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STRESS RESPONSES OF GENETICALLY MODIFIED *MUCOR CIRCINELLOIDES* STRAINS

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Abstract

The stress-related responses of *Mucor circinelloides* transformants altered in carotenoid production have been investigated. In zygomycetous fungi β-carotene is the predominant carotenoid, however, in transformants containing the *crtW* and *crtZ* astaxanthin biosynthesis genes from *Agrobacterium aurantiacum* new carotenoid compounds are present. *Mucor* strains with altered carotenoid content were treated with different concentrations of copper, cadmium, chromium and the oxidative stress-inducing agents, menadione, *tert*-butyl hydroperoxide and hydrogen peroxide. Results suggest that the intermediers of the β-carotene-astaxanthin pathway more efficiently increase the stress tolerance of the fungal cells as the astaxanthin, the end-product of the biosynthesis.

Keywords

astaxanthin, carotenoid biosynthesis, *Mucor circinelloides*, oxidative stress, transformation

1. INTRODUCTION

Carotenoids pigments are widely distributed in the nature. They are important, high-value additives in the cosmetic, food, and pharmaceutical industry. Their beneficial effects on human and animal health are also well documented. Among others, their antioxidant property linked to a preventive action on various types of cancer and an enhancement of the immune response makes them important in the human diet [5, 11].

Though some of these pigments could be manufactured synthetically, the demand for exploitation of natural source is continuously increasing. Microbial production is especially promising for the orange-red ketocarotenoids (e.g. astaxanthin, canthaxanthin) not available in other cheap and exploitable natural sources [2, 8, 13]. Metabolic engineering could assist for the development of commercially utilizable microbial carotene production. Recently, de novo carotenoid biosynthesis was performed in otherwise colourless organisms, such as *Escherichia coli* [17], or *Candida utilis* [10] by introduction of bacterial carotenogenic genes.

The β-carotene producer zygomycete fungus *Mucor circinelloides* is a favoured organism when fungal carotenogenesis has to be investigated. The existence of an efficient transformation system [1, 16], the capacity to express exogenous genes [6] and the ability to grow in a yeast-like form [12] are its most attractive characteristics.

Although, oxidative stress response has been extensively studied in pro- and eukaryotes, the information about filamentous fungi is fragmentary. The main objective of the present work was to investigate the stress response of various *M. circinelloides* transformants modified in their carotenoid production.

2. THE STUDY

MS12, a *leuA*-, *pyrG*-mutant of the wild-type *M. circinelloides* strain (CBS277.49) and its transformants were used in the experiments. The *crtZ* and *crtW* genes of *Agrobacterium aurantiacum* (encoding β-carotene hydroxylase and β-carotene ketolase, respectively) [9] were used for obtaining

transformants with modified carotenoid content. Transformants MS12-Z, MS12-W and the co-transformants MS12-ZW. harboured heterologous *crtZ*, *crtW*, and both of them, respectively [14].

Pigment samples were obtained as described by Papp et al [14]. Measurements of the pigment contents and pigment compositions were carried out by recording the absorbance at 492 nm and with thin layer chromatography (TLC) or with high pressure liquid chromatography (HPLC) analysis, respectively [14].

Genetically modified *Mucor* strains with altered carotenoid content were treated with different concentrations of copper, cadmium, chromium and the oxidative stress-inducing agents menadione, *tert*-butyl hydroperoxide (tBOOH) and hydrogen peroxide. For oxidative stress experiments, 20 ml of YNB (glucose 1%, ammonium sulphate 0.15%, glutamate 0.15%, leucine and/or uracil 0.02%, agar 3%, pH 4.5) supplemented with the required stressor was poured in Petri dishes. Before inoculation each fungal species was grown on YNB for 10-14 days at 28°C. A disk was cut using a cork borer from the actively growing margin of the source of fungus and transferred to the centre of each study plate. Tolerance against stress conditions was analyzed by measuring the colony diameters after 5 days incubation (25°C). Minimum inhibitory concentration (MIC) values were taken as the stressor concentrations causing >95% growth inhibition. All experiments were carried out in triplicates.

3. ANALYSIS AND DISCUSSION

Misawa et al. [14] isolated a gene cluster responsible for the synthesis of astaxanthin from the marine bacteria *A. aurantiacum*. In a previous study, plasmid constructs with the genes *crtZ* (encoding β-carotene hydroxylase) and *crtW* (encoding β-carotene ketolase) used to transform *M. circinelloides*. These enzymes mediate the oxigenation reactions from β-carotene to astaxanthin thus allowing the formation of many intermediates of astaxanthin, i.e., β-cryptoxanthin, zeaxanthin, adonixanthin, phoenicoxanthin, canthaxanthin and echinenone [14].

The *Mucor* transformants with modified carotenoid content (Table 1.) were subjected to the effect of copper, cadmium, chromium and some chemical compounds (menadione, *tert*-butyl hydroperoxide and hydrogen peroxide). The toxic manifestations of heavy metals and certain chemicals are caused primarily due to imbalance between pro-oxidant and antioxidant homeostasis of the cells which is termed as oxidative stress. This oxidative stress is a disparity between free radical production and the antioxidant defence of the cell [3]. Carotenoids are well-known antioxidants and therefore a modified carotenoid content has to result an altered sensitivity of the cells against the oxidative damage.

Table 1. The relative carotenoid composition of the *M. circinelloides* Ms12 strain and its transformants

Strain	astaxanthin	canthaxanthin	zeaxanthin	β-cryptoxanthin	echinenone	β-carotene
MS12	-	-	+	+	-	++
MS12-Z	-	-	++	++	-	++
MS12-W	++	+	-	+	++	++
MS12-ZW	+	++	+	++	++	++

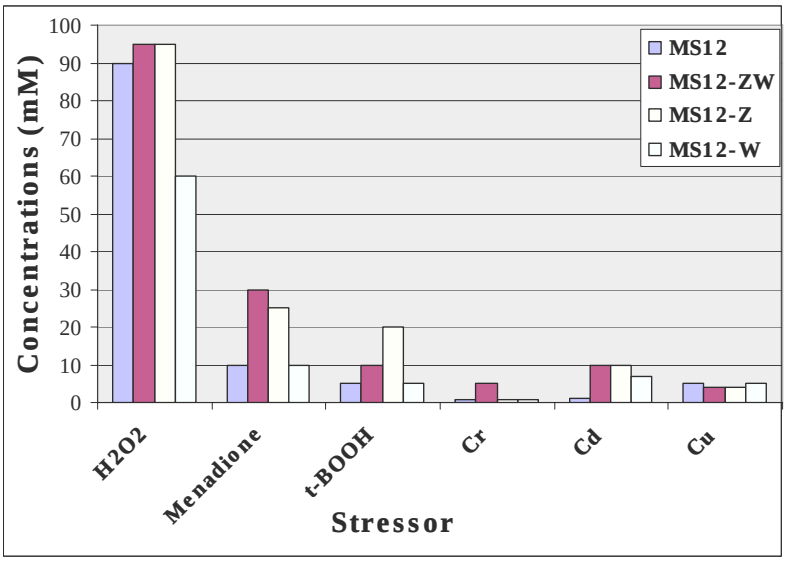

Figure 1. MIC-values against oxidative stressors for *Mucor* MS12 strain and its transformants. The values plotted represent the averages of triplicate samples. Individual values varied less than 10%

Metal induced toxicity is very well reported in the literature [7]. One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress [3]. Cadmium, unlike other heavy metals is unable to generate free radicals by itself, however, reports have indicated superoxide radical, hydroxyl radical and nitric oxide radicals could be generated indirectly [4]. Watanabe et al [18] showed generation of non-radical hydrogen peroxide which by itself became a significant source of free radicals via the Fenton chemistry. In the case of the investigated *Mucor* strains there were no difference detected against copper for the strain MS12 and its transformants. However, with chromium and cadmium treatment Ms12-ZW (containing both of the transforming bacterial genes) revealed substantially higher MIC values than the parental strain.

When oxidative stress inducing chemicals were tested, menadione and tBOOH treatment resulted in higher MIC values for MS12-ZW and MS12-Z than for MS12. There was no such difference for hydrogene-peroxide. Surprisingly, practically for all stressors MS12-W demonstrated similar or lower MIC values than the parental strain.

4. CONCLUSIONS

Several studies have shown metals like copper, cadmium, iron, mercury, nickel, lead and arsenic possess the ability to generate reactive radicals, resulting in cellular damage like depletion of enzyme activities, damage to lipid bilayer and DNA [15]. Similarly, there are a broad range of chemicals which impair cells through similar mechanisms. Carotenoids able to "quench" singlet oxygen primarily by a physical mechanism, in which the excess energy of singlet oxygen is transferred to the carotenoid's electron-rich structure: due to this feature they are well known antioxidants. The presented results reinforce that in metabolically engineered fungal cells new carotenoids express protective effect against oxidative stress. Surprisingly, various intermediers of the β-carotene-astaxanthin pathway seem to be more important from this respect than the end-product astaxanthin.

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