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Ustilago maydis accumulates β-carotene at levels determined by a retinal-forming carotenoid oxygenase

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ABSTRACT

The basidiomycete *Ustilago maydis*, the causative agent of corn smut disease, has emerged as a model organism for dimorphism and fungal phytopathogenicity. In this work, we line out the key conserved enzymes for β-carotene biosynthesis encoded by the *U. maydis* genome and show that this biotrophic fungus accumulates β-carotene. The amount of this pigment depended on culture pH and aeration but was not affected by light and was not increased by oxidative stress. Moreover, we identified the *U. maydis* gene, *cco1*, encoding a putative β-carotene cleavage oxygenase. Heterologous overexpression and *in vitro* analyses of purified enzyme demonstrated that Cco1 catalyzes the symmetrical cleavage of β-carotene to yield two molecules of retinal. Analyses of β-carotene and retinal contents in *U. maydis cco1* deletion and over-expression strains confirmed the enzymatic function of Cco1, and revealed that Cco1 determines the β-carotene content. Our data indicate that carotenoid biosynthesis in *U. maydis* is carried out to provide retinal rather than to deliver protective pigments. The *U. maydis* genome also encodes three potential opsins, a family of photoactive proteins that use retinal as chromophore. Two opsin genes showed different light-regulated expression patterns, suggesting specialized roles in photobiology, while no mRNA was detected for the third opsin gene in the same experiments. However, deletion of the *cco1* gene, which should abolish function of all the retinal-dependent opsins, did not affect growth, morphology or pathogenicity, suggesting that retinal and opsin proteins play no relevant role in *U. maydis* under the tested conditions.

1. Introduction

Carotenoids are widely spread terpenoid pigments synthesized by all photosynthetic species and many non-photosynthetic microorganisms, such as heterotrophic bacteria and fungi (Britton et al., 1998; Sandmann and Misawa, 2002; Fraser and Bramley, 2004). In photosynthetic organisms, carotenoids fulfill an essential role in protecting the photosynthetic apparatus from photooxidation and represent indispensable components of the light-harvesting and reaction center complexes (for review see, Cunningham and Gantt, 1998; Hirschberg, 2001; DellaPenna and Pogson, 2006). In addition, carotenoids are responsible for the bright colors of many fruits and flowers as well as of some animals. A well known example is the pink color of flamingos or salmons, due to the accumulation of astaxanthin. Animals are unable to synthesize carotenoids and depend on their diet to obtain these pigments, which function as radical scavengers and, more important, as precursors of the vision pigment retinal and the vertebrate morphogen retinoic acid (Blomhoff and Blomhoff, 2006). Some carotenoids are also used in cosmetics and food industry, where algae and fungi are employed as biotechnological carotenoid producers (Avalos and Cerdá-Olmedo, 2004; Del Campo et al., 2007).

Carotenoids are fat-soluble compounds consisting of an aliphatic polyene chain usually composed of eight isoprene units. Their synthesis is initiated by the condensation of two geranylgeranyl pyrophosphate units to generate the colorless carotene phytoene (Fig. 1). The following introductions of four conjugated double bonds and two β-ionone rings yield β-carotene, one of the most widely distributed of more than 600 known carotenoids. The oxidation of carotenes results in the formation of the second carotenoid sub-group, the xanthophylls.

The genes and enzymes involved in carotenoid biosynthesis have been identified in several fungi, i.e. the ascomycetes *Neurospora crassa* and *Fusarium fujikuroi*, the zygomycetes *Phycomyces*

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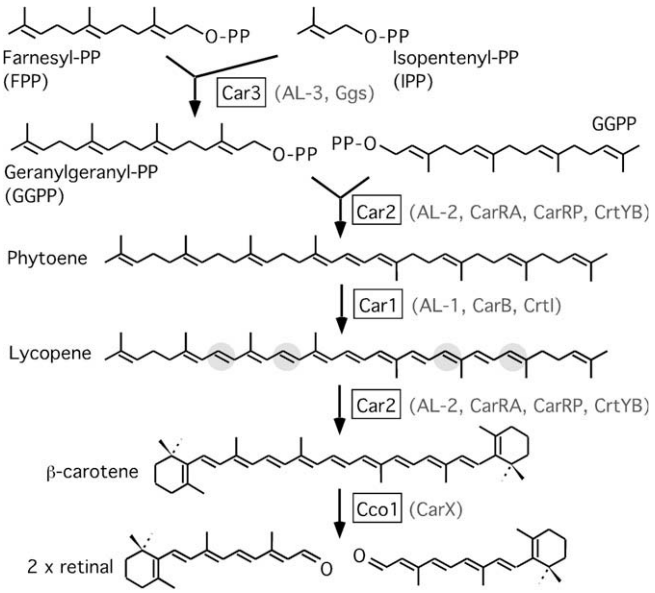

Fig. 1. Biosynthetic pathway for β-carotene (Avalos and Cerdá-Olmedo, 2004) and retinal (Prado-Cabrero et al., 2007b). Putative *U. maydis* enzymes are boxed and fungal orthologs are indicated in parentheses. Desaturation steps leading to lycopene formation are shaded. The function of Cco1 as a β-carotene-cleaving, retinal-forming enzyme is shown in this work.

blakesleeanus, *Mucor circinelloides*, and *Blakeslea trispora*, and the basidiomycete *Xanthophyllomyces dendrorhous,* formerly *Phaffia rhodozyma* (reviewed by Avalos and Cerdá-Olmedo, 2004). In contrast to photosynthetic species, fungal phytoene synthase and cyclase activities reside on a single bifunctional polypeptide encoded by a highly conserved gene, called *carRA* in *P. blakesleeanus*, *B. trispora* and *F. fujikuroi*, *carRP* in *M. circinelloides*, *al-2* in *N. crassa*, and *crtYB* in *X. dendrorhous*. The desaturation steps leading from phytoene to lycopene are catalyzed by a conserved phytoene desaturase called Al-1 in *N. crassa*, CrtI in *X. dendrorhous* and CarB in the other mentioned fungi. The sequential action of phytoene synthase/cyclase and phytoene desaturase generates β-carotene, the end-product of the pathway in the cited zygomycete species. Other fungi employ additional enzymes mediating further oxidation steps to yield different carotenoid derivatives. For instance, the carotenoid pathways in *F. fujikuroi* and *N. crassa* involve a fifth desaturation step instead of the second cyclization, followed by two oxidative reactions leading to the carboxylic apocarotenoid neurosporaxanthin (Prado-Cabrero et al., 2007a; Saelices et al., 2007; Estrada et al., 2008). In *X. dendrorhous*, β-carotene is converted into astaxanthin by introduction of keto and hydroxy groups in the β-ionone rings (Visser et al., 2003; Alvarez et al., 2006; Ojima et al., 2006).

Carotenoids are precursors of different physiologically important compounds termed apocarotenoids. The synthesis of apocarotenoids is generally initiated by cleaving of double bonds in the carotenoid backbone, catalyzed by carotenoid oxygenases (Moise et al., 2005; Auldridge et al., 2006; Bouvier et al., 2005). One of the best known apocarotenoids is retinal, which in animals is synthesized by the central cleavage of β-carotene (Wyss, 2004). Retinal serves as the chromophore of opsins, ubiquitous photoreactive transmembrane proteins (Sharma et al., 2006; Spudich, 2006). Opsins act as light-driven ion pumps and mediate different photoreceptor functions (Sharma et al., 2006), such as phototaxis in algae (Ridge, 2002; Sineshchekov et al., 2002) or vision in animals (Menon et al., 2001). Putative opsin-encoding genes occur also in fungal genomes (Brown, 2004). Many of them are potentially photoactive, as indicated by the presence of a highly conserved lysine residue required for covalent binding of the retinal chromophore. A thoroughly investigated example is *nop-1* from *N. crassa* (Bieszke et al., 1999a), which was shown to bind retinal and to undergo a photochemical reaction cycle (Bieszke et al., 1999b; Brown et al., 2001).

Carotenoids are not essential for fungi, as deduced from maintained viability upon mutational or chemical block of the pathway in different species, e.g. in *F. fujikuroi* (Avalos and Cerdá-Olmedo, 1986, 1987) and in *P. blakesleeanus* (Cerdá-Olmedo, 1987). However, carotenoids provide protection against oxidative stress (Edge et al., 1997). Consistently, hydrogen peroxide treatment results in enhanced production of astaxanthin in *X. dendrorhous* (Liu and Wu, 2006) and of neurosporaxanthin in *N. crassa* (Iigusa et al., 2005). Furthermore, carotenoid biosynthesis is stimulated by light in different fungi (Avalos et al., 1993). This induction was attributed to the protective properties of carotenoids in *N. crassa* (Yoshida and Hasunuma, 2004; Iigusa et al., 2005). In addition to the protective role, fungal carotenoids function as precursors of several compounds with distinct features and activities. In zygomycetes, β-carotene is used for the synthesis of sexual hormones, the trisporic acids (Burmester et al., 2007). Similarly, the recent characterization of the carotenoid oxygenase CarX from *F. fujikuroi* demonstrated that carotenoids also serve as retinal precursors in fungi (Prado-Cabrero et al., 2007b). The *carX* gene is a constituent of a co-regulated gene cluster harboring the two β-carotene biosynthesis genes, *carRA* and *carB*, in addition to *carO* that encodes one of the two potentially photoactive opsins of this fungus (Prado et al., 2004; Thewes et al., 2005).

In recent years, genome databases for an increasing number of fungi have become available, allowing the identification of carotenoid genes in model fungi. A relevant example is the biotrophic basidiomycete *Ustilago maydis*, the causative agent of corn smut disease (Kahmann et al., 2000). Signaling pathways involved in different developmental stages in *U. maydis* life cycle have been subjects of detailed analyses (Feldbrügge et al., 2004; Klosterman et al., 2007; Nadal et al., 2008; García-Pedrajas et al., 2008), establishing this basidiomycete as a leading model in the research of the molecular mechanisms involved in dimorphism (Sánchez-Martínez and Pérez-Martín, 2001), mating (Bakkeren et al., 2008) and other basic biological processes (Steinberg and Pérez-Martín, 2008). Furthermore, the identification of secreted effectors for pathogenic development predestined *U. maydis* to be a valuable model system for the elucidation of the molecular mechanisms of fungal biotrophy (Kämper et al., 2006).

U. maydis produces different secondary metabolites, such as ferrichrome or ustilagic acid, that contribute to its survival in natural habitats (Bölker et al., 2008). Carotenoid biosynthesis has not received attention in this fungus, probably due to the absence of a patent pigmentation. However, carotenoids were formerly found in *Ustilago violacea*, the causing agent of anther smut in *Silene alba*. Strains from this species show different colors, ranging from pink to yellow, and accumulate different mixtures of intermediates of the pathway from phytoene to β-carotene (Fig. 1; Will et al., 1984).

In this study, we investigated carotenoid biosynthesis in *U. maydis* and performed experiments to elucidate its relevance for different aspects of *U. maydis* biology. Based on sequence comparisons, we identified candidate genes responsible for the formation of β-carotene and retinal. Our data show that *U. maydis* accumulates moderate amounts of β-carotene, and that this sole detected carotenoid mainly serves as a retinal precursor. Using biochemical and genetic approaches, we identified and characterized the *U. maydis* enzyme Cco1, and show that it is responsible for the formation of retinal by mediating the symmetrical cleavage of β-carotene. Consistently, targeted deletion of *cco1* resulted in the absence of retinal and in higher β-carotene accumulation. Surprisingly, neither *cco1* deletion nor overexpression resulted in additional evident phenotypes. This indicates that retinal and the potential three photoactive opsins of