrin (PE), (B) phycocyanin (PC), (C) allophycocyanin (AP), (D) chlorophyll \(a\) (chl\(a\)) and (E) carotenoids (car) were determined for cells growing in BG-11 culture medium containing 20 mM HEPES at 27°C with shaking at ~175 rpm under increasing intensities of RL of 10, 50, 75 or 100 \(\mu\)molm\(^{-2}\)s\(^{-1}\) for the number of dilutions indicated (see key, i.e., 8, 16, 24 or 40) or cells grown under 100 \(\mu\)molm\(^{-2}\)s\(^{-1}\) for 25 dilutions before transfer to 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\) (100–10) for 15 dilutions.

Increased RL intensity results in the accumulation of reactive oxygen species (ROS) in *F. diplosiphon*. In prior studies we demonstrated that the spherical shape of *F. diplosiphon* that is observable under RL conditions is correlated with a RL-mediated elevation of ROS levels, in contrast to the elongated rod- or brick-shaped cells characteristic of growth under GL, where ROS levels are lower.\(^15\) To determine whether the induction of a more spherical shape under increasing intensities of RL is correlated with altered ROS levels, we quantified ROS in cells growing at various intensities of RL. ROS levels were indeed significantly higher in cells grown at higher RL fluences (i.e., 50, 75 or 100 \(\mu\)molm\(^{-2}\)s\(^{-1}\)) than in cells grown at 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\) (Fig. 3A).

The impacts of increased light intensity on *F. diplosiphon* cells is reversible. The observed impact of increased RL intensity on decreasing cell length and the promotion of a more spherical morphology (Fig. 1), increased carotenoid levels (Fig. 2) and increased ROS levels (Fig. 3) are reversible when cells grown at a high RL intensity of 100 \(\mu\)molm\(^{-2}\)s\(^{-1}\) are transitioned to a lower intensity of RL at 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\). The transition of the more spherical morphology at 100 \(\mu\)molm\(^{-2}\)s\(^{-1}\) to a less spherical shape observed at 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\) of RL becomes more apparent after several generations of growth of high light-adapted cells at a lower intensity of RL (Fig. 4). Likewise, when cells acclimated to high light intensity were transitioned to lower intensity RL, carotenoid levels in the transitioned cells were indistinguishable from cells maintained constantly at low RL (Fig. 2E). This change in carotenoid levels that is correlated with light and is reversible is consistent with intensity-dependent changes in carotenoid levels reported for cells grown in varying intensity of GL.\(^5\) Notably for cells transitioned from 100 to 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\) of RL, ROS levels also returned to a level that was not significantly different from cells maintained at 10 \(\mu\)molm\(^{-2}\)s\(^{-1}\) (Fig. 3). Taken together, these results confirm a prior observation that light-induced elevations...
of ROS levels and an induction of spherical morphology are correlated in *F. diplosiphon*.12

**Discussion**

Our recent results described here and in prior work6,12 have established that light-induced changes in ROS, which can be mediated by light quality or light intensity, appear causative for inducing a more spherical shape of *F. diplosiphon* cells. In prior work, we showed that the light quality-dependent changes in morphology under GL vs. RL is regulated by the same photoreceptor RcaE that controls changes in pigmentation in response to these conditions.3 RcaE also has a role in light-intensity regulated accumulation of ROS.12 These studies are providing new insights into the photoregulatory mechanism(s) controlling cellular morphology and filament length in cyanobacteria.

**Materials and Methods**

**Experimental organism and growth conditions.** Wild-type (WT) UTEX481 strain of *F. diplosiphon* was used in this study. Cultures were grown in autoclaved BG-11 medium (Fluka) containing 20 mM HEPES (hereafter BG-11/HEPES) at pH 8.0 at 27°C with continuous shaking at ~175 rpm under RL, using a 660 nm 90 watt RL LED source (model 2506RD, LEDwholesalers). A Li-Cor light meter (model LI-250, Li-Cor) with a coupled Li-Cor quantum sensor (model LI-190SA) was used to determine light intensities.

**Reiterative dilution of cells.** Initial cultures were derived from a plate of WT cells grown under fluorescent white room lights at room temperature for reiterative dilution studies as described previously.6 Cultures were grown in autoclaved BG-11/HEPES as described above under RL at 10 μmol m<sup>−2</sup> s<sup>−1</sup>. Cultures were then diluted to an optical density of 750 nm (OD<sub>750</sub>) of ~0.1 and grown under RL at 10, 50, 75 or 100 μmol m<sup>−2</sup> s<sup>−1</sup>. After the initial dilution and the placement of cultures under the indicated light conditions, the OD<sub>750</sub> was measured twice weekly (once every 3–4 d) and cultures diluted to an OD<sub>750</sub> of ~0.1. Cells were collected for confocal imaging analyses after the fifth, thirteenth, twenty-first, thirty-seventh and forty-fifth dilutions. After iterative dilution, a culture of cells that had been maintained at 100 μmol m<sup>−2</sup> s<sup>−1</sup> was diluted to OD<sub>750</sub> of ~0.1 and transitioned to 10 μmol m<sup>−2</sup> s<sup>−1</sup> and maintained for many reiterative dilutions to determine the impact of reverting from high intensity RL to low intensity RL.

**Confocal microscopy-based analysis of cellular morphology.** Slides for cellular morphology analyses using confocal microscopy were prepared and imaged as previously detailed.3,16 We acquired differential interference contrast (DIC) and autofluorescence images using an inverted Axiovert 200 Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM: Carl Zeiss MicroImaging).

**Figure 3.** Measurement of reactive oxygen species (ROS) accumulation in *Fremyella diplosiphon* strain UTEX481 grown under increasing intensities of RL. Levels of ROS were determined for cells growing in BG-11 culture medium containing 20 mM HEPES at 27°C with shaking at ~175 rpm for > 25 dilutions and then moved to 10 μmol m<sup>−2</sup> s<sup>−1</sup>. Cells were imaged after the first or 19th dilution after transfer from high to low intensity RL, i.e., 100 →10. Cells at 100 μmol m<sup>−2</sup> s<sup>−1</sup> for 45 dilutions are the same images as shown for this condition in Figure 1. Representative optical slices from a Z-series of differential interference contrast (DIC) images (left) and maximum intensity projection of phycobiliprotein autofluorescence (auto) images (right). All images were acquired with a 40× oil objective with 2× zoom. Bar represents 10 μm.

**Figure 4.** Confocal laser scanning microscopy analyses of cellular morphology of *Fremyella diplosiphon* strain UTEX481 transitioned from high intensity red light (RL) to low RL intensity. *F. diplosiphon* WT was grown in BG-11 culture medium containing 20 mM HEPES at 10 μmol m<sup>−2</sup> s<sup>−1</sup> at 27°C with shaking at ~175 rpm for > 25 dilutions and then moved to 10 μmol m<sup>−2</sup> s<sup>−1</sup>. Cells were imaged after the first or 19th dilution after transfer from high to low intensity RL, i.e., 100 →10. *Cells at 100 μmol m<sup>−2</sup> s<sup>−1</sup> for 45 dilutions are the same images as shown for this condition in Figure 1. Representative optical slices from a Z-series of differential interference contrast (DIC) images (left) and maximum intensity projection of phycobiliprotein autofluorescence (auto) images (right). All images were acquired with a 40× oil objective with 2× zoom. Bar represents 10 μm.
Photosynthetic pigment extraction and quantification. Phycobiliproteins (PBPs), chlorophyll a (chl a) and carotenoids were extracted from *F. diplosiphon* cells grown in liquid cultures and quantified. PBPs were extracted using the protocol of Kahn et al. Individual PBP concentrations were quantified according to Tandeau de Marsac and Houmard. Chlorophyll content was determined as previously described and carotenoids extracted and quantified as previously detailed.

Reactive oxygen species (ROS) detection. ROS levels were determined using the fluorescent dye 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as described in He and Häder. An aliquot of *F. diplosiphon* cells was incubated with DCFH-DA (final concentration of 10 μM) in darkness for 1 h at room temperature as previously detailed. The mixture was then excited at 485 nm and fluorescence detected at 520 nm using a SpectraMax M2 microplate reader (Molecular Devices). The fluorescence of the reaction mixture without added cells was measured as a negative control and the autofluorescence of cells alone measured as a background comparison, as previously described.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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