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Cyclooxygenases and lipoxygenases are used by the fungus *Podospora anserina* to repel nematodes

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ABSTRACT

Oxylipins are secondary messengers used universally in the living world for communication and defense. The paradigm is that they are produced enzymatically for the eicosanoids and non-enzymatically for the isoprostanoids. They are supposed to be degraded into volatile organic compounds (VOCs) and to participate in aroma production. Some such chemicals composed of eight carbons are also envisoned as alternatives to fossil fuels. In fungi, oxylipins have been mostly studied in *Aspergilli* and shown to be involved in signalling asexual *versus* sexual development, mycotoxin production and interaction with the host for pathogenic species. Through targeted gene deletions of genes encoding oxylipin-producing enzymes and chemical analysis of oxylipins and volatile organic compounds, we show that in the distantly-related ascomycete *Podospora anserina*, isoprostanoids are likely produced enzymatically. We show the disappearance in the mutants lacking lipoxygenases and cyclooxygenases of the production of 10-hydroxy-octadecadienoic acid and that of 1-octen-3-ol, a common volatile compound. Importantly, this was correlated with the inability of the mutants to repel nematodes as efficiently as the wild type. Overall, our data show that in this fungus, oxylipins are not involved in signalling development but may rather be used directly or as precursors in the production of odors against potential agressors.

Significance: We analyzse the role in inter-kingdom communication of lipoxygenase (lox) and cyclooxygenase (cox) genes in the model fungus Podospora anserina.

Through chemical analysis we define the oxylipins and volatile organic compounds (VOCs)produce by wild type and mutants for cox and lox genes,

We show that the COX and LOX genes are required for the production of some eight carbon VOCs.

We show that COX and LOX genes are involved in the production of chemicals repelling nematodes.

This role is very different from the ones previously evidenced in other fungi.

1. Introduction

The complete arsenal of molecules used by microorganisms to sense and adapt to their biotic and abiotic environment is far from being known. Oxylipins derived from the oxidation of polyunstaurated fatty acids (PUFAs) are used throughout the plant, animal and fungal kingdoms to signal defense mechanisms, but also development and reproduction [1]. In fungi, their roles are well studied in the genus *Aspergillus* mostly through the deletion of cyclooxygenase genes (Table 1) and in this genus oxylipins are at the crossroads of several biologically-significant mechanisms, such as reproduction and growth, pathogen interactions and secondary metabolite production [2, 3]. Few genes involved in oxylipin production have been analyzed in other fungi and developmental roles may or may not be found in the studied

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Table 1
Lipoxygenase and cyclooxygenase genes in selected fungal genomes.

	Lox Val-group	Lox Ile-group	Cox	Abm	Phenotypes	References
Podospora anserina		**PaLox1** (Pa_2_4370) **PaLox2** (Pa_6_8140)	**PaCox1** (Pa_1_4690) **PaCox2** (Pa_5_1240)		See text.	This paper
Magnaporthe grisea	MGG_08499		**MGG_13239** MGG_10859	**MGG_04777**	MGG_13239: No phenotype for mycelium, conidia, appressoria and plant invasion. MGG_04777: reduced invasive growth in rice	[9]
Fusarium verticillioides	FVEG_05726	FVEG_09897 FVEG_03347	FvLDS1a (FVEG_09294) **FvLDS1b** (FVEG_09294) FVEG_11670 FVEG_12540		Thin, leathery & pink mycelium; conidia more numerous with faster germination, fewer perithecia when crossed with WT, enhanced virulence.	[58]
Aspergillus nidulans			**ppoA** (AN1967) **ppoB** (AN6320) **ppoC** (AN5028)		Alteration of the ratio between cleisthothecia *vs* conidia.	[7]
Aspergillus flavus	**XP_002379215**		**ppoA** (ACO57610) **ppoB** (ACO57611) **ppoC** (ACO57612) **ppoD** (ACO57613)		Alteration of the ration of sclerotia *vs* conidia.	
Aspergillus fumigatus	Afu4g02770	Afu7g00860	**PpoA** (Afu4g10770) **PpoB** (Afu4g00180) **PpoC** (Afu3g12120)		PpoC alone:Fewer conidia with faster germination, altered stress resistance PpoA, PpoB: no phenotype PpoABC-RNAi: increased virulence & altered stress response.	
Ustilago maydis			**Ssp1** (Ustma1_4571)		No phenotype for yeast morphology, teliospore formation, mating and plant invasion.	[59]

This table gives the genes present in selected fungal genomes. Darkened cell: no gene could be found by BLAST analysis. In bold, genes that have been inactivated by targeted gene deletion or in the case of the triple *PpoABC* mutant of *A. fumigatus* by RNAi.

fungi (Table 1). For the model ascomycete *Aspergillus nidulans*, it has been shown that specific oxylipins, known as Precocious Sexual Inducers (psi factors), play a crucial role in the balance between sexual and asexual reproduction [4, 5]. Similar roles were observed in *Aspergillus flavus* [6, 7], *Aspergillus fumigatus* [8] and *Fusarium verticillioides* [9]. In *Trichoderma atroviride*, oxylipins have been hypothesized to play a role in wounding response [10], and in *Ascocoryne sarcoides* in the production of eight-carbon volatile organic compounds (VOCs) [11]. Many microorganisms, both prokaryotic and eukaryotic, generate VOCs [12, 13]. Fungal VOCs can have a variety of applications ranging from the control of bacteria and fungi [14] to clean biofuels [15]. Like oxylipins, in nature, VOCs are responsible for inter- and intra-organismic communication, leading to attraction, repulsion, as well as growth and differentiation enhancement [16]. The volatile emission profile is a consequence of specific metabolic activities of each microorganism. Fungi produce VOCs as mixtures of alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and derivatives originating from a variety of precursors [17]. They especially synthesize many VOCs with eight carbons that are responsible for the fungal odor [18, 19]. The exact pathways used by fungi to produce these eight-carbon VOCs are not well-known [20, 21] and fungi, like plants, may utilize PUFAs and/or oxylipins to produce volatile compounds, because PUFAs may first be oxidized and then cleaved to produce the short-chain volatiles [21].

Oxylipins are synthesized in two ways, directly chemically by reactive oxygen species (ROS) [22] and indirectly by enzymes belonging to the dioxygenase family like cyclooxygenases (COX) [22], lipoxygenases (LOX) [22] or the monooxygenase family like the recently-discovered Abm monooxygenase [23]. Eicosanoids are supposed to be produced mainly enzymatically, while isoprostanoids mainly non-enzymatically [24–27]. Presently, the exact contribution of the enzymatic *versus* non-enzymatic oxidation of lipids *in vivo* is not well known, nor it is proven that oxylipins are indeed important precursors of VOCs in fungi [21, 28]. Here, we describe the role of two LOX and two COX by systematic targeted gene deletion in the production of oxylipins and VOCs, as well as in the general physiology of the model fungus *Podospora anserina*. This fungus inhabits herbivore dung and, thanks to its rapid and easy culture and manipulation in the laboratory, is frequently used to rapidly address the role of genes [29]. We provide evidence that (1) this fungus produces isoprostanoids by an enzymatic route, (2) oxygenases are necessary for the production of some of the eight-carbon VOCs, especially 1-octen-3-ol, and (3) oxylipins and/or VOCs are used by the fungus to repel nematodes.

2. Materials and methods

2.1. Strains and growth conditions

The *P. anserina* strains (Table S1) used in this study derived from the "S" (uppercase S) wild-type strain [30] used for sequencing [31, 32]. Standard culture conditions, media and genetic methods for *P. anserina* have been described [29, 33]. The M2 medium had the following composition KH_2PO_4 0.25 g/l, K_2HPO_4 0.3 g/l, $MgSO_4$/7H2O 0.25 g/l, Urea 0.5 g/l, Thiamine 0.05 mg/l, Biotine 0.25 µg/l, Citric Acid 2.5 mg/l, $ZnSO_4$ 2.5 mg/l, $CuSO_4$ 0.5 mg/l, $MnSO_4$ 125 µg/l, Boric Acid 25 µg/l, Sodium Molybdate 25 µg/l, Iron Alum 25 µg/l, Dextrine 5 g/l, Agar 12,5 g/l. The paper medium has the same composition as M2 except that dextrin was replace by 3 cm × 3 cm "3 MM" Whatman paper. M0 is the same as M2 except that dextrin is omitted. The N2 Bristol *C. elegans* strain was raised on *Escherichia coli* OP50.

2.2. Chemicals

9-hydroxy-octadecadienoic acid (9-HODE), 10-hydroxy-octadecadienoic acid (10-HODE) and 13-hydroxy-octadecadienoic acid (13-HODE) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). 16-F_{1t}-phytoprostanes, and 9-F_{1t}-phytoprostanes were synthesized according to published procedures [34–36].

2.3. Gene deletions and phenotypic analysis

The COX and LOX genes were deleted as described [29]. The primers used are reported in Table S2. After verification of the deletions by Southern blot analyses (Fig. S1), two successive crosses of the mutants