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CONFERENCE PROCEEDINGS

Oxidative Stress and Differentiation in *Neurospora crassa*

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Abstract—Environmental stress factors induce oxidative stress in fungi by increasing the intracellular concentrations of reactive oxygen species (ROS). In the mycelium, ROS act as signal molecules needed for cytodifferentiation at certain stages of the development of fungi. Generation of ROS in cells induces the activation of antioxidant protective mechanisms. The purpose of this communication is to analyze the role of ROS in light signal transduction, mediated in *Neurospora crassa* cells by the White Collar Complex.

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Under natural conditions, fungi, a group of lower eukaryotic organisms, are constantly subjected to physical and chemical stresses. Such external factors as starvation, light and temperature conditions, ionizing radiation, changes in the gas composition and osmotic pressure of the medium, as well as mechanical damage of the mycelium, may induce oxidative stress in fungal cells and trigger the process of cytodifferentiation. Reactive oxygen species (ROS), inevitably generated under the influence of internal and external factors, are represented by oxygen in its excited singlet state $^1O_2$, the superoxide radical ($^\bullet O_2^-$), peroxide radical ($HO_2^\bullet$), peroxide ion (HO_2^-), hydrogen peroxide (H_2O_2), nitric oxide ($^\bullet NO$), and hydroxyl radical ($^\bullet OH$). These radicals are extremely unstable (the lifetime of $^\bullet O_2^-$ and $^\bullet OH$ is 10^{-6} and 10^{-9} sec). However, owing to their high reactivity, they can destroy lipids, proteins, and DNA [1]. It has been shown that reactive oxygen species can play the role of secondary messengers in bacterial, plant, and animal cells by modulating the activity of transcription factors [1].

The oxidative stress response, induced by external factors that provoke oxygen radical formation, exhibits instability and is followed by profound changes in cell metabolism, which may eventually lead to cell death. Growth ceases, and morphogenetic changes, observed at various stages of cell development, are associated mainly with cell adaptation to the new conditions by decreasing endogenous oxygen concentrations [2].

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Neurospora crassa and Differentiation

The ascomycete *Neurospora crassa* (red bread mold) is an excellent model for investigating the regulation processes of cytodifferentiation because of its small genome (decoded in 2003), which contains only ten thousand genes (twice as many as the genome of yeast and 25% less than that of *Drosophila*). Also important is the limited number of cell types appearing during *Neurospora crassa* ontogenesis and the fact that its cells therewith retain all morphological and biochemical features of eukaryotic cells.

The mycelium of *N. crassa* is composed of multinuclear branched hyphae which show apical polar growth. The hyphae are divided into compartments (100 × 20 μm) by septa, each having a central pore 0.5 μm in diameter. The pore is permeable to cytoplasm, nuclei, and mitochondria. The septal pores of *N. crassa* are considered to be functional analogues of gap junctions of animal cells, plasmodesmata of plants, and microplasmodesmata of filamentous cyanobacteria [4]. The diffusional and electric relationships between hyphal cells are local, as it is in other organisms, and involve three or four cells along the hypha. These relationships are genetically determined and controlled by the gradient of potential between the cells and by light of the blue–violet spectral area [4].

In response to external signals (carbon and nitrogen starvation, light), transition between the growth phases occurs, growth stops, and cytodifferentiation begins. Depending on the external conditions, *N. crassa* may produce macroconidia, microconidia (asexual reproduction), or ascospores (the sexual process) [5]. In *N. crassa* cells, oxidation of proteins and their subsequent degradation, as well as a loss of reducing equivalents, glutathione oxidation, and excretion of glu-

tathione disulfide to the extracellular medium [2] occur at certain stages of macroconidium differentiation during surface growth under starvation conditions (cell aggregation, third–fourth hour; aerial hyphae formation on newly aggregated hyphae, tenth hour; macroconidium formation on aerial hyphae, sixteenth–eighteenth hour). Furthermore, a low level of lucigenin- and luminol-dependent chemiluminescence was recorded before each differentiation stage. Antioxidants inhibited chemiluminescence and stopped cytodifferentiation; this supports the assumption that ROS are generated before each stage of the development cycle [6].

Among all the stress factors, the effect of light on *N. crassa* cytodifferentiation is the best understood. Thus far, it has been found that light of the blue–violet range of the visible spectrum controls the branching rate of *N. crassa* hyphae, stimulates carotenogenesis, speeds up the processes of asexual and sexual reproduction, and shifts the circadian rhythms of conidiation. The beaks of female sex organs (protoperithecia) exhibit positive phototropism [5]. It was shown that, in the course of light-signal transduction, initiating the process of *N. crassa* differentiation, the functions of individual cells, which exhibit considerable electrophysical heterogeneity, are synchronized [4].

Molecular Mechanisms of Stress Signal Transduction

Molecular mechanisms of differential expression of the *N. crassa* genome during cytodifferentiation induced by stress factors (glucose and nitrogen starvation and the effect of light) have been characterized in part [5]. The list of genes associated with the development of *N. crassa* is certain to include the genes encoding the components of antioxidant protective systems (PS), primarily hydrophobins and carotenoid pigments [5]. In hyphae, light induces the expression of carotenogenic genes and, consequently, the accumulation of neurosporoxanthin and other orange pigments in the mycelium. The genes *al–1* (encoding phytoene dehydrogenase), *al–2* (encoding phytoene synthase), and *al–3* (encoding geranylgeranyl pyrophosphate synthase) were cloned and sequenced. These genes control the three successive stages of carotenoid biosynthesis during conidiation, although carotenogenesis may also be induced in the vegetative mycelium by light and oxygen stress [5].

Photoinduction of carotenoid biosynthesis in *N. crassa* is the most advanced model for studying the light signal transduction. Perkins and his colleagues have isolated two *N. crassa white collar* (*wc*) mutants. The *wc-1* and *wc-2* mutants do not differ in their phenotypes and produce pigmented conidia and white mycelium [5]. The *wc-1* and *wc-2* mutants are completely "blind" in respect to blue light responses of *N. crassa*. In addition, electrophysiological investigations of the *wc-1* mutant (the *wc-2* mutant was not investigated) have revealed constitutive changes in input resistance, membrane potential, and electric coupling between the cells; the changes in electric reactions of the plasmalemma were blocked by light [4]. Hence, these mutants exhibit a pleiotropic "blind" phenotype [5]. The products of the *white collar-1* and *white collar-2* genes are the PAS-domain-containing polypeptides WC-1 and WC-2, which form the heterodimeric photoreceptor White Collar Complex (WCC). WCC controls all the light-dependent reactions of *N. crassa*, including the expression of the *albino* genes [5]. The PAS designation is an acronym formed from the names of *Drosophila period clock protein* (PER), mammal *aryl hydrocarbon receptor nuclear translocator* (ARNT), and *Drosophila single-minded protein* (SIM), in which these domains were identified for the first time [7]. PAS domains include 100–120 amino acids. Conformational changes within these domains are part of the regulatory function. PAS domains promote intermolecular aggregation of polypeptides, including aggregation with other protein molecules that contain similar domains (i.e., WC-1 and WC-2 in *N. crassa*). Despite the differences in their amino acid sequences, the secondary structures and spatial organization of these domains are similar. The PAS domain-containing proteins are known to detect changes in light intensity, redox status, oxygen, and small ligand levels, as well as in the energetic state of the cell. PAS domains have been identified in histidine and serine/threonine kinases, chemoreceptors, photoreceptors for taxis and tropism, circadian clock proteins, voltage-activated ion channels, cyclic nucleotide phosphodiesterases, regulators of responses to hypoxia, and regulators of embryological development of nerve cells. PAS domains are combined with a variety of regulatory modules in multidomain proteins [5].

LOV (Light, Oxygen, Voltage) domains are modifications of PAS domains. These domains bind flavin cofactors (FMN or FAD) and may undergo redox transformations [7]. In *N. crassa*, WC-1, a LOV domain–containing protein, is a polypeptide composed of 1168 amino acids and the blue light photoreceptor, whereas WC-2 (530 amino acids) provides a stabilizing function in this photoreceptor complex [8]. The WC-2 protein does not contain the LOV domain and contains only one PAS domain. The primary structures of these proteins share considerable similarity (47%), and their sequences are 26% identical. Dimerization of the WC-1 and WC-2 proteins and formation of WCC do not depend on light intensity conditions [8].

Hence, the functionally active photoreceptor is a combination of the WC-1 and WC-2 polypeptides and the FAD chromophore bound noncovalently to WC-1. It is the WC-1 protein that is responsible for photon absorption [8]. The fact that the WC-1 and WC-2 proteins contain "Zn-fingers" that recognize GATA sequences in promoter regions suggests that they are probable transcription factors [5].

Reactive Oxygen Species and Differentiation in Fungi

When we consider the effect of light on *N. crassa* as a cytodifferentiation trigger, we should realize that light is a strong stress agent, since fungal cells contain a great quantity of sensitizers such as flavins, pterins, and porphyrins. When these molecules are stimulated by a light quantum, this leads to the generation of reactive oxygen species. The incomplete reduction of oxygen to water during cell respiration is a source of $^{\bullet}O_2^-$. Another pathway of $^{\bullet}O_2^-$ production by the cell is the activity of NADPH oxidases. These enzymes were previously viewed only as sources of $^{\bullet}O_2^-$; now, members of the NOX family are considered as sources of signal molecules as well. In particular, it has been shown that fungi (e.g., *Aspergillus*) require such a signalling system for the sexual process [6]. The close association of the differentiation processes (conidial germination, transition to the stationary growth phase, hypha aggregation, formation of aerial hyphae on newly aggregated ones, conidiation on aerial hyphae) with the factors that promote the ROS formation in cells, as well as suppression of differentiation in the presence of antioxidants, has resulted in recognition of the role that reactive oxygen species play as signal molecules in the cell transition to the next growth phase. [2, 6]. An increase in the ROS content before the next stage of differentiation, which leads to the formation of a new type of cells, has been shown in various species of fungi and myxomycetes (*Dictiostelium discoideum*, *N. crassa*, *Sclerotium rolfsii*, *Flammulina velutipes*) [9–11]. Hence, free radicals are probably necessary mediators of cell differentiation in fungi. The enhanced reactivity of ROS and their participation in the regulation of fungal growth results in the necessity of systems that could control the concentration of reactive oxygen species in fungal cells.

Reactive Oxygen Species and Antioxidant Protection in N. crassa Cells

In any cell, there are a number of enzymes and low-molecular-weight compounds involved in keeping the ROS concentration at a level favorable for the cell development. Mechanisms of external oxygen isolation, including cell surface modification (cell aggregation during cytodifferentiation and hydrophobin synthesis) and accumulation of low-molecular-weight compounds (trehalose, proline, etc.) also play a protective role [2, 12].

Components of the antioxidant protective mechanisms capable of reducing the content of primary ROS ($^1O_2$, $^{\bullet}O_2^-$, H_2O_2), produced in the cell under the influence of external factors, play an important role in the prevention of oxidative stress. Enzyme systems capable of reducing the level of $^{\bullet}O_2^-$ and the H_2O_2 catalytically produced from it are represented by superoxide dismutases (SOD) (EC 1.11.1.6) and catalases (EC 1.15.1.1). Carotenoids can provide effective protection of the cell against $^1O_2$ produced on exposure to light.

Superoxide dismutases (SOD) catalyze the transformation of $^{\bullet}O_2^-$ into H_2O_2 and O_2. As in other eukaryotes, in *N. crassa*, the principal superoxide dismutases are Cu, Zn SOD (SOD1), located in the cytosol, and Mn SOD (SOD2), contained in the mitochondrial matrix, each encoded by a separate gene. The total SOD activity increases during the germination of conidia and transition to the stationary phase [13]. The lifespan of SOD mutants is shorter, their sexual process is reduced, and their capacity for conidiation is lowered. [14]. Moreover, SOD1 mutants show high rates of spontaneous mutations and carotenoid production [15], which points to the role of these enzymes in cell protection against ROS and their important function in the regulation of the *N. crassa* development.

Catalases are heme-containing enzymes that decompose H_2O_2 into water and O_2. Several forms of catalases have been discovered during the study of *N. crassa* development [6]. The most active catalase, Cat-1, was found in *N. crassa* conidia; its activity decreases markedly during the germination of conidia [6, 13]. The activity of Cat-2 (a catalase that can also fulfill peroxidase functions) is associated with the late stationary phase, cell lysis during conidiation, as well as with sexual reproduction, whereas Cat-3 is associated with mycelium growth and spore formation [6]. Cat-3 is induced by stress factors—starvation, heat shock, redox mediators, and metals with variable valence [16]. Oxidation of Cat-1 and Cat-3 under the conditions of high ROS concentrations (both in vitro and in vivo) and intensification of electrophoretic mobility of the oxidized forms of the enzyme have been demonstrated [16].

Thiol-containing compounds (glutathione, thioredoxin, as well as the cysteine SH groups of proteins) are thought to be particularly important for cell protection against oxidative stress. The SH groups of thiols can react with O_2^- and H_2O_2 and influence the activity of the antioxidant protective mechanisms at the level of signal transduction and the regulation of gene expression [17]. Reductases of glutathione and thioredoxin are NADPH-dependent; thus, the maintenance of the thiol level is closely related to the redox balance of the cell.

Carotenoids. The results of a series of investigations into the fungal cell point to the relationship between carotenoid synthesis and cell protection against the photodynamic effect of the reactive oxygen species produced in the presence of light and oxygen [5]. β-Carotene, neurosporaxanthin, and astaxanthin are the major carotenoids found in fungi. In has been

demonstrated that ROS regulate carotenogenesis in *Phaffia rhodosyma* and *Fusarium aqueductum* [5].

In *N. crassa*, the production of carotenoids is associated with the effect of light on the expression of the *al-1*, *al-2*, and *al-3* genes controlled by the WCC. Oxygen and H_2O_2 also stimulate carotenogenesis in *N. crassa* in the presence of light [18]. It is noteworthy that, in the *cat-3* and *sod-1* mutants of *N. crassa* [18, 19], an increase in the carotenoid production in the presence of light and a decrease in transcript *al-1* accumulation in the presence of light and antioxidants were revealed. Cycloheximide did not affect the accumulation of the mRNA of *albino* genes in *sod-1* mutants; i.e., no new WC-1 molecules were needed for their expression. The double mutants *wc-1xsod-1* and *wc-2xsod-2*, like the *wc-1* and *wc-2* mutants, do not synthesize carotenoids in the presence of light [15]. This indicates that carotenoid biosynthesis in *sod-1* mutants is regulated by the WCC proteins [15, 18]. The redox state of WC-1 may affect the DNA-binding activity [15, 18] of the WCC. Hence, it seems likely that ROS regulate light-induced carotenogenesis in *N. crassa* via the WCC.

Changes in the constituents of *N. crassa* antioxidant protective mechanisms at particular growth stages. The photoreceptor WCC in the nucleus of *N. crassa*, involved in the reception and transduction of light signals, modulates, in accordance with the ROS level in the cell, the expression of the light-dependent genes responsible for carotenoid biosynthesis [15, 18]. The lack of carotenoid synthesis resulting from abnormalities in the ROS signal transduction in WCC mutants may lead to changes in the activity of other constituents of the antioxidant protective mechanisms. Unlike the wild type, in the WCC mutants of *N. crassa*, the transition to the stationary phase was accompanied by the activation of catalase and production of its forms with higher electrophoretic mobility, as well as by an increase in the level of SH-groups involved in the decomposition of H_2O_2; that is, it occurred under more severe oxidative stress [13]. The activation of catalase, as well as increased concentrations of thiol compounds in the WCC mutants of *N. crassa* suggest the possible involvement of H_2O_2 in the growth phase transition as a signal molecule.

The Assumed Mechanisms of the ROS Signal Transduction in N. crassa

The mechanisms for controlling the redox balance and ROS signal transduction play a central role in balancing the constituents of the antioxidant protective mechanism.

The transduction of redox signals is based on the reaction of reactive oxygen species with a regulatory protein and consequent changes in its conformation and activity. The most frequent conformational changes in transcriptional factors involve oxidative modification of cysteine residues, just as in yeast, where H_2O_2 signal transduction is mediated by glutathione peroxidase. However, other amino acids might also be sensors of the redox-state of the cell [17]. Oxidation of amino acid residues, induced by ROS, changes the properties of some proteins involved in signal transduction (protein kinases, protein phosphatases, and transcription factors) [1]. In *N. crassa*, the ROS signal may be transduced via several pathways: the two-component histidine kinase system, the sensory module of which contains the PAS domain, the MAP kinase cascade, and the G-protein–cAMP–protein kinase A signaling pathway [3].

The light signal perception in *N. crassa* is accompanied by the oxidation of the FAD chromophore bound noncovalently to WC-1 in the photoreceptor White Collar Complex. It can be assumed that the redox state of WC-1 may influence the DNA-binding capacity of WCC [18]. When we discuss the mechanism of signal transduction via WCC, we cannot but emphasize that, in the *wc-1* mutant, a decrease in the membrane potential together with an increase in input resistance and a greater electric coupling coefficient between cells (as compared with the wild type) have been demonstrated in addition to the absence of all known light responses (carotenoid synthesis in the mycelium, photomorphogenesis, phototropism, phase shifting of the circadian clock of conidiation). The fact that the electric parameters of the *wc-1* plasma membrane remain unchanged in the presence of light suggests that the White Collar Complex may regulate the interactions between *N. crassa* cells.

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