Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*

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Abstract  Previously, we found that intracellular reactive oxygen species (ROS) affect photomorphogenesis in *Neurospora crassa*. In this study, we investigated the physiological roles of ROS in the response to light and found that the exposure of mycelia to air was important for the light-induced carotenogenesis. Mycelia treated with a high concentration of O2 gas and H2O2 to release ROS showed an enhancement of light-induced carotenoid accumulation and the expression of gene related to light-inducible carotenogenesis. These results suggested that stimuli caused by the exposure of the mycelia to air containing O2 gas triggered the light-induced carotenoid synthesis.

Keywords: Reactive oxygen species; Carotenoid; Oxygen; Hydrogen peroxide; *Neurospora crassa*

1. Introduction

The filamentous fungus *Neurospora crassa*, a member of the ascomyce, has been used in photo-biological research for some time. In *N. crassa*, blue light regulates the following biological phenomena: (i) induction of carotenoid synthesis in mycelia [1], (ii) induction of conidiation [1], (iii) protoperithecial formation under nitrogen-limited conditions [2], (iv) light-dependent perithecial polarity [3], (v) positive phototropism of perithecial beaks [4] and (vi) phase shift in the circadian rhythm of conidiation [5].

Two mutants, white-collar (wc)-1 and wc-2, lacking most of the light-induced responses of the organism have been isolated and characterized [6,7]. The protein products of WC-1 and WC-2 for the wild-type genes of wc-1 and wc-2 are suggested to be a light receptor and transcription factor. These proteins possess a zinc-finger DNA-binding domain, a PAS (PER, ARNT, and SJM) dimerization domain, and a glutamine-rich putative transcription activation domain [8,9]. WC-1 and WC-2 form heterodimers (White Collar Complex; WCC), presumably through their PAS domain [10]. WCC can bind the promoter region of light-regulated genes via the zinc-finger DNA-binding domain and acts as a transcription factor [8,9,11,12]. WC-1 contains a LOV (light, oxygen, voltage) domain, which contains a PAS dimerization domain [8]. WCC has also been reported to bind to FAD (flavin-adenine dinucleotide) via the LOV domain in place of FMN (flavine mononucleotide) in phototropins [13]. Therefore, WC proteins have been thought to play a critical role in the responses to light and act as a photoreceptor and transcription factor. Several genes identified as genetic components for these light-evoked responses, have been isolated and characterized. Three albino genes, *albino-1* (al-1), *al-2*, and *al-3*, encode enzymes essential for the synthesis of carotenoids [7,14,15].

Recently, it has reported that a sod-1 null mutant, which is defective in Cu-Zn-type superoxide dismutase (SOD-1), showed hyperaccumulation of light-induced carotenoids and the expression of WC-controlled genes [16]. These results suggest that intracellular ROS are one of a key components for light signal transduction. However, how and why ROS are able to regulate responses to light, are still unknown. In this study, we obtained the results that intracellular ROS, levels of which increased with the contact between mycelia and air containing oxygen, could have the ability to enhance several light-evoked responses as controlling factors.

2. Materials and methods

2.1. Strains and growth conditions

The *N. crassa*, wild-type strain 74-OR23-1A (Fungal Genetics Stock Center (FGSC) number 987 was obtained from FGSC, Department of Microbiology, University of Kansas Medical Center, Kansas City), was used in this study. Vogel's minimal medium (1.5% sucrose) was used for the culture of mycelia [17], and glycerol complete slant medium was used for the stock of strains and harvesting of conidia [18].

2.2. Quantification of mycelial carotenoid accumulation

Conidia (1 x 10⁶) were inoculated into 50 ml of Vogel's minimal medium in 200-ml flasks and shaken (100 rpm/min) at 30°C for 2 days in darkness. Mycelia were harvested on filter paper by vacuum filtration to form mycelial mats. In the air-exposure experiment, mycelial mats were put on petri dishes or soaked with 50 ml of Vogel's medium. The plates were exposed to light (TOSHIBA, FL20SS-N/18, 40 μE m⁻² s⁻¹) for 1.5 h. In the gas treatment experiment, mycelial mats were fragmented (4.5 cm x 1.5 cm), put into 50-ml tissue culture dishes (IWAKI). Gasses (nitrogen, oxygen, and carbon dioxide) were exchanged in plastic Roux flasks for about 30 s. The flasks were exposed to light (40 μE m⁻² s⁻¹) for 1.5 h. In H2O2 treatment, mycelial mats were soaked with 50 ml of Vogel's medium containing 10 mM H2O2 on petri dishes and exposed to light (40 μE m⁻² s⁻¹) for 1.5 h. After mycelia were harvested, carotenoid accumulation was determined as described by Yoshida and Hasunuma [16].

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method [19]. 32P-labeled probes were mixed with the membrane and nitrogen. Total RNA was prepared by a small-scale RNA extraction method [19]. 32P-labeled probes were mixed with the membrane and hybridized with 30 ml of Church/C213 X-Omat AR film.

Frozen mycelia were powdered with a pestle and mortar in liquid paper by vacuum filtration and immediately frozen in liquid nitrogen. Mycelia was determined based on the absorbance at 470/650 nm.

3. Results

3.1. Light-induced carotenoid accumulation enhanced by the contact of mycelia with air

Formation of conidia from mycelia has been reported to require the contact of mycelia with air [21]. To examine whether the induction of the light-induced carotenoid accumulation in the mycelia depends on the contact of mycelia with air, mycelial mats were completely immersed in the liquid medium to avoid any contact with air. The mycelia were illuminated with or without exposure to air. The accumulation of carotenoids in the air-exposed mycelia was about 10-fold higher after 1.5 h of illumination than that in the air-exposed mycelia grown in the dark (Fig. 1). However, the light-induced carotenoid accumulation in the immersed mycelia was reduced to twofold of that observed in the dark-cultured immersed mycelia. These data suggested that light-induced carotenoid accumulation in the mycelia was enhanced by the exposure of mycelia to air.

3.2. Effects of gas exposure on the light-induced carotenoid accumulation

To investigate which gas in the air is responsible for the light-induced carotenoid accumulation, we examined the amount of carotenoid after 1.5 h of illumination in the mycelia, which were exposed to either oxygen, nitrogen or carbon dioxide gas. Untreated mycelia exposed to air were used as a positive control of the response to light. The accumulation of carotenoids in oxygen-exposed mycelia was about threefold that in untreated air-exposed mycelia. Light-induced carotenoid accumulation was not detected in the nitrogen- or carbon dioxide-exposed mycelia similar to the dark-cultured mycelia (Fig. 2). These results indicate that a high concentration of oxygen enhanced the accumulation of light-induced carotenoids.

3.3. Effects of H2O2 on light-induced carotenoid accumulation

To investigate whether ROS directly affect the light-induced carotenoid accumulation, we examined the accumulation in the mycelial mats immersed in the liquid medium containing H2O2. We used H2O2 as a reagent to release ROS. Mycelia treated with 10 mM H2O2 showed an enhancement of light-induced carotenoid accumulation, although the mycelia were immersed in the liquid medium (Fig. 3). As a control, the mycelia were exposed to air or immersed in the liquid medium without ROS. The mycelia immersed in the liquid medium without H2O2 did not show the carotenoid accumulation (Fig. 3). These results indicated that extracellular H2O2 enhanced the accumulation of carotenoid, although mycelia were kept without exposure to air.

3.4. Effects of exposure to gasses and H2O2 on al-1 gene expression

To investigate whether the response to oxygen-exposure results from the expression of light-inducible genes encoding carotenoid synthetic enzymes, the accumulation of al-1 mRNA in the gas-exposed mycelia was determined by Northern blot analysis. The al-1 transcripts in each treatment were not detected in the mycelia grown in darkness (Fig. 4). The al-1 mRNA accumulation in oxygen-exposed mycelia was fivefold higher than that of untreated mycelia. However, light-induced carotenoid accumulation was not detected in the nitrogen- or carbon dioxide-exposed mycelia similar to the dark-cultured mycelia (Fig. 2). These results indicate that a high concentration of oxygen enhanced the accumulation of light-induced carotenoids.
expression in the mycelia exposed to carbon dioxide was not detected. Unexpectedly, mycelia exposed to nitrogen showed an accumulation of al-1 mRNA similar to that observed in the untreated mycelia. These results indicated that oxygen in air enhanced the light-dependent carotenoid synthesis and the expression of al-1 mRNA. Although nitrogen-exposure did not affect the expression of the al-1 gene, it was suggested that nitrogen affected carotenoid synthesis at the post-transcriptional level. In contrast, the expression of al-1 mRNA was repressed by carbon dioxide-exposure. This result indicated that carbon dioxide in the air inhibited the carotenogenesis.

The accumulation of al-1 transcripts in ROS (H₂O₂)-treated mycelia after 10 min of illumination was determined by Northern blot analysis. In the liquid-immersed mycelia, al-1 transcripts were detected slightly under light, but not in darkness (Fig. 5). The level of al-1 mRNA in H₂O₂-treated mycelia was about twofold that in the untreated mycelia and about half that in the air-exposed mycelia. These results indicated that ROS such as H₂O₂ have the ability to induce the expression of carotenoid synthetic genes and could induce carotenoid accumulation (Fig. 5).

4. Discussion

We analyzed the biological function of ROS during the light-induced carotenoid accumulation in the mycelia of N. crassa. Photoconidiation has been reported to be enhanced by the exposure of mycelia to air [21]. We found that the contact of mycelia with air, oxygen and H₂O₂ was important for the light-induced carotenoid accumulation (Figs. 1–3). Furthermore, in Northern blot analysis, the expression of al-1 mRNA, encoding the enzyme involved in the synthesis of carotenoid,
was enhanced by oxygen-exposure and H2O2-treatment (Figs. 4 and 5). These results suggested that ROS, levels of which increased with exposure to air containing oxygen, could induce light-induced carotenogenesis and may act as a controlling factor in the WC-signaling cascade, because the light-induced expression of al-1 mRNA depends on WC proteins.

In mammals, Clock:BMAL1 and NPAS2:BMAL1, heterodimeric transcription factors, regulate the expression of genes encoding molecular clock components by controlling DNA-binding activity [22,23]. This binding activity was controlled encoding molecular clock components by controlling DNA-expression of creased with exposure to air containing oxygen, could induce and 5). These results suggested that ROS, levels of which increased by oxygen-exposure and H2O2-treatment (Figs. 4 and 5). These results suggested that ROS, levels of which increased with exposure to air containing oxygen, could induce light-induced carotenogenesis and may act as a controlling factor in the WC-signaling cascade, because the light-induced expression of al-1 mRNA depends on WC proteins.

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