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Cleavage of resveratrol in fungi: Characterization of the enzyme Rco1 from *Ustilago maydis*

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ABSTRACT

Ustilago maydis, the causative agent of corn smut disease, contains two genes encoding members of the carotenoid cleavage oxygenase family, a group of enzymes that cleave double bonds in different substrates. One of them, Cco1, was formerly identified as a β-carotene cleaving enzyme. Here we elucidate the function of the protein encoded by the second gene, termed here as *Ustilago maydis* Resveratrol cleavage oxygenase 1 (Um Rco1). *In vitro* incubations of heterologously expressed and purified UM Rco1 with different carotenoid and stilbene substrates demonstrate that it cleaves the interphenyl Cα–Cβ double bond of the phytoalexin resveratrol and its derivative piceatannol. Um Rco1 exhibits a high degree of substrate specificity, as suggested by the lack of activity on carotenoids and the other resveratrol-related compounds tested. The activity of Um Rco1 was confirmed by incubation of *U. maydis rco1* deletion and over-expression strains with resveratrol. Furthermore, treatment with resveratrol resulted in striking alterations of cell morphology. However, pathogenicity assays indicated that *Um rco1* is largely dispensable for biotrophic development. Our work reveals Um Rco1 as the first eukaryotic resveratrol cleavage enzyme identified so far. Moreover, Um Rco1 represents a subfamily of fungal enzymes likely involved in the degradation of stilbene compounds, as suggested by the cleavage of resveratrol by homologs from *Aspergillus fumigatus*, *Chaetomium globosum* and *Botryotinia fuckeliana*.

1. Introduction

Resveratrol, 3,5,4′-trihydroxy-*trans*-stilbene (for structure, see Fig. 1), is a biphenolic phytocompound accumulated in the fruits of more than 70 plant species, including grape, peanut and several berries. Since its discovery in 1963 in Japanese knot weed (*Polygonum cuspidatum*), a traditional medicinal plant, versatile health benefits have been attributed to resveratrol. In addition to its functions in the inhibition of lipid peroxidation, the downregulation of platelet aggregations and the reduction of cholesterol levels, resveratrol prevents different stages of carcinogenesis and to modulate signal transduction cascades that mediate cellular growth, cell division, apoptosis, inflammation, angiogenesis and metastasis. Moreover, resveratrol is considered to be an anti-aging molecule prolonging the life span of several animal species (for review, see King et al., 2006; Harikumar and Aggarwal, 2008; Halls and Yu, 2008).

Resveratrol belongs to the stilbenes, a group of phytocompounds consisting of two aromatic rings joined by a methylene bridge. Like other phenylpropanoids, stilbenes are derived from cinnamic acid synthesized by phenylalanine-ammonia lyase (PAL), the branch point enzyme between primary and secondary metabolism in plants. In the next step, cinnamic acid is hydroxylated by the enzyme cinnamate-4-hydroxylase yielding *p*-coumaric acid, which is then activated by *p*-coumaroyl:CoA ligase to form the ester *p*-coumaroyl-coenzyme A (CoA). Finally, resveratrol is formed through condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA, which is accompanied by four decarboxylation steps. These reactions are catalyzed by stilbene synthases (STS), type III polyketide synthases (for review, see Halls and Yu, 2008; Dixon and Paiva, 1995).

Stilbene derivatives act as phytoalexins, antimicrobial compounds synthesized by plants in response to pathogen attack (Dixon and Paiva, 1995). For instance, the phytoalexins of grapevine and other *Vitaceae* belong mainly to the stilbene family with a *trans*-resveratrol skeleton (for review see, Jeandet et al., 2002). Moreover, several studies on various phytopathogens, like *Pyricularia oryzae*,

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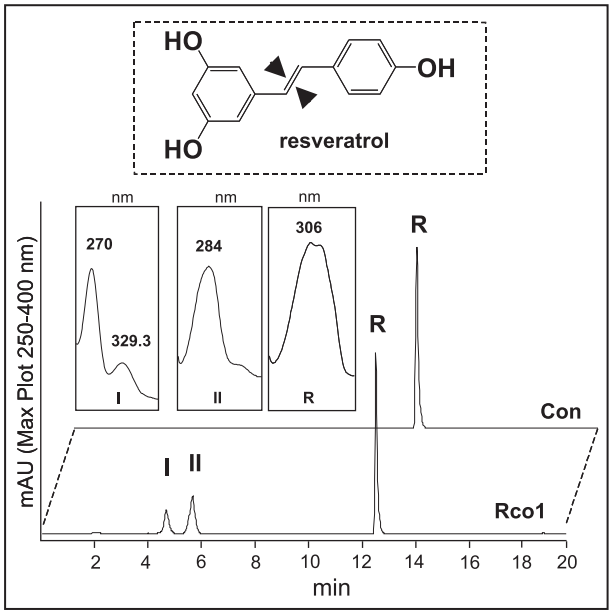

Fig. 1. HPLC analysis of incubation of purified Um Rco1 with resveratrol. The *in vitro* assay was performed for 2 h with 40 μg purified Um Rco1 (Rco1) or with the corresponding volume of the control (Con) in a total volume of 200 μl. The enzyme cleaved resveratrol (R; structure shown in the dashed inset) into the compounds I and II, supposed to correspond to 3,4-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde, respectively, indicating the cleavage of the Cα–Cβ interphenyl double bond (arrows). UV/Vis spectra of the substrate (R) and the products are shown in the insets.

Plasmopara viticola, and *Sphaeropsis sapine*, suggest antifungal activities for stilbenes (for review, see Jeandet et al., 2002). In particular, resveratrol was shown to inhibit conidial germination of the necrotrophic fungus *Botrytis cinerea* (Adrian et al., 1997), whose teleomorph is called *Botryotinia fuckeliana*. Even higher antifungal activities were reported for the stilbene derivative pinosylvin (Seppänen et al., 2004).

Ustilago maydis, the causative agent of corn smut disease, has emerged as a model system to investigate the molecular mechanisms and signaling cascades in fungal dimorphism, sexual and pathogenic development (García-Pedrajas et al., 2008; Klosterman et al., 2007; Nadal et al., 2008) as well as for studies on the intimate interactions between biotrophic basidiomycetous fungi and their host plants (Kämper et al., 2006; Brefort et al., 2009). As a facultatively biotrophic fungus, *U. maydis* encounters very different environmental conditions. The saprophytic haploid cells have to compete with other microorganisms for nutrients, while the pathogenic filamentous form has to cope with defense reactions of its host plant and redirect plant metabolism and development to satisfy its own needs and complete its life cycle (Doehlemann et al., 2008; Brefort et al., 2009). During these distinct interactions, bioactive secondary metabolites produced by the fungus play important roles. Pathways for the production of iron-chelating siderophores, tryptophan-derived indole pigments, the plant hormone indole acetic acid as well as for the synthesis of extracellular glycolipids that serve as biosurfactants with antimicrobial activity have been characterized (for review, see Bölker et al., 2008).

Like several other fungi, *U. maydis* produces β-carotene, a tetraterpene (C_{40}) pigment that serves as precursor of the visual chromophore retinal (Estrada et al., 2009). Retinal belongs to apocarotenoids, a diverse group of physiologically important compounds that includes, besides the retinal derivative retinoic acid, the fungal pheromone trisporic acid and the phytohormone abscisic acid (ABA). In general, the initial step in the synthesis of apocarotenoids is mediated by carotenoid cleavage oxygenases, which constitute a ubiquitous enzyme family catalyzing the cleavage of conjugated double bonds by the introduction of molecular oxygen (for review, see Bouvier et al., 2005; Moise et al., 2005; Auldridge et al., 2006). In addition to their role in ABA biosynthesis, plant carotenoid cleavage oxygenases are also involved in fungus-plant interactions. For instance, the development of arbuscular mycorrhiza is accompanied by the accumulation of cyclohexenone (C_{13}) and derivatives of the C_{14}-compound mycorradicin (Schliemann et al., 2008), synthesized by the plant carotenoid cleavage dioxygenase 1 (Floss et al., 2008; Sun et al., 2008). In addition, the establishment of arbuscular mycorrhiza involves further plant apocarotenoids, the strigolactones, crucial chemo-attractants for both symbiotic arbuscular mycorrhizal fungi and parasitic plants (Akiyama, 2007; Bouwmeester et al., 2007). Strigolactones act also as plant hormones that regulate shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). Biochemical characterization of carotenoid cleavage dioxygenase 8 (CCD8) enzymes from different plant species pointed to β-apo-13-carotenone as a precursor of these phytohormones (Schwartz et al., 2004; Alder et al., 2008). The C_{18}-compound β-apo-13-carotenone was also shown to be the precursor of trisporic acids in the zygomycete *Blakeslea trispora* (Schachtschabel et al., 2008). Recently, we characterized the carotenoid cleavage oxygenases involved in the synthesis of retinal in *Fusarium fujikuroi* (Prado-Cabrero et al., 2007b) and of the acidic C_{35}-apocarotenoid neurosporaxanthin in *F. fujikuroi* (Prado-Cabrero et al., 2007a) and *Neurospora crassa* (Saelices et al., 2007), and elucidated the final reactions of neurosporaxanthin biosynthesis in the latter fungus (Estrada et al., 2008a,b).

U. maydis contains two members of the carotenoid cleavage oxygenase family, encoded by the genes *um00965* and *um05084*. Recently, we reported on the *um00965* encoded enzyme, the carotenoid cleavage oxygenase 1 (Cco1), which acts as a β-carotene-cleaving and retinal-forming oxygenase that determines the β-carotene content in *U. maydis* cells (Estrada et al., 2009). In this work, we explored the function of the *um05084* gene product (accession no.: XM_756138). Based on previous studies showing cleavage of stilbene derivatives by bacterial homologs, e.g. lignostilbene by the lignostilbene-α,β-dioxygenase (LSD) from *Sphingomonas paucimobilis* (Kamoda and Saburi, 1993) and resveratrol by the enzymes Nov1 and Nov2 from *Novosphingobium aromaticivorans* DSM 12444 (Marasco and Schmidt-Dannert, 2008), we investigated the enzymatic activity of the heterologously expressed and purified *um05084* gene product designated here as *U. maydis* Resveratrol cleavage oxygenase 1, Um Rco1. Incubation with various substrates revealed a novel eukaryotic enzymatic activity mediating the cleavage of resveratrol and piceatannol at the Cα–Cβ double bond, while carotenoids and other stilbenes tested were not cleaved. This specific activity was confirmed by incubation with cell lysates from *U. maydis rco1* deletion and over-expression strains. Moreover, investigation of the activity of heterogously expressed and purified homologs from *Aspergillus fumigatus* (Af Rco1; accession no.: XP_746307), *Chaetomium globosum* (Cg Rco1; accession no.: XP_001219451) and *B. fuckeliana* (Bf Rco1; accession no.: XP_001548426) suggests that Um Rco1 represents a subfamily of fungal enzymes likely involved in the degradation of stilbene compounds. Exposure of *U. maydis* to resveratrol resulted only in slight growth inhibition, but induced dramatic alterations of cell morphology. However, these effects were not impacted by the Um Rco1 status of the cell; and pathogenicity assays indicated that *Um rco1* is largely dispensable for biotrophic development.

2. Materials and methods

2.1. Strains and growth conditions

U. maydis strains used in this study are listed in Table 1. *A. fumigatus* Af293 (FGSC A1100) was obtained from the Fungal Genetics