Characterization of oxylipin signaling in the chemical interaction between the endophyte Paraconiothyrium variabile and the phytopathogen Fusarium oxysporum

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Caractérisation de la signalisation par les oxylipines dans la communication chimique entre l'endophyte *Paraconiothyrium variabile* et le phytopathogène *Fusarium oxysporum*

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Characterization of oxylipin signaling in the chemical interaction between the endophyte *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum*

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Abreviations list

13-HPODE : 13-hydroperoxyoctadecadienoic acid

13-oxo-ODE : 13-hydroxyoctadecadienoic acid

BEA : beauvericin

BLAST : basic local alignment search tool

cDNA : complementary DNA

COX : cyclooxygenase

CP450 : cytochrome P450

CRISPR : clustered regularly interspaced short palindromic repeat

DAPI : 4',6'-diamino-2-phenylindole

DOX : dioxygenase

DSBs : double strand breaks

f. sp : *formae specialis*

FeLOX : iron lipoxygenase

GFP : green fluorescent protein

GPCR : G-protein coupled receptor

hph : hygromycin phosphotransferase gene

HPOTrE : hydroperoxyoctadecatrienoic acid

HR : homologous recombination

IPTG : isopropyl-Beta-d-Thiogalactopyranoside

JA : jasmonic acid

LC-MS : mass spectrometry coupled to liquid chromatography

LCP : Fungal Strain Collection, Laboratory of Cryptogamy (MNHN)

LOX : lipoxygenase

MNHN : Museum of Natural History

MnLOX : manganese lipoxygenase

MOTUs : Molecular operational taxonomic units

mRNA : messenger RNA

NHEJ : non-homologous end joining

NRPS : non-ribosomal peptide synthetase

ORF : open reading frame

PCR : polymerase chain reaction

PDA : potato dextrose agar

PEG : polyethylene glycol

PMT : protoplast mediated transformation

PUFAs : polyunsaturated fatty acids

RNAi : RNA interference

RT-qPCR : retro-transcription quantitative PCR

SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis

sgRNA : single guide RNA

tRNA : transfer RNA

Prelude

1. *P. variabile*, a "multi-faceted" endophyte studied in the laboratory

Almost all plants lineages, from all ecosystems live in close association with microorganisms such as bacteria, fungi and viruses (Hardoim *et al.,* 2015). Some of them colonize plant tissues without visible symptoms, and have gained strong interest among the scientific community since they have been shown to protect plants against various aggressions (Rodriguez *et al.,* 2009). Such microorganisms are termed "endophytes". Recently, the idea has emerged that selective forces act on the whole plant, including its associated microbial community (microbiota), and that the plant phenotype is an extended phenotype of its microbiota (Rosenberg *et al.,* 2009). Plant fitness, that is, its ability to survive and reproduce in an environment, is strongly linked with its microbiota, and collectively they form a "holobiont" (Vanderkoornhuyse *et al.,* 2015).

However, foliar endophytes are poorly investigated and our apprehension of their ecological role in plant communities is biased by the fact that a high percentage of microorganisms are not culturable (Amann *et al.,* 1995). Progress of sequencing technologies, indeed, allowed "decoding" of plant microbiota assemblages and has led to the accumulation of an enormous amount of sequencing data. "Omic" tools are offering very promising prospects in the study of endophyte-plant interactions (Kaul *et al.*, 2016). These data are mostly descriptive and need additional information about physiological functions of these microorganisms within the plant holobiont. Studying microbe-microbe interactions is therefore a necessary step before addressing more complicated questions about plant microbiomes (Figure P-1).

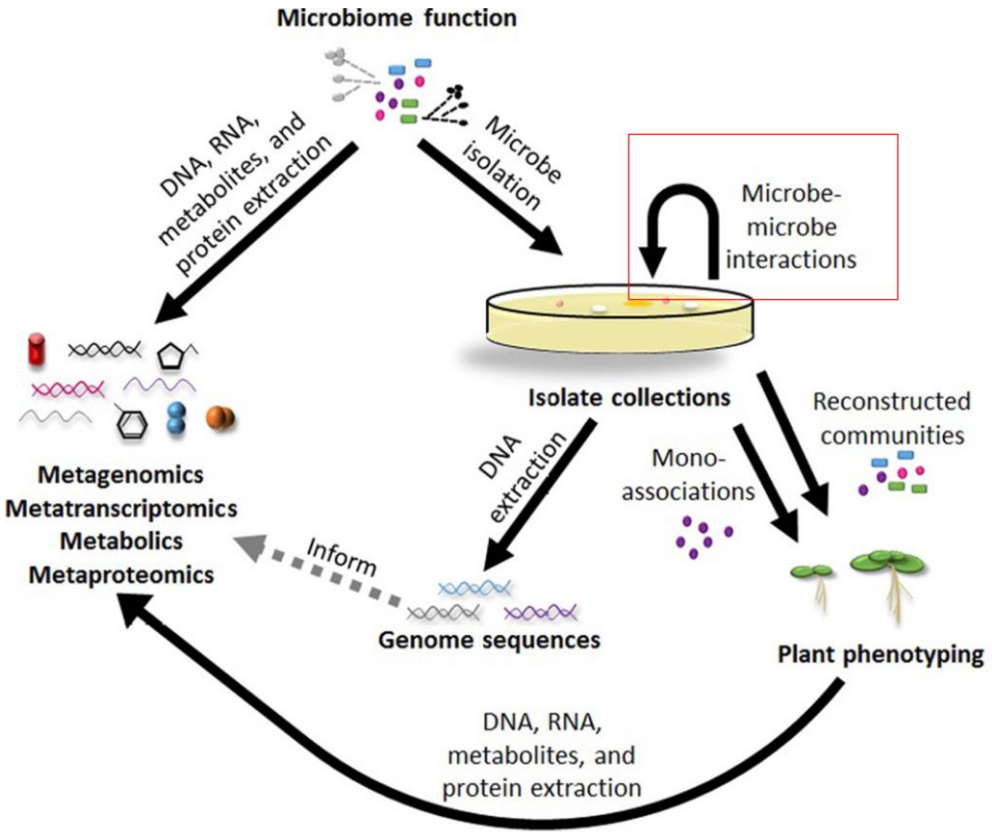


Figure P-1: Proposed combinatorial methods to address functions of plant microbiomes (Lebeis, 2014). Identification of microbial community members, analysis of their interactions, and characterization of their impact on the plant phenotype, collectively form a "pipeline" to decipher microbiome function.

The difficulty in a more functional approach to comprehend interactions within the plant's microbiota, is to choose the microorganisms to analyze, within their vast diversity, and to decide on a plant host. One can either select agronomically relevant species, plant models or poorly investigated taxons to decipher physiological, genetic and biochemical traits of microbe-microbe communication within the plant host. This knowledge is necessary to pinpoint mechanisms responsible for plant protection against pathogens.

I will clarify below our choice to dissect the interaction between the foliar endophyte *Paraconiothyrium variabile* and the ubiquitous and common soil-born pathogen *Fusarium oxysporum*.

1.1 Cultivable microbiota from the conifer *Cephalotaxus harringtonia* and discovery of the endophyte *Paraconiothyrium variabile*

Cephalotaxus harringtonia (Japanese Plum Yew) is an Asian medicinal plant rich in cytotoxic compounds well-known to our laboratory, the "Chemistry of Fungal Natural Products (CPNF)" team (figure P-2). *C. harringtonia* produces a highly diverse set of secondary metabolites in the alkaloid series (Bocar *et al.,* 2003 - CPNF), diterpene and flavonoid series (Abdelkhafi & Nay, 2012 - CPNF), and some of them have already been characterized for antifungal activities against *Candida tropicalis* (Evanno *et al.,* 2008 - CPNF). A project was launched in the CPNF laboratory in collaboration with J. Dupont ("Diversité, Evolution Végétale et fongique", UMR 7205 CNRS/MNHN), in the perspective of acquiring a large collection of culturable endophytes from the compelling, but little studied conifer tree *C. harringtonia*. Langenfeld *et al.* (2013) sampled foliar and stem endophytes to further investigate their chemistry, their interactions with pathogens, and their functional diversity (Figure P-2).

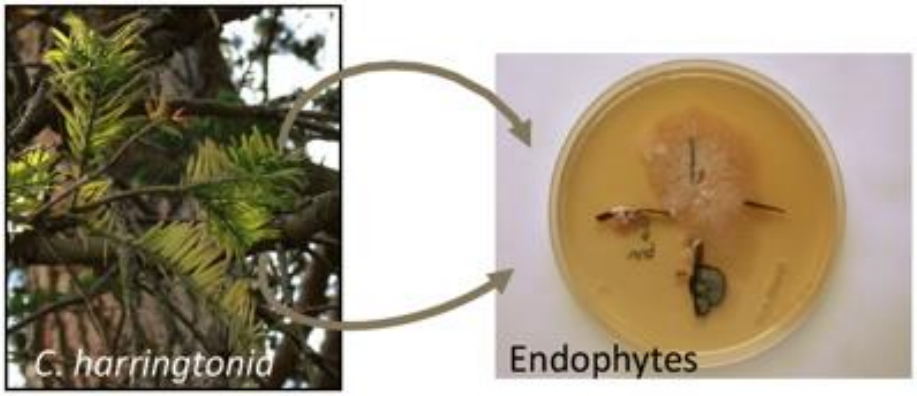


Figure P-2: Isolation of endophytes from *C. harringtonia* (From *Langenfeld et al.,* 2013 - CPNF). Stem and needle fragments were surface-sterilized and plated on 2% malt extract agar.

They isolated endophytes from needles and stems from different individuals, some of which were localized in Japan within the native area of the species, and others that had been introduced into France. From 554 isolates, 104 Molecular Operational Taxonomic Units (MOTU) were identified and 52 were named at the species level. The majority of endophytes isolated were Sordariomycetes (62.5 %) with Dothideomycetes the next most common group (24%). *Paraconiothyrium* sp. (Pleosporales) isolates were found in both Japan and France but they belonged to distinct species. *Paraconiothyrium variabile* occurred with high frequencies in France, in conifer trees sampled from urban and rural sites, and was not observed in the neighbouring tree species (unpublished data).

P. variabile is an Ascomycota, Pezizomycotina, Dothideomycetes, Pleosporomycetidae, Pleosporales (see Appendix 1 for a phylogenetic tree). The mycelium is slowly developing, from whitish to dark brown and it forms dark pycnidia (fruiting body), containing small conidia which are the asexual spores (2-4 µm, see figure P-3). *P. variabile* was first described by Damm *et al.*. (2008), who isolated several *Paraconiothyrium* species (*P. africanum, P. brasiliense, P. hawaiiense* and *P. variabile*) on stone fruit trees (*Prunus sp*) and other woody hosts. The genus *Paraconiothyrium* belongs to the order Pleosporales which contains several dominant fungal tree endophytes (Arnold, 2007; Sieber, 2007) but also important necrotrophic plant pathogens such as *Stagonospora nodorum* on wheat and *Cochliobolus heterostrophus* on maize. In the same genus, we also find *P. minitans* (previous name *Coniothyrium minitans*), which hyperparasitises sclerotia from *Sclerotinia* rots and is used as a biological control agent (Contans®, Bayer; Mathieu & Von Erfia, 2008). However, *P. minitans* can also cause wood rot of birch and pine (Nilsson, 1973). In the same way, *P. variabile* has also been reported to cause leaf spot disease on *Phoenix theophrasti,* a palm species extensively used as ornamentals in Greece (Ligoxigakis *et al.*, 2013). Diverse lifestyles can therefore be attributed to different species of the genus *Paraconiothyrium* and within the species itself. Recently, *P. variabile* has been isolated from coral reefs in La Réunion (Fouillaud *et al.,* 2017).

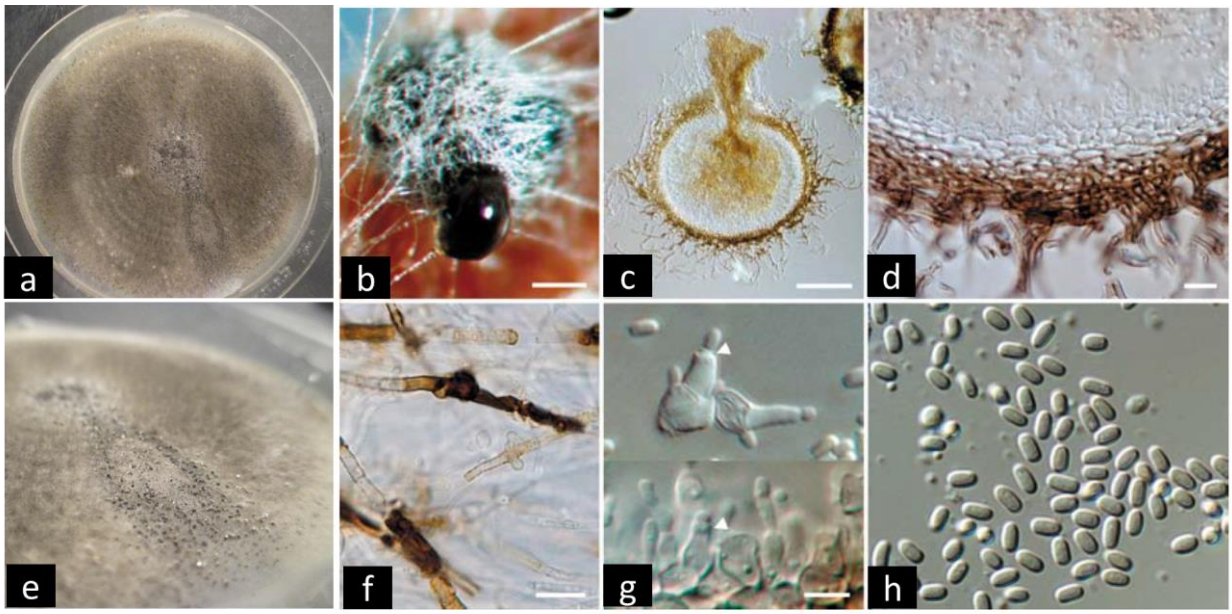


Figure P-3: *Paraconiothyrium variabile* . (a) 20-day old colony on potato dextrose medium; (b) conidia oozing from pycnidia; (c) longitudinal section through a pycnidium; (d) pycnidial wall with conidiophores; (e) focus on pycnidia; (f) dark-walled hyphae; (g) conidiophores; (h) conidia. Scale bars: b,c =100 µm; d, f=10 µm; g,h = 5 µm. Pictures b, c, d, f, g, h are credited to Damm *et al.,* 2008.

Our team demonstrated that out of the hundred metabolites produced by *C. harringtonia*, the endophyte *P. variabile* was able to metabolize three different flavonoids from the plant (Tian *et al.,* 2014). Interestingly, those flavonoids were deglycosylated to obtain two aglycones (apigenin and chrysoeriol) and both compounds were found to promote the growth of *P. variabile*. Apigenin has been reported to possess antibacterial activity (Cushnie & Lamb, 2005), highlighting here an advantage to the plant in hosting *P. variabile*. It nicely demonstrates how the endophyte can metabolize the plant's compounds and how both partners can benefit from it.

Combès *et al.,* (2012) investigated the antagonistic effect of *P. variabile* towards several pathogens including *Sphaeropsis sapinea*, *Alternaria alternata*, *Fusarium moniliforme* and *Fusarium oxysporum*. They were all inhibited by the endophyte in dual assays on solid medium as illustrated in figure P-4.

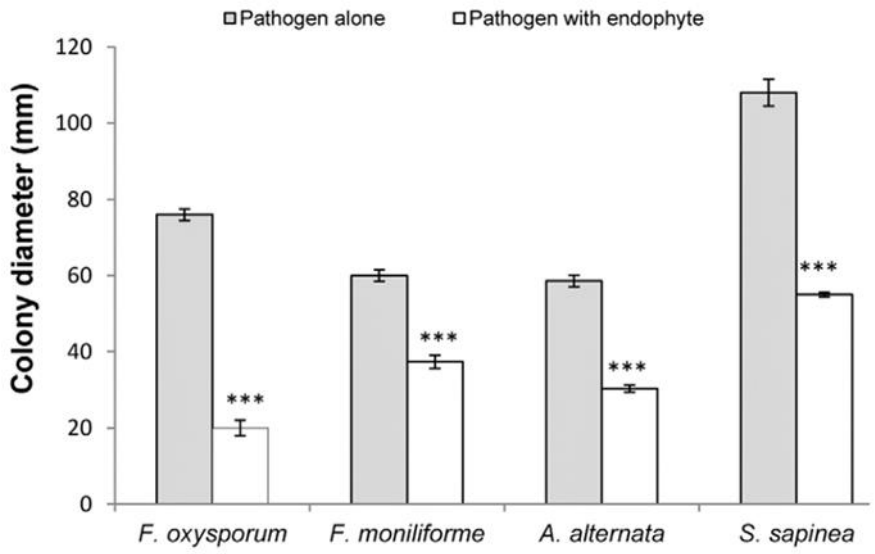


Figure P-4: Antagonism observed in dual culture of *P. variabile* with different strains of phytopathogens (from Combès *et al.*, 2012). Asteriks indicate a statistically significant decrease of the growth of the phytopathogen in the presence of the endophyte by t-test (***p<0.001). F. = *Fusarium*, A. = *Alternaria*, S. =*Sphareopsis*.

The strongest antagonism was achieved towards the phytopathogen *Fusarium oxysporum f.sp medicaginis*. *Fusarium* is a genus of filamentous Ascomycota (Sordariomycetes) that encompasses many toxin-producing plant phytopathogens (Ma *et al.,* 2013). Owing to its broad host range (>100) and economic importance, *F. oxysporum* has been rated among the top 10 fungal pathogens (Dean *et al.,* 2012). It provokes severe crop losses in melon, tomato, banana and cotton, among others,

due to vascular wilting and defoliation, eventually leading to plant death. The *F. oxysporum* species complex includes different *formae specialis (f. sp.),* named according to their ability to cause disease on specific host plants (Armstrong, 1981).

1.2 Interaction between *P. variabile* and *Fusarium oxysporum*

In order to identify potential metabolites responsible for the antagonism of *P. variabile* against *F. oxysporum*, the main compounds isolated from *P. variabile* (organic extract) were tested alone or in combination (Combès *et al.,* 2012), as well as the crude extract, but none of them were effective. In the same way, the hypothesis that volatile organic compounds could be involved was rejected. Finally, the LC/MS profiles of the metabolites produced by each strain in competition were compared with the metabolites produced by each strain alone, in order to identify specific metabolites deregulated in the interaction (the principle is illustrated on figure P-5).

Interestingly, two compounds were specifically up-regulated in the crude extract of *P. variabile* in competition and could be identified as 13-oxo-9,11-octadecadienoic acid (13-oxo-ODE) and 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE). Their structures are given on figure P-6, A.

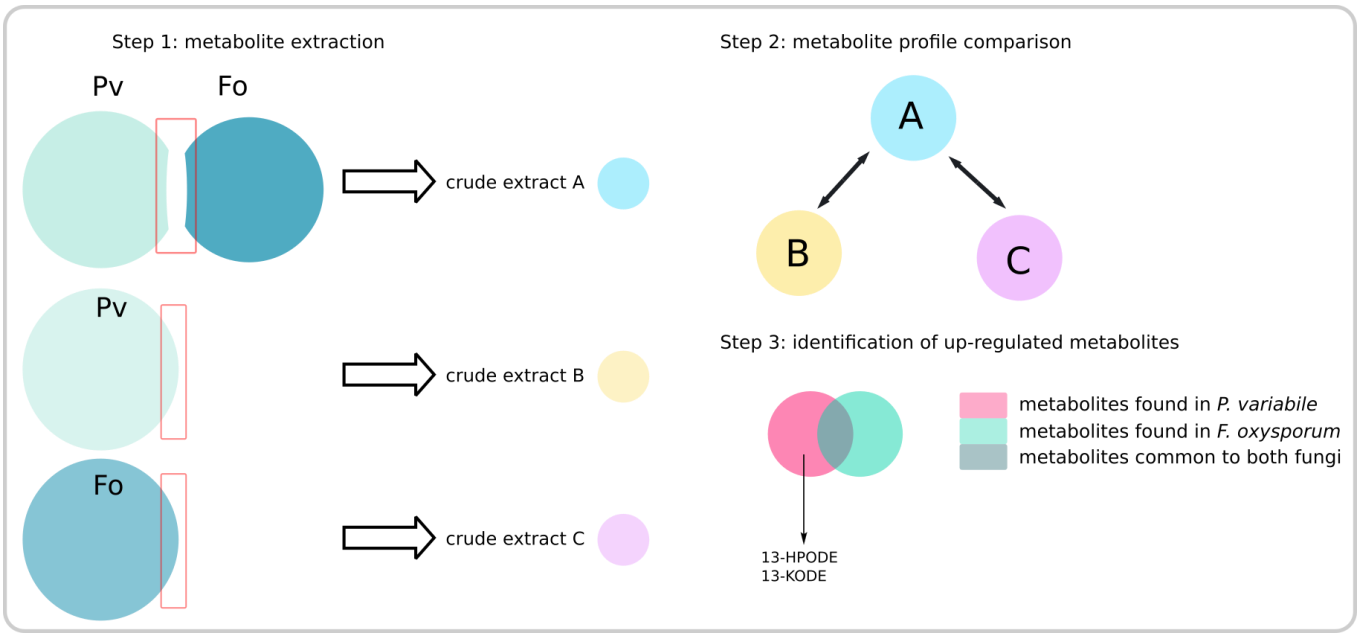


Figure P-5: Comparison of metabolomic profiles from *P. variabile*, *F. oxysporum* and the competition zone. Step 1 illustrates the sampling zones of mycelia for metabolite extractions. Each crude extract was then analyzed by LC-MS and the metabolomic profiles were compared two-by-two, to identify the upregulated metabolites. Within the upregulated metabolites, those belonging to *F. oxysporum* or common to both fungi were put aside to focus on metabolites specifically induced in *P. variabile*. Among those, 13-HPODE and 13-oxo-ODE were identified.

The two metabolites, 13-HPODE and 13-oxo-ODE, belong to the well-known family of oxylipins, which are oxygenated fatty acids. Oxylipins are ubiquitous molecules produced by eukayotes and prokaryotes, and they have been extensively studied as lipid mediators involved in inflammation responses in mammals and defense mechanisms in plants (Fisher & Keller, 2016). In fungi, they have been described to regulate (positively or negatively) the synthesis of secondary metabolites such as the mycotoxin sterigmatocystin in *Aspergillus nidulans* (Tsitsigiannis *et al.,* 2005). In this context, Combès *et al.* explored the modulation of mycotoxin production by *F. oxysporum* alone or in competition with *P. variabile*, and found a 90% decrease in beauvericin concentration (Figure P-6, D). To a lesser extent, 13-oxo-ODE (13-HPODE was not tested) also diminished beauvericin production by *F. oxysporum*. Beauvericin (BEA) shown in figure P-6, is a cyclic hexadepsipeptide mycotoxin (Hamill *et al.,* 1969), produced by many different *Fusarium* species (Logrieco *et al.,* 1998). It exhibits antibacterial and antiviral activities (Wang & Xu, 2012), in addition to its mammalian toxicity (Lemmens-Gruber *et al.,* 2000). Zhang *et al.,* (2007) also reported the antifungal acitivity of beauvericin in combination with ketoconazole towards *Candida albicans*. Kouri *et al.* (2002) provided experimental evidence of its cellular toxicity and claimed that BEA could integrate into cellular membranes and act as a ionophore, disturbing the physiological concentration of important cations. It could explain its non-specific toxicity towards mammals, plants, insects and microbes. Recently, BEA was shown to act as a virulence factor on tomato plants (Lopez-Berges *et al.,* 2013). Inhibition of this mycotoxin during the infection process of the phytopathogen could therefore be advantageous for plants.

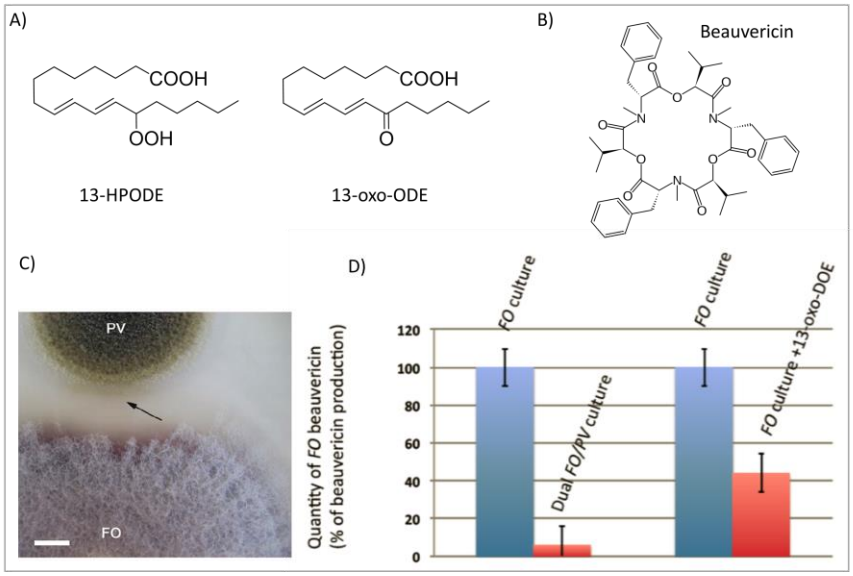


Figure P-6: *P. variabile* – *F. oxysporum* interaction, results from Combès *et al.,* (2012). A) Structures of the two oxylipins induced in the dual zone. **B)** Structure of the mycotoxin beauvericin. **C)** Mutualistic

antagonism of *P. variabile* and *F. oxysporum* on potato dextrose agar. Scale bar = 1 cm. D) Decrease of beauvericin production in *F. oxysporum* in the presence of *P. variabile* or 13-oxo-ODE.

1.3 Genome sequencing of *P. variabile* and identification of two lipoxygenase genes

In October 2013, the CPNF team decided to advance the research on the endophytic fungus *P. variabile* and to sequence its genome. The original *P. variabile* isolate from a needle of *Cephalotaxus harringtonia* is maintained at the LCP culture collection of the MNHN (Paris) under the number LCP5644. A monospore strain (MS1) from LCP5644 strain was obtained to have pure starting material. High quality DNA was then extracted in large amounts from strain MS1 for the genome sequencing accomplished by BGI Tech Solutions Co., Ltd (China) in December 2014. The *P. variabile* MS1 data was assembled by BGI and showed a genome size of 40.42 Mb, GC content of 51.68 % and a number of scaffolds and contigs of 453 and 517, respectively. The genome was not annotated.

Oxylipins are formed by diverse enzymatic reactions. 13-oxo-ODE and 13-HPODE in particular are synthesized by lipoxygenase enzymes (LOX). The best characterized fungal lipoxygenase producing 13-oxylipins is the 13-LOX from *Gaeummanomyces graminis* (Q8X150.2). Therefore, during my Masters study, orthologs for *13-lox* genes were searched with BLAST (Basic Local Alignment Search Tool) in the genome of *P. variabile*. We found two ORFs (Open reading frames) on two different contigs, encoding two hypothetical lipoxygenase proteins. One coding for a protein with 36% identity to the *G. graminis* MnLOX, that will be named *pvlox1* (2062 bp), the other coding for a protein with a sequence identity of 39%, that will be named *pvlox2* (2168 bp). In *F. oxysporum*, two LOXs producing 13-HPODE have been purified and characterized, and a third putative LOX mentioned. Their features are given in table P-1. Since *F. oxysporum f. sp medicaginis* has been sequenced, orthologs for those identified *lox* genes will be searched in our isolate.

Locus tag (NCBI)	Organism	Corresponding enzyme	Accession number	Products from linoleic acid	Reference
FOXG_0487	*F. oxysporum f. sp lycopersici* 4287	FoxLOX	KNB01601.1	13*S*-HPODE 9*R*-HPODE	Brodhun *et al.*, 2013
FOXB_09004	*F. oxysporum* Fo5176	Fo-MnLOX	F9FRH4.1	11*R*-HPODE 9(*R/S*)-HPODE 13*S*-HPODE	Wennman *et al.*, 2015
FOXG_02545	*F. oxysporum f. sp lycopersici* 4287	F. oxsyporum-LOX1	KNA98118.1	Not characterized	Brodhun *et al.*, 2013

Table P-1: Lipoxygenases enzymes characterized in *F. oxysporum*

2. Working hypothesis

To date, an extensive bibliography can be found on the role of oxylipins in cross-kingdom interactions (Tsitsigiannis *et al.,* 2007; Christensen & Kolomiets, 2011; Pohl & Kock, 2014., Fisher & Keller, 2016), especially in plant - pathogen interactions, but no work has focused on the issue of oxylipin signaling in microbe-microbe interactions. How fungal endophytes communicate with invading plant pathogens, and what role oxylipins play in this "chemical language" is the topic of my research project. In particular, I will study the role of oxylipins in the crosstalk between *P. variabile* and *F. oxysporum*. Based on the results presented above and results obtained during my Master thesis, we propose the following working hypothesis (Figure P-7): **the presence of *F. oxysporum* induces in *P. variabile* biosynthesis genes responsible for oxylipin production, which in turn inhibit beauvericin synthesis in *F. oxysporum*.**

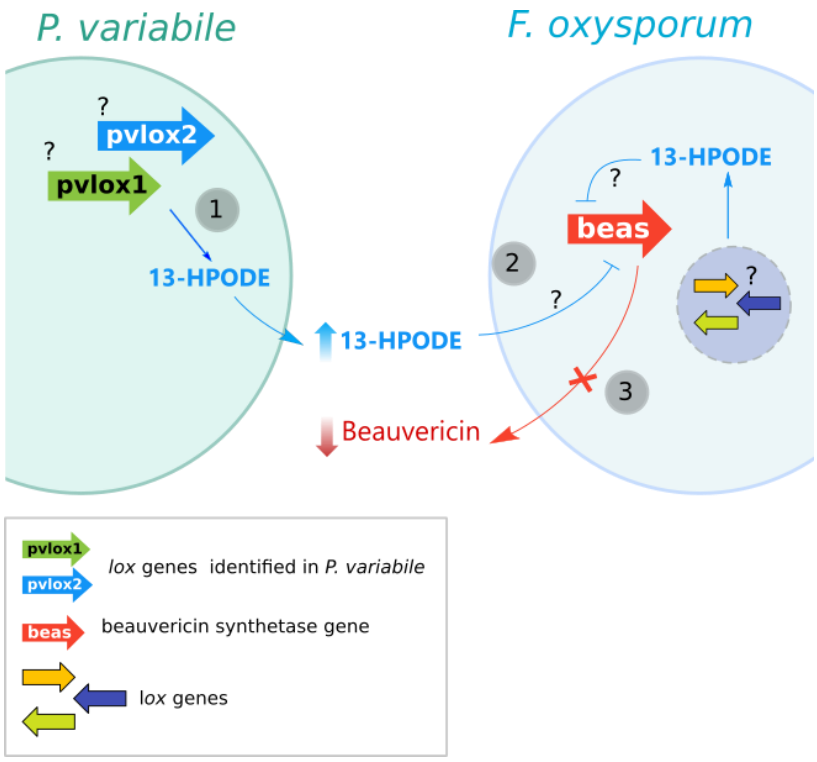


Figure P-7: Working hypothesis model. **1:** lipoxygenase genes *pvlox1* and/or *pvlox2* in *P. variabile* are induced during the interaction which leads to higher amount of 13-HPODE in its mycelium. **2:** 13-HPODE repress *beas* gene encoding beauvericin synthetase in *F. oxysporum*. **3:** Lower amounts of beauvericin are synthesized and secreted. Possible induction of *lox* genes in *F. oxysporum f. sp. medicaginis* is shown.

3. Thesis objectives

The objective of the present work was to understand the mechanisms leading to beauvericin decrease in the interaction between *F. oxysporum* and *P. variabile*, and explore the possible role of oxylipins in mycotoxin modulation.

In this perspective, my first project was to establish a genetic transformation system to obtain oxylipin deficient mutants (⊿*pvlox1* and ⊿*pvlox2*) in the endophyte *P. variabile*, and in the long run, a genetic tool to study in more depth the functional role of this foliar endophyte.
The results will be shown and discussed in Chapter I.

In parallel, my second project was to develop a heterologous expression system to characterize biochemically the two putative endophytic lipoxygenases PVLOX1 and PVLOX2 with a two-fold-objective: (i), confirm the biosynthesis of 13-HPODE and 13-oxo-ODE by these enzymes and (ii), get new insights into the sequence-to-structure relation in fungal LOXs.
The results will be shown and discussed in Chapter II.

Finally, my third project was to characterize the transcriptional regulation of *lox* genes in both partners, and of the beauvericin synthetase gene in *F. oxysporum* by a RT-qPCR approach. In parallel, I assessed the corresponding metabolites, oxylipins and beauvericin, produced by the antagonists, by LC-MS. An underlying project was to determine beauvericin toxicity for the endophyte *P. variabile*.
The results will be shown and discussed in Chapter III and will give rise to a publication.

Introduction

1. Endophytes : symbiotic lifestyles and ecological success

The first definition of endophytes is attributed to Petrini (1991), who qualified them as organisms inhabiting plant organs for at least part of their life cycle, without causing apparent harm to their host. The word "symbiosis" will be used here to describe the long-term biological interaction of unlike organisms living together in close physical associations, including mutualists, parasites, and commensalists (De Bary, 1879; Hentschl *et al.,* 2000)

1.1 Endophytes, who are they?

Endophytes are mutualistic organisms (reciprocally beneficials) found entirely in plant tissues, unlike mycorrhizal fungi that are rather "root extensions" for the plant. Rodriguez *et al.* (2009), in an extensive review, proposed a classification of endophytes into four major groups, based on their host range, plant colonization pattern and role in plant ecophysiology. These four groups are illustrated on figure I-1. Clavicipitaceous endophytes are surely the most described endophytes as they show significant agricultural impact (Saikkonen *et al.,* 1998). Indeed, they are known to produce alkaloids, causing toxic syndromes in cattle and horse herds grazing fescue. Endophyte-infected fescue can contain high levels of alkaloids, which give them the nickname "drunken horse grass" (Saikkonen *et al.,* 2013). Clavicipitaceous endophytes form a well defined taxonomic group among the Ascomycota with *Epichloë* (teleomorph) and *Neothyphodium* (anamorph, only asexual form known*)* the most represented genera. They are characterized by their narrow host range, as they establish symbioses almost exclusively with Gramineae and Graminoids (herbaceous plants with a grass-like morphology), herbaceaous plants such as rush, fescue grass and sedge (Bacon *et al.,* 2000). Most importantly, as "obligate" endophytes they are unable to compete outside the plant host.

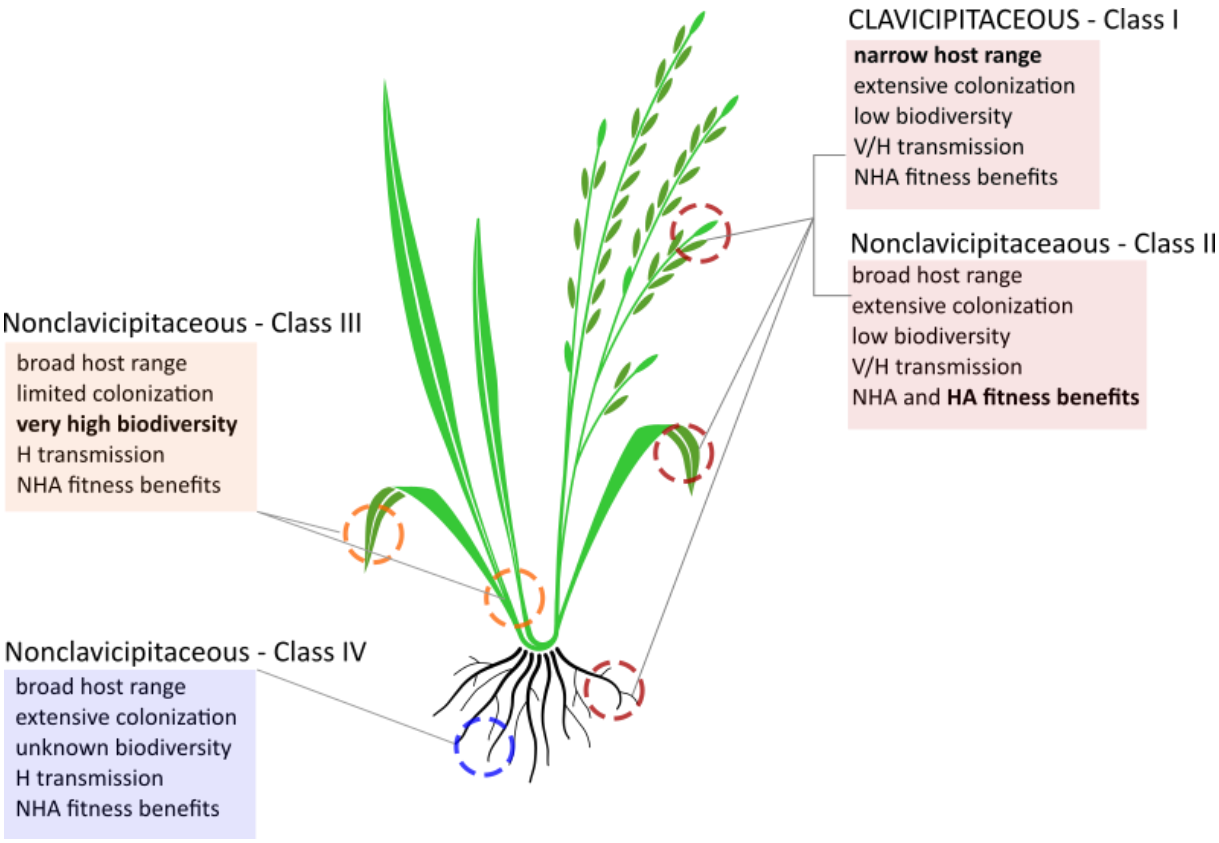


Figure I-1: Criteria used to characterize fungal endophytes and plant colonization pattern (adapted from Rodriguez *et al.,* 2009). H and V transmission refer to "horizontal" and "vertical" transmission modes. "NHA" stands for non-habitat adapted benefits (such as protection against herbivory) and "HA" for habitat adapted benefits (such as drought tolerance).

Clavicipitaceous endophytes colonize systemically the leaves, the rhizomes and the culms and proliferate in the shoot meristem (Clay *et al.,* 2002). Most species can be transmitted vertically through seeds to offspring (Ahlholm *et al.,* 2002). It seems that they have evolved in strong relationship with their plant host and they protect plants against herbivores, by deterring feeding (Clay, 1988). The remaining three groups are usually termed "nonclavicipitaceous" endophytes. They are mainly horizontally transmitted and they can switch between endophytic and free-living lifestyles, as evidenced by their abundance in soils.

Class II endophytes are similar to the Clavicipitaceous ones in their extensive colonization of plants, but in contrast they have a broad host range and confer, for some species, "habitat-specific" benefits (Rodriguez *et al.,* 2008), that is for exemple drought, heat or salt tolerance. The most studied species belong to the genera *Colletotrichum*, *Arthrobotrys* and *Fusarium*.

Class III endophytes (such as *P. variabile*) are notable for their incredibly high diversity, surely underestimated. They are found exclusively in the above ground tissues like in flowers, leaves, shoots, wood, fruits, but are rarely isolated from seeds (Arnold *et al.,* 2003). A single plant can harbor thousands of them and their assemblage seems to vary a lot within the plant organs, and also with the seasons (U'Ren *et al.,* 2012). They have been mainly isolated from tropical leaves. Herre *et al.* (2007) proposed the life cycle illustrated on figure I-2 whereas airborne spores, highly prevalent, land on the leaf surface and eventually penetrate into leaf tissues after germination. Endophytes can sporulate again after leaf abscission and infect new plants.

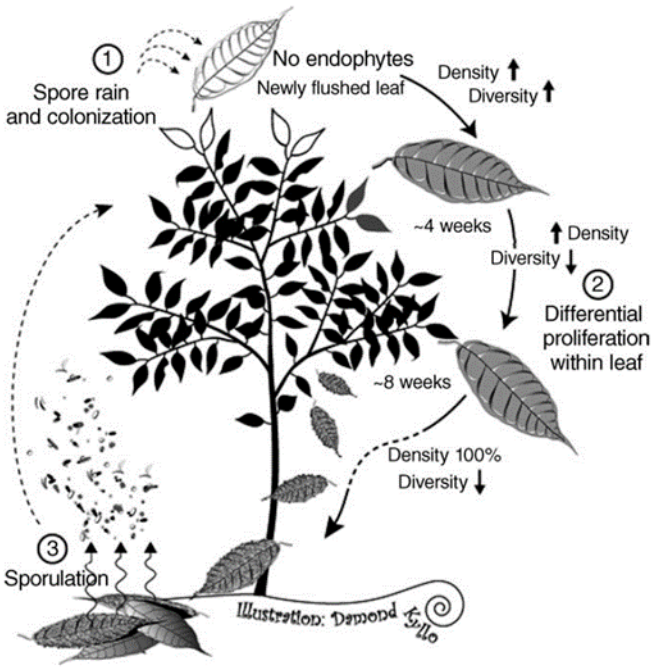


Figure I-2: Proposed life cycle for tropical foliar endophytic fungi (FEF) and their host plants. (From Herre *et al.,* 2007). 1) Leaves flushed by rain are free of FEF. Spores contained in the air can then land on the leaf surface and, upon wetting, germinate and penetrate the leaf cuticle. After a few weeks, the density of FEF infection within the leaf appears to saturate with a very high diversity. 2) Over several months, FEF diversity usually declines. 3) After leaf senescence and abscission, FEF sporulate and the cycle begins anew.

Finally, Class IV endophytes form a group less well defined of previously called "dark septate endophytes", because of their high melanin content. They are restricted to plant roots, extensively colonize them, and are proposed to increase plant growth through nutritional acquisition, as well as protecting plants from pathogens and herbivores (Mandyam *et al.,* 2005).

1.2 Where do they come from?

Plants are constantly surrounded by microorganisms, and their habitat can be divided into several compartments that are sources for endophytes. Root-associated microorganisms are recruited from the soil and the rhizosphere, whereas endophytes from air-borne parts are supposed to be mainly recruited from the phyllosphere (Vandenkoornhuyse *et al.,* 2015). The leaf litter could be a major source of inoculum for both compartments (Arnold & Herre, 2003). Figure I-3 illustrates those

different habitats. Plant-microbe interaction starts as early as microbe recruitment in the surrounding environment of the plant. Indeed, in the rhizosphere which is a thin layer of soil in close association with plant roots, the microbial community is claimed to be influenced by root exudates. Concerning leaves, Vorholt *et al.,* (2012) estimated the abundance of microorganisms in the phyllosphere as high as 10^{26} cells on a leaf surface. In the same way as root exudates drive microbe recruitment, compounds produced by plants on the leaf surface could as well influence the epiphytic community (microbes growing on the plant surface) and indirectly the endophytic diversity (Vandenkoornhuyse *et al.,* 2015).

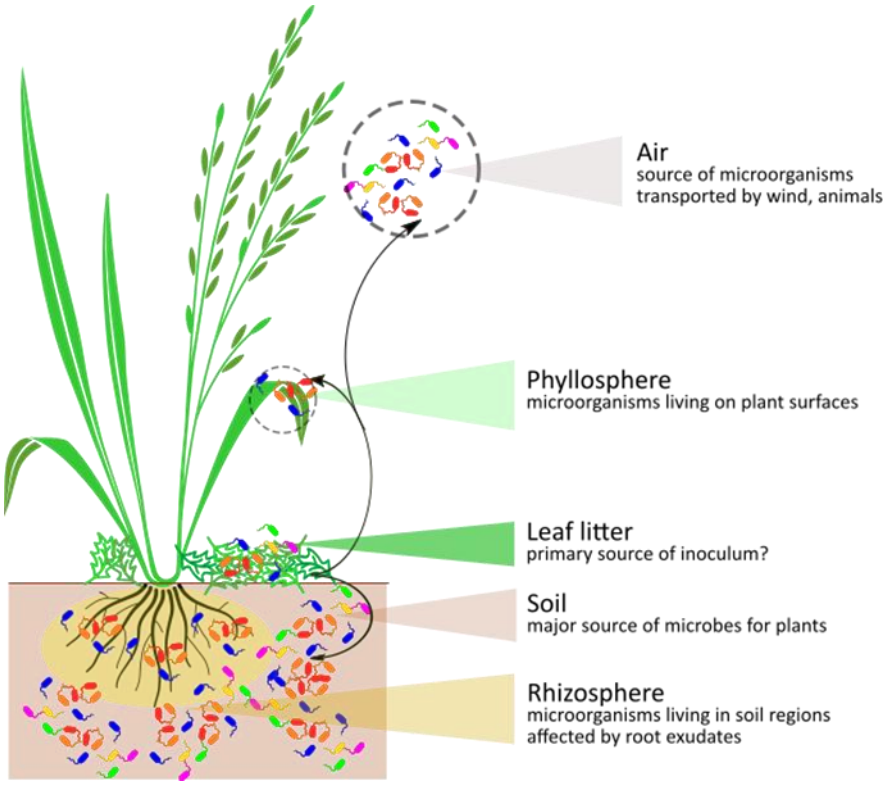


Figure I-3: Continuum of microbial habitats surrounding the plant. The phyllosphere refers to the total above-ground portions of plants as a habitat for microorganisms. The rhizosphere refers to the soil area in near contact with roots, rich in nutrients and root exudates.

1.3 How did endophytes evolve as plant mutualists?

Evolutionary origins

Plant-fungus symbioses are known to have occurred since the colonization of land by terrestrial plants and the oldest fossil record indicates that plants harbor symbionts since at least >400 Million years (Krings *et al.,* 2007). Different theories have been developed to explain the origin of endophytes. Torres *et al.* (2007) proposed that Clavicipitaceous endophytes were first insect-parasites that finally colonized plants by infection through insect stylets. The author hypothesized that those fungi did not possess enzymes or toxins to degrade plant tissues, and therefore were able to establish asymptomatically in plant tissues. They were then able to diversify thanks to

multiple infection events in different plant species. He also suggested that the endophyte's ancestors already produced alkaloids and that this functional trait was maintained as it conferred them, later in the evolution, the ability to deter herbivore feeding. By switching to endophytic lifestyles, those endophytes would have gained increasing dependence on the host plant for their nutrition, explaining why they are now "obligate" microbes. This hypothesis is supported by the existence of fungal strains still capable nowadays to infect both insects, on which they are pathogenic, and plants, on which they behave as endophytes, alike the entomopathogen and plant endophyte *Beauveria bassiana* (Barelli *et al.,* 2016).

Aside from their possible introduction via insects, other features of endophytes can explain why they remain closely associated to plants. A striking example is the one of *Epichloë* species. Some fungi among this genus are responsible for the "choke disease" where they form fruiting body structures visible at the naked eyed outside the plant through sexual reproduction. This structure inhibit the formation of the plant's reproductive organs (inflorescences and seeds) in cool-season grass infected (see figure I-4). As a result, the plant dies without offspring. However, some *Epichloë* species are unable to perform reproduction and as a result, they are harmless to the plant that can produce seeds, and the fungus is transmitted vertically through those seeds, explaining its ecological success compared to pathogenic species (Clay *et al.,* 2002). In a way, we could say that the plant is dictating the fungus its endophytic lifestyle that is, to be non-pathogenic to be propagated (Selosse & Schardl, 2007).

Figure I-4: *Epichloë bromicola* causing "choke disease" on infected stems of grass (Rector *et al.,* 2016)

In contrast, the nonclavicipitaceous fungi can switch from endophytic to free-living lifestyles. Porras-Alfaro *et al.* (2011) suggested that mutualistic lifestyles evolved from pathogenic and saprotrophic ancestors (organisms that feed on nonliving organic matter). Indeed, numerous Ascomycota plant pathogens and saprotrophs are derived from the same lineages as common

endophytic fungi (Promputtha *et al.,* 2007). Isolates of endophytic *Colletotrichum sp.*, *Fusarium sp.*, and *Phomopsis sp.* were suggested to adopt a saprotrophic lifestyle when leaves die. Colonization of leaves as endophytes could therefore be a strategy to gain a competitive advantage over other saprotrophs (Schulz & Boyle, 2005). These endophytes have possibly evolved, among other things, via the loss of genes. By comparing *Colletotrichum tofieldae,* a beneficial root endophyte, and its pathogenic relative, *C. incanum*, Hacquard *et al.* (2016) found that the endophyte has kept all his wall degrading enzymes, but possesses a reduced set of effector proteins (molecules that trigger plant defense responses). In the same way, it was reported that a single gene mutation could turn the cucurbit pathogen *Colletotrichum magna* into an endophytic mutualist (Freeman *et al.,* 1993).

Strategies of plant colonization

These particular evolutionary records are important to understand the plant colonization patterns by endophytes. Because of their ancestral background, some of them possess an enzymatic "toolkit" to degrade plant cell walls and penetrate into plant tissues. *Epichloë festucae*, *Serendipita indica* (syn. *Piriformospora indica),* or the root endophyte *Colletotrichum tofieldae,* have all extended gene regions encoding hydrolytic enzymes and carbohydrate binding enzymes, as a heritage from their pathogenic or saprotrophic ancestors (Fesel & Zuccaro, 2016).

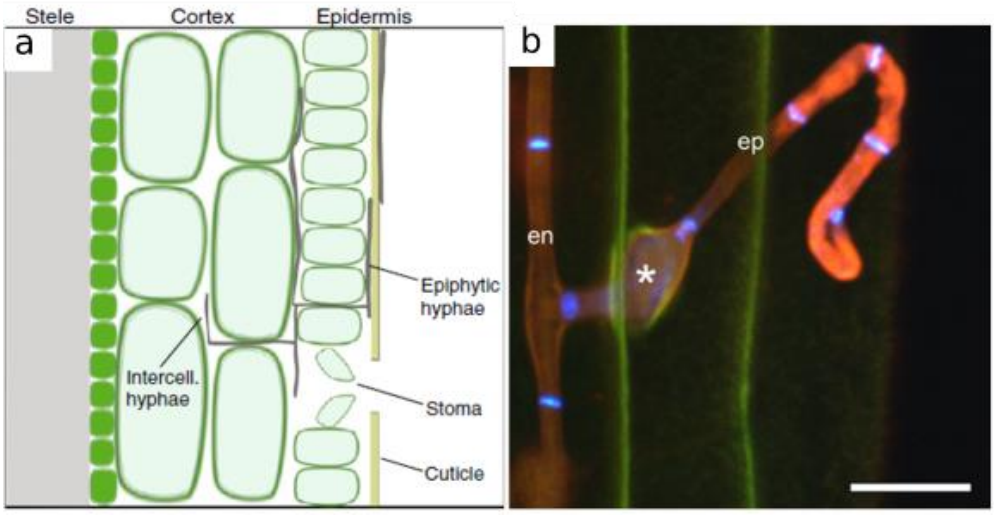


Figure I-5: Colonization strategy of *Epichloë festucae* (adapted from Hessel and Zuccaro, 2016; picture credited to Becker *et al.,* 2016)**.** A) The diagram illustrates the mode of infection of *Epichloë festucae*. B) Endophytic hypha (en) from *E. festucae* colonizing epidermal cells from a leaf of *Lolium perenne*. The emerging epiphyllous hyphae (ep) is visible and forms an appresorium-like (*) structure (scale bar: 10 μm).

Endophytes are supposed to penetrate (and exit) plant tissues via the formation of appressoria (appressoria are flattened, "pressing" organs, using turgor pressure to help the fungus penetrate the plant tissues) or directly via hyphae, entering through stomata and other natural openings or

small lesions (Ernst *et al.,* 2003). A nice example of an appressorium-like structure is given on picture I-5, where we can observe a hypha from *Epichloë festucae* getting out of a leaf from the grass *Lolium perenne*. The growth of Clavicipitaceous endophytes is exclusively intercellular as illustrated here.

Class III endophytes, which are mostly foliar, sporadically enter the plant endosphere and create small localized infections (Rodriguez *et al.,* 2009). As a result, few studies have visualized endophytic fungi in leaf tissues. They are thought to grow slowly between epidermal cells (Deckert *et al.,* 2001). Recently, Huang *et al.* (2018) observed that the endophyte *Cladosporium tenuissimum* could readily colonize the interiors of leaves from the poplar tree (see figure I-6).

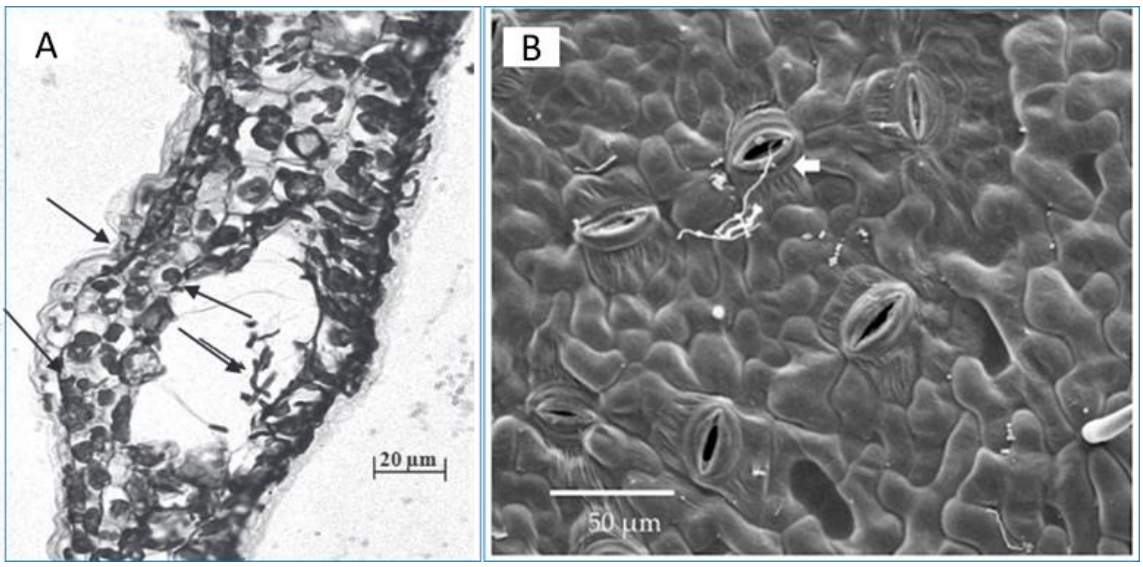


Figure I-6: Presence of endophytes in leaves revealed by microscopy. A. Transversal cut of a leaf from *Sapindus saponaria* (tropical tree) observed by light microscopy showing the presence of a fungus endophyte (arrows) growing in parenchyma tissues (Garcia *et al.,* 2012). **B.** Epidermis of a leaf from *Populus trichocarpa* observed by scanning electron microscopy, showing a *Cladosporium* endophyte entering a stomata (Huang *et al.;* 2018).

1.4 Host specificity and microbiota assemblage

Currently, we do not have a comprehensive view of factors explaining microbiota assemblage inside plants. Why are particular endophytes recruited or not, are they host specific or opportunistic mutualists, which are the determinants of microbe community structures? These questions remain mostly unanswered. Studies have shown that composition of endophyte communities is governed by the host genotype, the developmental stage, and mainly by the environment (Hardoim *et al.,*

2015). Endophyte diversity increases along a latitudinal gradient, from the artic to the tropics, and tropical leaves can be qualified as "hotspots" for fungal diversity, with many species occurring only once (Arnold *et al.,* 2007). Arnold & Herre (2003) proposed that the leaf chemistry was a dominant driver in endophyte assemblage. In poplar trees, species richness was decreasing with tree age (Martin-Garcia *et al.,* 2011). Interestingly, a study using axenic *A. thaliana* demonstrated that the soil is the major factor explaining microbe assemblages (Lundberg *et al.,* 2012), whereas studies comparing the microbiota from five different species under natural conditions, tend to see a dominant "host-effect", prevailing over sample location (Ding *et al.,* 2013). In the end, it appears that the environment (season, geography, rainfall, temperatures, wind) influences the air and soil microbial community, and that more host-specific mechanisms further drive the recruitment of microorganisms in the phyllosphere and the rhizosphere, directly impacting the endophytic communities. Most importantly, the already present microbiota strongly influences the recruitment of new endophytes (Bulgarelli *et al.,* 2013).

New-sequencing technologies have given a better access to the high percentage of non-cultivable microbes. Attempts to apprehend the microbiota of plants mainly concern bacterial communities (Van der Heijden & Hartmann, 2016) and seldom concern fungi (see De Souza *et al.,* 2016 for fungal microbiome in sugarcane). These studies have revealed that plants preferentially associate with a core of microbes and "hub species", surely providing important functions for the plant fitness (see figure I-7).

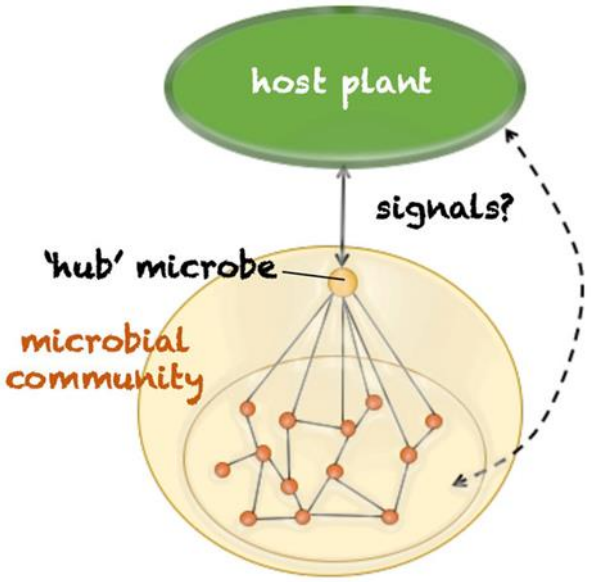


Figure I-7: "Hub" microbes shape plant-associated microbial community

(E. Kemen, http://www.mpipz.mpg.de/kemen)

Microbe "hubs" are defined as a small number of taxa which presence are determinant for microbial community structures and recruitment of other species in the plant (Agler *et al.,* 2016). Abiotic factors and host molecular signatures act directly on those "hub" microbes, which then transmit the effects to the microbial community. Study of the core microbiota and "hub species" will certainly be the next trend in plant-microbe interactions (Lundberg *et al.,* 2012; Vandenkoornhuyse *et al.,* 2015, Hamonts *et al.,* 2018).

1.5 Endophytes shape plant communities

Fitness benefits

Recently, ecologists came up with the idea that the symbiosis between the plant and the endophyte was building ecological success (Selosse *et al.,* 2004). This idea is now supported by an increasing number of studies suggesting that endophytes can shape plant communities, through plant protection from abiotic and biotic stresses. For example, Aschehoug *et al.* (2014) demonstrated that the invasive plant *Centaurea stoebe* was more competitive to a native North American grass sharing the same habitat, when inoculated with the fungal endophyte *Alternaria alternata.* The authors suggested that the endophyte was producing allelopathic compounds inhibiting the germination and growth of the native grass. However this is a rare example of study conducted at the ecosystem level, and most studies have explored the functional role of endophytes in artificial environments.

Adaptation to habitats

Avoiding abiotic stress is a first example of how endophytes can improve plant fitness. The endophyte *Neotyphodium* improved persistence of ryegrass in stressful habitats subject to drought and hot temperatures (Ravel *et al.,* 1997). Similarly, *Serendipita indica* (syn. *Piriformospora indica*) was shown to induce drought tolerance in cabbage through increase in antioxidant levels (Sun *et al.,* 2010). This endophyte could also increase the activities of antioxidant enzymes in barley roots under salt stress conditions (Baltruschat *et al.,* 2008). Endophytes have also been found associated to plants living in extreme habitats, like the grass *Dichantelium lanuginosum* growing in geothermal soils of Yellowstone. In these hot habitats, symbiotic plants inoculated with the fungus *Curvularia protuberata* could survive temperatures up to 65°C (Redman *et al.,* 2002). Inversely, the same authors (2011) found fungal endophytes improving cold tolerance in rice. Plants can also benefit from endophytes through an increased biomass, leading to more competitive plants. *S. indica* was shown to produce cytokinins, well-known phytohormones, promoting plant growth in *A. thaliana*

(Vadassery *et al.,* 2008) and *Penicillium sp.* could improve cucumber growth under saline stress by production of gibbereline, another plant hormone (Kang *et al.,* 2012). Plant growth-promoting effects can also be attributed to better nutrient influx and CO_2 uptake in the presence of endophytes (Spiering *et al.,* 2006).

Protection against herbivores and insects

Endophytes have been reported to deter feeding by herbivore and insects (Hardoim *et al.,* 2015) through the production of bioactive secondary metabolites. In many grasses, endophytes of the genus *Neotyphodium* and *Epichloë* produce alkaloids such as ergovaline, permine, loline and lolitrem (Spiteller, 2015). Those metabolites are highly toxic to insects such as loline (Rohlfs *et al.,* 2009) or responsible for toxicosis of mammals (Lyons *et al.,* 1986). Alkaloids usually interact as antagonist of neurotransmitters leading to disturbance in the central nervous system (Panaccione *et al.,* 2014). Production of toxic compounds is not restricted to Clavicipitaceous endophytes, and the quinone rugulosin has been identified in an endophyte of the balsam fir *Abies balsamea* (Calhoun *et al.,* 1992). This compound caused mortality of larvae from a defoliating insect attacking the conifer.

Protection against pathogens

Foliar endophytes seem to be generalists, establishing on many different species, and it is difficult to assess their ecological role as they have a small space and time window of opportunities for contact with plant pathogens. An increasing number of studies, however, tend to demonstrate their role in plant protection against phytopathogens and have allowed the discovery of new active metabolites in these endophytes (for reviews see Schulz *et al.,* 2002; Rodriguez *et al.,* 2009; Hardoim *et al.,* 2015). A persuasive example comes from the study of the endophytic microbiota associated to the cocoa tree, *Theobroma cacao*. Endophyte-free leaves re-inoculated with microorganisms isolated from healthy trees, were more resistant to the pathogen *Phytophtora* (Arnold *et al.,* 2003). Looking closer at the mechanisms involved in plant protection, resistance against pathogens is mainly attributable to: (i) antifungal and antibacterial compounds produced by the endophyte or by the plant in response to /or in collaboration with the endophyte, (ii) induction of plant defenses, (iii) fungal parasitism or (iv) trophic competition between endophytes an other microbes.

(i) Antimicrobial compounds

Increased interest in endophytes is mainly due to the discovery that they actively contribute to the synthesis of bioactive compounds, once considered exclusive to plants (Kusari *et al.,* 2011-a). Several hundreds of secondary metabolites with antimicrobial activities have been isolated from endophytes, including important chemical classes such as alkaloids, terpenoids and polyketides

(Strobel *et al.,* 2003; Gunatilaka *et al.,* 2006; Spiteller, 2015). *Pestalotipsis microspora*, a common rainforest endophyte, was found to produce ambuic acid, an antifungal agent against several *Fusarium* species, *Acremonium graminaerum* a pathogen of wheat and *Lasiodiplodia theobromae* a pathogen on citrus fruit (Li *et al.,* 2001a). In the same way, two pyrrocidines from the maize endophyte *Acremonium zeae* were found to inhibit the growth of maize pathogens such as *Fusarium verticillioides* (Donald *et al.,* 2005). Endophytes have also been described to produce antibacterial compounds, such as the phomopsichalasin. This cytochalasin-like compound was isolated from an endophyte of a Chinese willow tree and was highly active against *Bacillus subtilis* (Horn *et al.,* 1995). In addition, several secondary metabolites synthesized by endophytes can act synergistically. For example, the fungus *Muscodor albus*, from the cinnamon tree, produces a mixture of volatile compounds with little inhibitory effect if tested separately, but collectively able to cause death in a broad range of phytopathogens (Strobel *et al.,* 2001).

The alkaloid camptothecin is a widely-known anticancer agent originally isolated from a chinese tree that also displays strong antifungal activities against some of the tree pathogens, such as *Alternaria alternata* (Li *et al.,* 2005). Kusari *et al.* (2011-b) found that the endophyte *Fusarium solani*, inhabiting this tree, is producing precursors of camptothecin and further requires a key plant enzyme to fulfill its biosynthesis. This example nicely illustrates how endophytes and plants can cooperate to synthesize antimicrobial compounds through complementary biosynthesis pathways. More generally, endophytes can also produce metabolites that modify the symbiosis assemblage at the expense of pathogens (Schulz & Boyle, 2005).

(ii) Induction of plant defenses

Beneficial microbes from the plant microbiota have been shown to induce systemic resistance (ISR) in plants (Pieterse *et al.,* 2014). ISR is an important mechanism of plant immunity by which plants acquire an improved resistance to pathogens by enhancement of their defenses through prior infection. For exemple, non-pathogenic *Fusarium* isolates, inoculated in roots of banana seedlings, improved plant resistance against a deleterious nematode (Vu *et al.,* 2006). In the *Phytophtora capsici*-hot pepper pathosystem, isolates of endophytic *Trichoderma* could induce several genes related to plant defenses such as lipid transferase proteins (Bae *et al.,* 2011). These small proteins can bind jasmonic acid and activate downstream plant defenses.

(iii) Fungal parasitism

Several fungi can behave as mycoparasites to other fungal species. *Thichoderma* is a well-known mycoparasite able to degrade and kill other fungi. In cocoa trees, *Trichoderma* was able to suppress

the causal agent of the "witches broom" disease (so called because the pathogen induces the formation of numerous small branches), by preventing the formation of basidioscarps by the pathogen (Bastos *et al.,* 1996).

(iv) Trophic competition

Disease protection by endophytes, as an indirect result from competition for nutrients and niche space, has been proposed a long time ago in literature (Lockwood *et al.,* 1992). Indeed, hyphal network, by its physical presence only, could exclude other pathogenic microorganisms. In addition, endophytic fungi consume significant amount of carbohydrates and could limit their availability for plant pathogens (Mandyam, 2005).

1.6 Endophytes: from mutualism to parasitism and pathogenicity, or the importance of ecological context

The previous paragraph illustrated how endophytes are able to contribute to their host's fitness. However, it does not imply that this symbiosis occurs without any cost for the plant host in supporting those endophytes. Thus, several studies have shown that their beneficial effects are only relevant in a specific host and ecological context.

A balanced interaction

Endophytes have been hypothesized to be latent pathogens, as illustrated in the case of *Fusarium verticilloides*, behaving as an endophyte or a pathogen on maize depending on the stress conditions for the plant (Bacon *et al.,* 2008). In the same way, proanthocyanidins (phenolic compounds toxic to microorganisms), produced by strawberries, maintained *Botrytis cinerea* in a quiescent state (Jersh *et al.,* 1989), but their decrease under nitrogen stress (two and four times the normal concentration in the growth medium) led to an increased susceptibility to the fungus (Schulz *et al.,* 1999). This demonstrates that effects assigned to endophytes in healthy plants might change when host plants are grown under more stressful conditions (Hardoim *et al.,* 2015). Unexpectedly, Schulz *et al.*, (1999) identified a high proportion of herbicidal compounds produced by endophytes. Together with the previous examples, it shows that both the host plant and the endophyte must be wary of each other. Those observations led to the formulation of the "balanced antagonism" theory. This theory proposes the plant's defense mechanisms to be overcome in the case of a pathogen leading to disease, whereas the endophyte and the plant maintain a balanced antagonism, each one tolerating the other (Schulz *et al.,* 1999; Kusari *et al.,* 2012). The endophyte overthrows the plant's defenses in a controlled way only so that colonization of the plant is possible. Under stressful conditions,

when the plant is weakened, the endophyte, however, present as a latent pathogen, can overwhelm the plant defense. This model is illustrated on figure I-8.

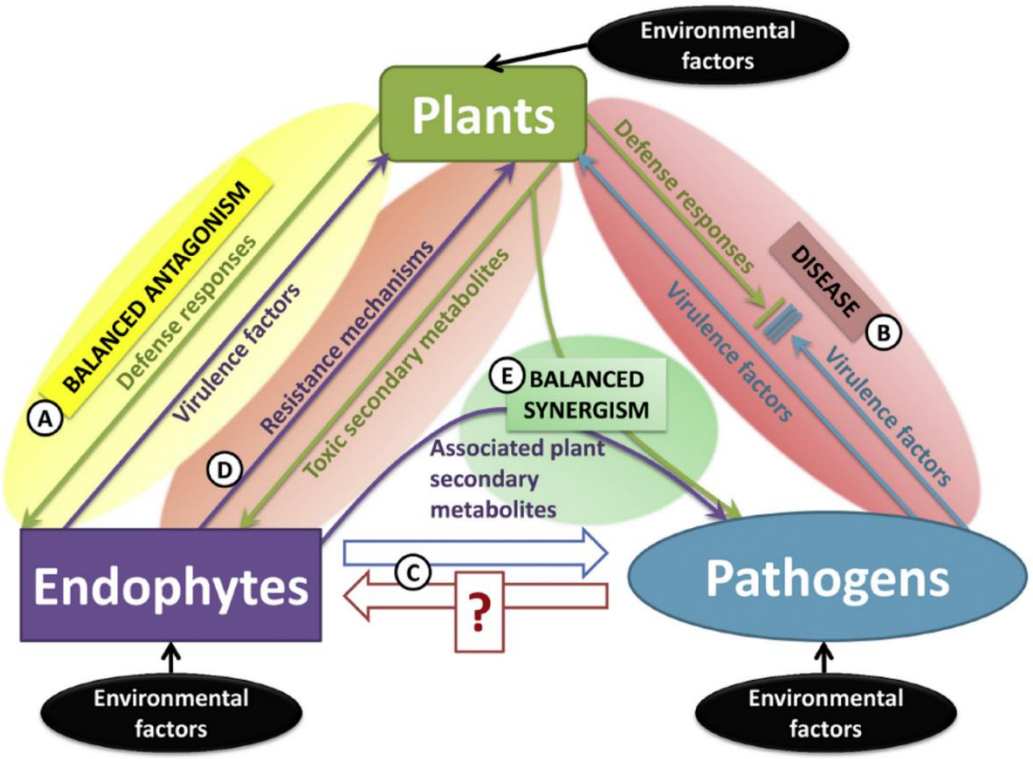


Figure I-8: Schematic interpretation of plant-fungus cost-benefit interactions (from Kusari *et al.,* 2012). A) Plant can tolerate virulence factors produced by the endophyte and the latter is able to colonize plants without symptoms. **B)** Pathogen counteracts plant defenses and the action of virulence factors leads to disease. **C)** Endophytes can switch to pathogenic lifestyle under particular environmental conditions. Question mark indicates that this phenomenon needs further investigation**. D)** Endophyte can survive plant toxic metabolites. **E)** Plant and endophyte can cooperate to protect the plant against pathogens.

Plant-Endophyte: a cost-benefit interaction

Symbiosis can range from mutualism, where benefits for the host exceed costs, to parasitism, where costs of the symbiont exceed benefits. Endophytes are no exception to that rule. A large fraction of the microbial community, indeed, can be defined as commensal organisms that find their niche in the association with plants but have no known function to their hosts (Brader *et al.,* 2017). They could be seen as opportunists, tolerated by the plant as long as their nutritional demand is not too costly for the plant (Saunders *et al.,* 2010). Wäli *et al.*, (2006) have shown that fitness benefits conferred on hosts were dependent on the habitat stress. For example, endophyte-infected ryegrass

was more susceptible to "snow mold" disease occurring in winter than endophyte-free plants, but recovered quickly in spring from the disease. Here, hosting the endophyte may be costly in winter when the plant experienced additional charge from cold stress.

So far, we have mostly insisted on plant benefits, but to complete reflections, endophytes might as well find advantages in this "cooperation". This is especially true in the case of horizontally transmitted facultative endophytes, which finally are the most prevalent endophytes. To date, very little has been published on this topic because most studies have focused on grass obligate endophytes and as a result, endophytism is too often associated with mutualism. However, it is not clear whether residing within plant tissues confers an advantage to foliar endophytic fungi over living freely in the soil, or on the plant surface as epiphytes. Plant habitat can be seen as a shelter for the endophyte, sparing it competition with other microorganisms or unfavorable climatic conditions, and giving it an easy access to photoassimilates.

An exciting aspect in studying endophytes, lies in the decoding of their chemical communication with the plant host, but also the plant microbiota. In particular, fungi have been proposed to use oxylipins as a common "currency" to elicit biological responses, and hijack plant and mammalian defenses (Fisher & Keller, 2016). Fatty acid derivatives, indeed, have ancient evolutionary origins as signaling molecules and are ideal candidates for inter-kingdom communication. Organisms from different kingdoms are, thus, able to "listen" and respond to oxylipin signals during interactions (Pohl & Kock, 2014). In the same way as oxylipins are understood by inter kingdom organisms, these "words" are certainly used for communication within fungal species, which to our knowledge, has not yet been explored.

2. Oxylipins as lipid mediators in host-fungi interactions

2.1 Oxylipins are cross-kingdom molecules

Oxylipins are ubiquitous and structurally diverse secondary metabolites derived from oxygenated fatty acids (Mosblech *et al.,* 2009). Compelling evidence has been found, that plants, animals and fungi use oxylipins as a common language (Schulz & Appel, 2004; Tsitsigiannis & Keller, 2007; Fischer & Keller, 2016). They have been identified in mammals, in plants ranging from algae to angiosperms, in fungi and even in bacteria (Noverr *et al.,* 2003; Wasternack & Feussner, 2018). Oxylipins are well documented in mammals were they mediate important immunological responses (Funk, 2001) and in plants, where jasmonic acid in particular has been extensively reviewed, with regard to its impact on growth processes, responses to abiotic stress and pathogen attacks (Blée,

2002). Fungi as well can produce a wide diversity of oxylipins as illustrated on figure I-9 (Brodhun & Feussner, 2011).

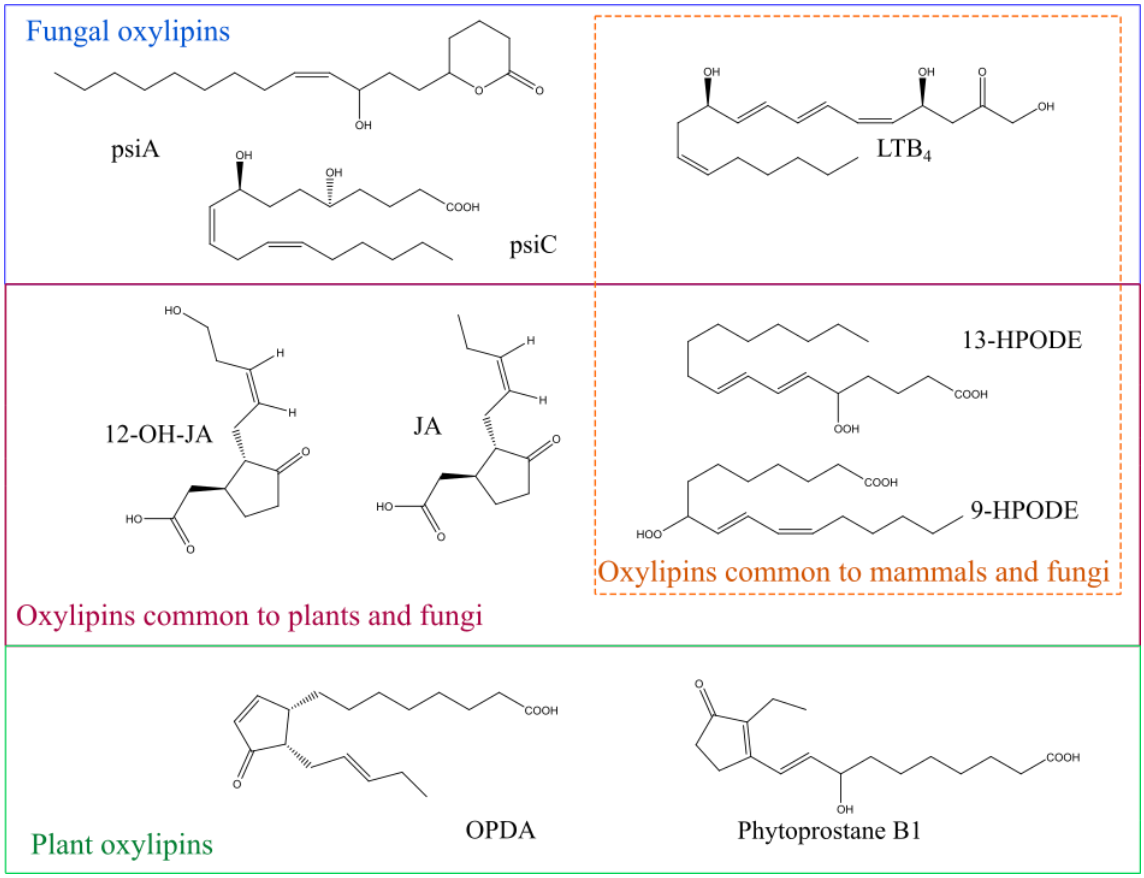


Figure I-9: Structural similarity of oxylipins found in fungi, plants and mammals. Fungi produce "psi factors" such as 8-hydroxy-octadecadienoic acid (psiA) and 5,8-dihydroxy-octadecadienoic acid (psiC). Fungi also produce leukotrienes such as LTB4, alike mammals. 12-oxophytodienoic acid (OPDA) and phytoprostane B1 are two typical plant oxylipins. 12-hydroxy jasmonate (12-0H-JA) and jasmonate (JA) are found in plants and fungi. 13-hydroperoxy-octadecadienoic acid (13-HPODE) and 9-hydroperoxy-octadecadienoic acid (9-HPODE) are common to plants, mammals and fungi.

2.2 Enzymes and biosynthesis pathways

Oxylipins are synthesized from polyunsaturated fatty acids (PUFAs) through three main pathways: the CP450 pathway involving cytochrome P450 enzymes, the dioxygenase (DOX) pathway and the lipoxygenase (LOX) pathway (Andreou *et al.,* 2009). Those pathways are summarized on figure I-10. In mammals, oxylipins are formed mainly via the arachidonic acid (AA) cascade, whereas in plants and fungi they mainly originate from linoleic (C18:2) and linolenic acid (C18:3). The CP450 pathway exists in plants and mammals, leading to the formation of hydroxides and epoxydes after insertion of a single oxygen atom.

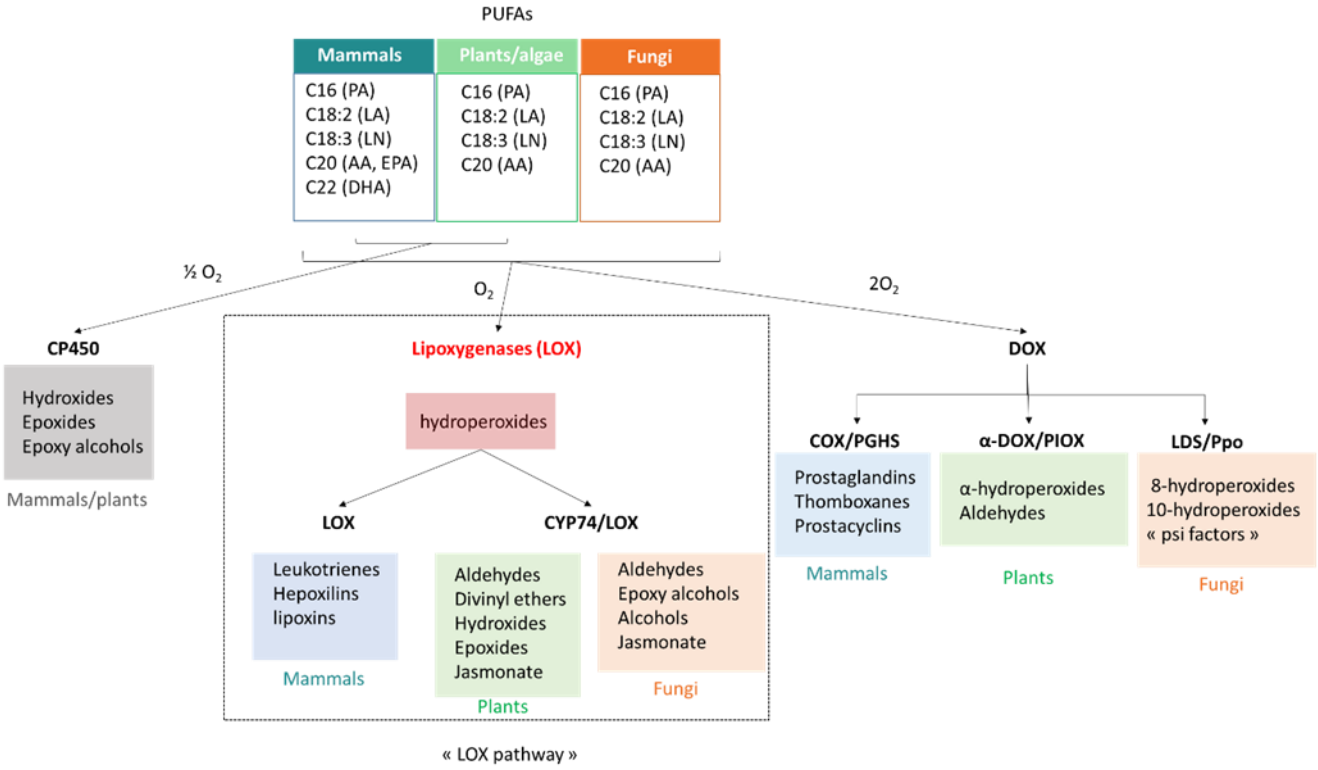


Figure I-10 : Oxylipin biosynthetic pathways in mammals, plants and fungi. (Adapted from Blée, 2002; Andreou *et al.,* 2009; Brodhun *et al.,* 2011.) PA : palmitic acid, LA : linoleic acid, LN : linolenic acid, AA : arachidonic acid, EPA : eicosapentaenoic acid, DHA : docosahexaenoic acid. Hydroperoxides can also serve, in plants and fungi, as substrates for an other unusual CP450 family called the CYP74. Those atypic enzymes can transform hydroperoxides into divinyl ethers (Divinyl Ether Synthetase), epoxy alcohols (Epoxy Alcohol Synthetase), aldehydes (Hydroperoxide lyase) or allene oxides (Allene Oxide Synthetase). In plants and in fungi, allene oxide is rapidly transformed to give the famous jasmonate.

In fungi, CP450 enzymes have not been described but instead, a new CP450 subfamily was identified (Brodhun & Feussner, 2011). Those enzymes possess a unique structure as they contain a fatty acid haem peroxidase/dioxygenase domain in the N-terminal region, fused to a CP450 domain in the C-terminal region (figure I-11). As a result, the dioxygenase domain catalyses the formation of 8-HPODE and the CP450 is responsible for the formation of their di-hydroperoxide derivatives. Those enzymes are usually referred to as LDS (linoleate diol synthase) or PpoA, for Psi-factor producing oxygenase, because they were first discovered in *Aspergillus nidulans*, were they induce premature sexual sporulation, "psi" standing for "precocious sexual inducer". PpoC are a different class of dioxygenases in which the CP450 domain is inactive. Therefore, they catalyse the production of hydroperoxides only (10-HPODE). Those enzymes (Ppo and LDS) are close to the mammalian cyclooxygenases (COX) and the plant α-dioxygenases (DOX). In mammals, they catalyse the synthesis of prostaglandins, also extensively studied because they have diverse hormone-like effects, ranging from cell growth control to inflammation regulation (Funk, 2001).

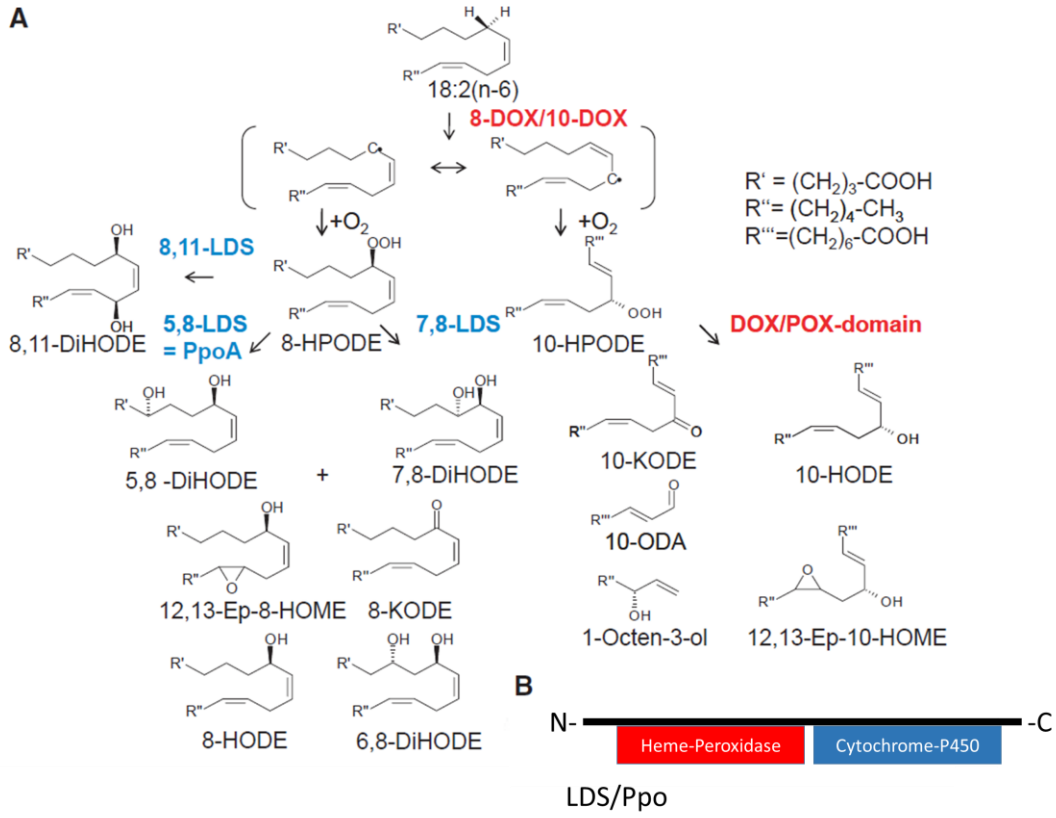


Figure I-11: Pathway of fungal oxylipin and Psi factor formation by Ppo enzymes (from Brodhun & Feussner, 2011). **A.** Reactions catalysed by Ppo enzyme heme-peroxidase(red) and CP450 (blue) domains. **B.** Domain structure of enzymes from the LDS/Ppo family

The last pathway is called the "LOX pathway" and involves lipoxygenases (LOXs) enzymes (Feussner & Waternack, 2002) . LOXs (EC 1.13.11.12), unlike dioxygenases and CP450, are non-haem enzymes that catalyse the insertion of molecular oxygen into fatty acid substrates leading to the formation of hydroperoxides (figure I-12). LOXs enzymes are diverse and named after the carbon at which oxygen is preferentially introduced. In mammals, we found mainly 15-LOX and 12-LOX, whereas in plants and fungi, mostly 13-LOX and 9-LOX were identified (Porta & Rocha-Sosa, 2002).

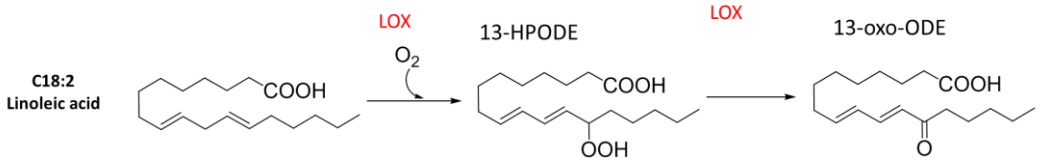


Figure I-12: Conversion of linoleic acid into 13-HPODE and 13-KODE by lipoxygenases

Synthesis of 13-hydroperoxides in fungi

Since Combès *et al.,* (2012) identified mostly 13-HPODE and 13-oxo-ODE in the interaction between *F. oxysporum* and *P. variabile*, we were interested in fungal LOXs with similar activities. In fungi as in plants and mammals, the formation of 13-HPODE is catalyzed by LOXs, from linoleic acid (18:2), whereas linolenic acid give rise to 13-HPOTrE (13-hydroperoxyoctadecatrienoic acid). In plants, it has been shown that the same lipoxygenase can convert linoleic acid into 13-HPODE and then 13-HPODE in 13-oxo-ODE (Künh *et al.,* 1991; see figure I-12). It should be noted, however, that those two oxylipins can also result from non-enzymatic oxidation processes (Christensen & Kolomiets, 2011).

Lipoxygenase structure

LOX are fatty acid dioxygenases with a non-heme catalytic metal, usually iron but they can also harbor manganese (Su & Oliw, 1998). They are monomer enzymes with a molecular mass between 94 to 104 kDa in plants (Brash, 1999). Soybean-LOX1 from *Glycine max* is the most studied LOX and served as a model for the determination of a typical LOX structure (Minor *et al.,* 1993). Mammalian and plant LOXs possess a small N-terminal domain known as PLAT (Polycystin-1, Lipoxygenase, Alpha-toxin) domain, similar to the catalytic domain of mammalian lipases (Newcomer & Brash, 2015), which suggests that they can interact with cellular membranes (Brash, 1999). This domain is lacking in fungal LOXs but they usually contain a secretion signal (Heshof *et al.,* 2014). The C-terminal domain is the catalytic domain which contains the active site of the enzyme and the metal cofactor, iron or manganese. Amino acids binding the metal cofactor are highly conserved between mammalian, plant and fungal LOXs (Oliw, 2002). The five conserved metal ligands are three histidines, an asparagin and the C-terminal amino-acid, which can be a valine or an isoleucine (figure I-13). The histidines and aspargine amino acids are found in specific sequences and the two motifs H-X3/4-H and H-X3-N are highly conserved among LOXs.

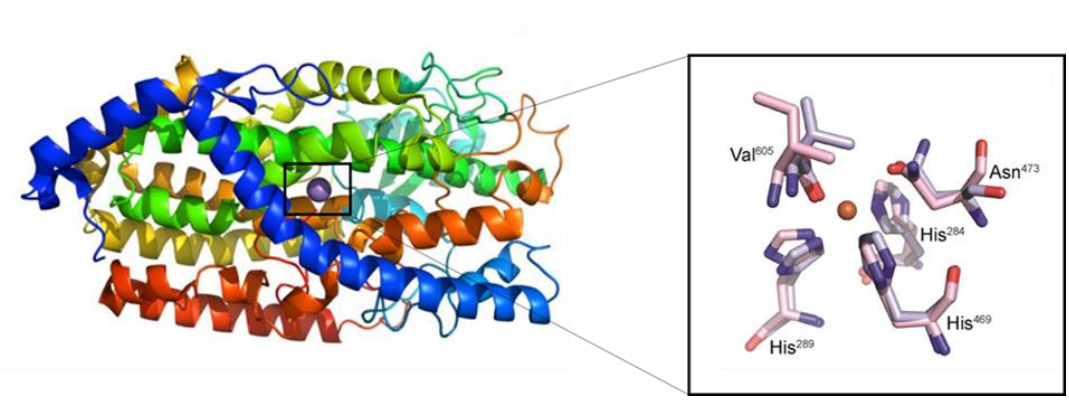


Figure I-13: Crystal structure of the manganese lipoxygenase from the rice blast fungus *Magnaporthe oryzae* (G4NAP4.2) with a focus on the conserved metal ligands. (From Wennman *et al.,* 2016)

Reaction mechanism

Very few lipoxygenases or enzymes with lipoxygenase activity have been purified and described in fungi so far and therefore we do not have a broad view of fungal biosynthetic routes involving lipoxygenases compared to plants and mammals. The best-characterized fungal LOX is by far the 13-MnLOX from *Gaeummanomyces graminis*. It was the first LOX discovered harboring manganese instead of iron in its catalytic center. This enzyme was able to convert linoleic acid into 13*R*-HPODE, the main metabolite (71%), and the reversible 11*S*-HPODE. Most of our knowledge about fungal LOX mechanisms originate from its study and figure I-14 illustrates a proposed mechanism.

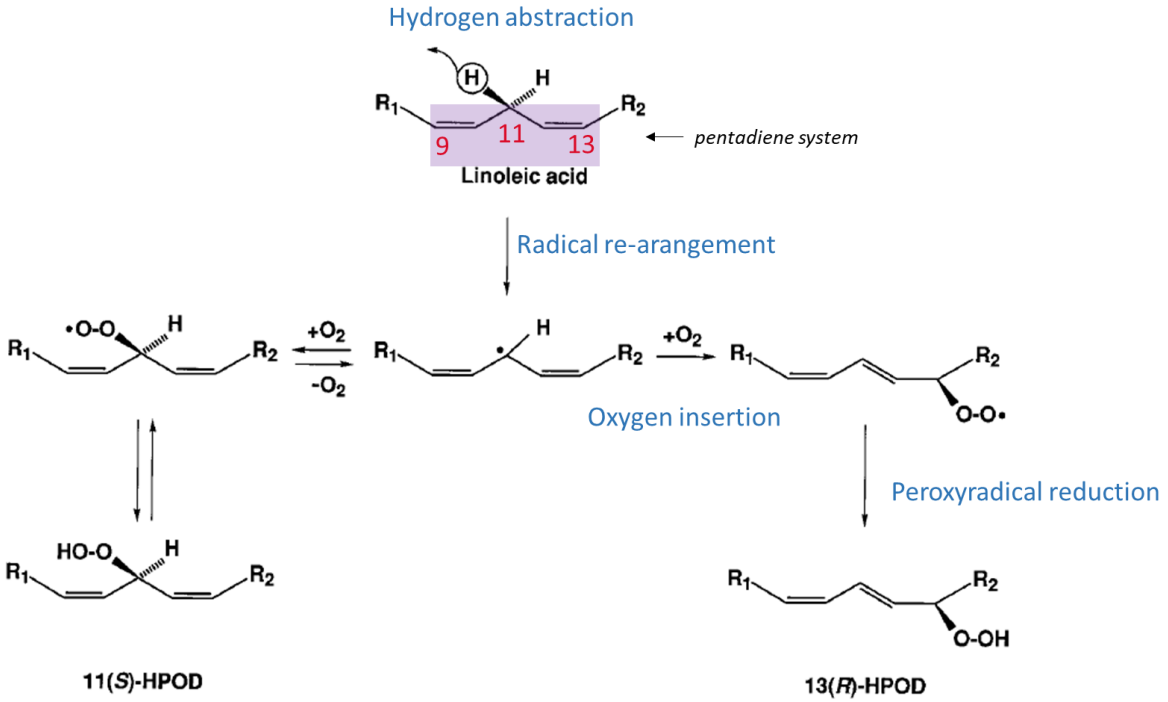


Figure I-14: Proposed mechanism for oxygenation of linoleic acid by the 13R-MnLOX from *G. graminis* (adapted from Hamberg *et al.,* 1998).

LOXs catalyze the insertion of oxygen into fatty acids containing a 1Z,4Z-pentadiene motif. In the 13R-MnLOX from *G. graminis*, the pro-S hydrogen is abstracted first, leading to the formation of a radical. At this stage, oxygen is either inserted at C-11 or at C-13, in an antarafacial way, after delocalisation of the radical. Then the peroxyradical forms the final 13R-hydroperoxide or 11S-HPODE (Hamberg *et al.,* 1998). In iron LOX, the first step is identical but then the oxygen is inserted in a suprafacial way either at the [+2]-position or [-2]-position, yielding the 13*S*-HPODE or the 9*R*-HPODE (see chapter II for more details).

2.3 Role of oxylipins in plant-fungi communication and mycotoxin regulation

In fungi, the first oxylipins to be described derived from psi factor-producing oxygenases (Ppo enzymes) in *Aspergillus sp*. and were shown to coordinate its development through sexual and asexual sporogenesis (Champe *et al.,* 1987). Apart from their role in sporulation, fungal oxylipins can also regulate secondary metabolite production such as mycotoxin production (Brodhagen & Keller, 2006).

In fungi, Tsitsigiannis *et al*., (2006) found that a *ppo* mutant in *Aspergillus nidulans* was impaired for the mycotoxin sterigmatocystin synthesis but produced more penicillin. In the same way, oxylipins can up or downregulate mycotoxin production in other fungi. The deletion of *ppo* led to decreased

levels in T2-toxin in *Fusarium sporotrichoides* (McDonald *et al.,* 2004) and 13*S*-HPODE repressed the synthesis of aflatoxin and sterigmatocystin in *Aspergilii* (Scarpari *et al.,* 2014). As observed in plants and mammals, fungal oxilipins are perceived by G-protein coupled receptors (Affeldt *et al.,* 2012). GPCRs reside in the cell membrane were they perceive extracellular signals and trigger signaling cascades regulating both sporulation and mycotoxin production in *Aspergilii* (Brodhagen & Keller, 2006). In plant-pathogen interactions, plant and fungi can mimic the other partner's oxylipins (Christensen & Kolomiets, 2011) leading to increased virulence or reduced plant colonization by the fungus. Some of these examples are illustrated on figure I-15 together with examples showing the role of oxylipins in mycotoxin regulation.

This oxyilipin crosstalk has been studied in particular in the two pathosystems *Aspergillus sp.* and *Fusarium sp.* in maize. Gao *et al.,* (2007; 2009) demonstrated that maize oxylipins synthesized by a 9-LOX (*ZmLOX3*) are perceived by the pathogen *F. verticilloides* and lead to increased levels of fumonisin, contributing to the pathogen virulence (see figure I-15, n°5). Scala *et al*., (2014) observed that a *lds1* (*ppo* homolog) mutant in *F. verticilloides* no longer triggered the expression of *ZmLOX3* and *ZmPR1*, a maize chitinase. Thus, although this mutant was producing less mycotoxin, it exhibited increased virulence in contrast with the wild type, due to the non-induction of plant defenses (see figure I-15, n°8). In this example, the plant can perceive the fungal oxylipins and activates defense mechanisms (chitinases). However, the presence of the pathogen also induces the formation of plant oxylipins, which in return trigger the synthesis of mycotoxins by *F. verticilloides*. Recently, a fungal monooxygenase from *Magnaporthe oryzae* was reported to be secreted during infection by the pathogen and to enter the rice plant (Patkar *et al.,* 2015). This enzyme can hydroxylate jasmonic acid from the plant, preventing induction of jasmonate signaling, which explains how the pathogen can evade the plant defenses.

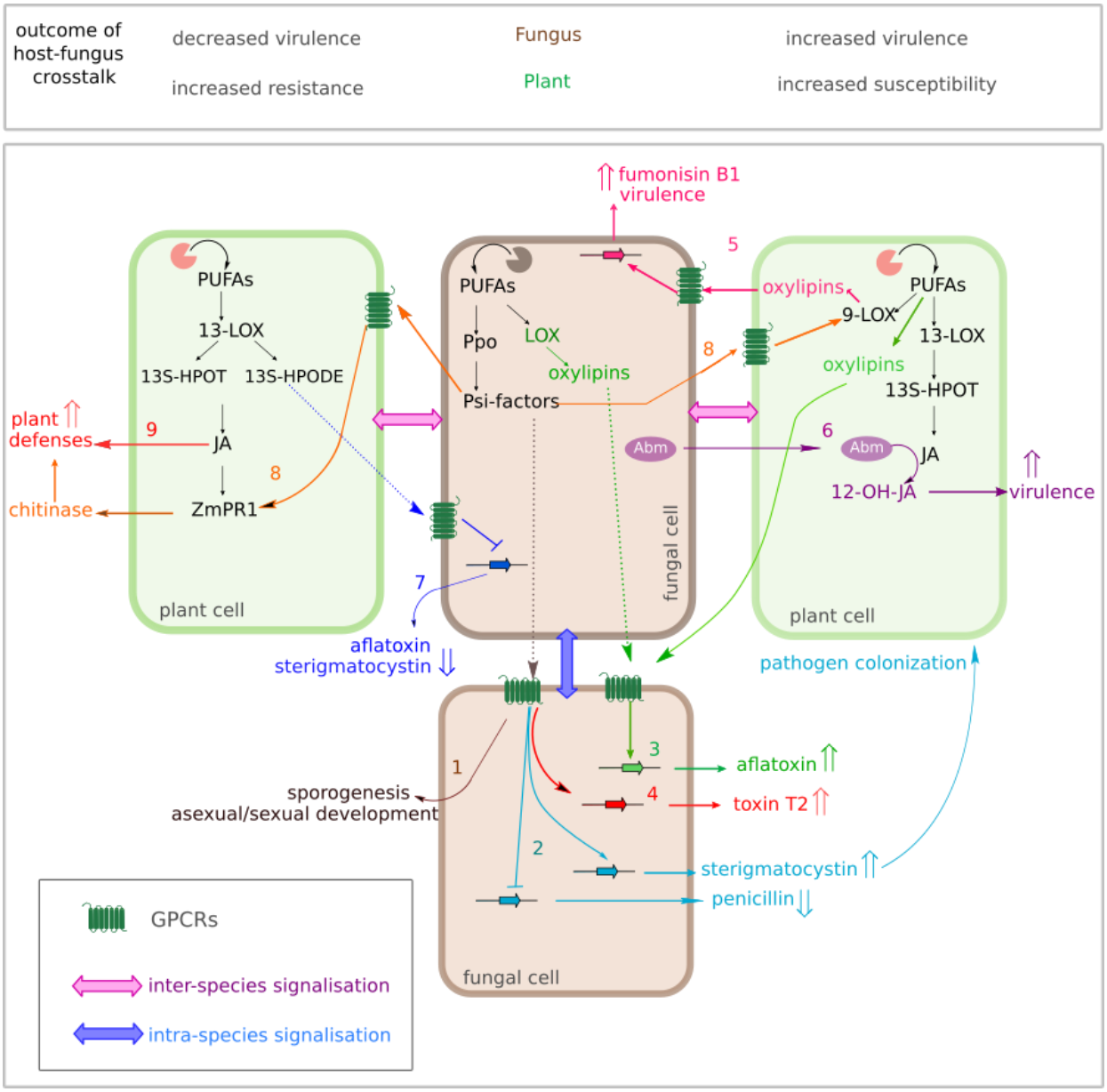


Figure I-15: Mutual oxylipin crosstalk between plants and fungal pathogens and proposed resulting mechanisms.

1-Deletion of *ppoA* in *Aspergillus nidulans* increases the ratio of asexual to sexual spores (Tsitsigiannis *et al.,* 2004). 2-*ppo* mutants in *A. nidulans* are unable to produce sterigmatocystin but overproduce penicillin. They are unable to colonize peanut seeds (Tsitsigiannis et *al.,* 2006). 3-Aflatoxin synthesis is hampered in *lox1* mutant in *A. flavus* but restored after inoculation on maize, suggesting that plant oxylipins can replace fungal oxylipins (Scarpari *et al.,* 2014). 4-T2-toxin synthesis is hampered in *ppo* mutants in *Fusarium sporotrichoides* (McDonald *et al.,* 2004).5-Fumonisin B1 synthesis by *F. verticilloides* was drastically reduced in maize kernels of *lox3* (9-LOX) mutants (Gao *et al.,* 2007). 6-A monooxygenase (Abm) from *Magnaporthe oryzae* converts JA into 12-OH-JA in rice, evading the plant defenses. *Abm* mutants are unable to colonize rice tissues. (Patkar *et al.,* 2015). 7-13*S*-HPODE from soybean represses aflatoxin synthesis in *A. parasiticus* and sterigmatocystin production in *A. nidulans* (Burow *et al.,* 1997). 8-A *lds1* (ppo homolog) mutant in *F. verticilloides* no longer triggers the expression of the lipoxygenase LOX3 (9-LOX) and the chitinase ZmPR1 in maize. *lds1* mutants are more virulent than the WT (Scala *et al.,* 2014). 9-JA, derived from 13-hydroperoxy-octadecatrienoic acid (13-HPOT),

induces resistance against necrotrophic pathogens in plants (Wasternack & Hause, 2013). **JA**: jasmonic acid; **LOX:** lipoxygenases; **Ppo**: Psi-factor producing enzymes; **PUFAs**: polyunsaturated fatty acids; **GPCRs**: G-protein coupled receptors

3. The mycotoxin beauvericin

3.1 Biosynthesis pathway

Mycotoxin beauvericin (BEA) production has been reported in several species of *Fusarium sp.* including different formae speciales of *F. oxysporum* as well as in the entomopathogens *Beauveria bassiana* and *Isaria sp.* (Logrieco *et al.,* 1998; Moretti *et al.,* 2007; Liuzzi *et al.,* 2017). BEA biosynthesis is catalyzed by beauvericin synthetase, a multimodular enzyme belonging to the Nonribosomal peptide synthetase (NRPs) family, with each module containing domains catalyzing an individual step of BEA synthesis (Finking & Marahiel, 2004). This enzyme utilizes D-2-hydroxyvalerate (D-hiv) and L-phenylalanine to form a dipeptidol monomer (Grove *et al.,* 1980). Three monomers are then assembled by cyclooligomerization to give a final cyclic trimeric ester. Xu *et al.,* (2008) proposed the biosynthesis pathway illustrated on figure I-16.

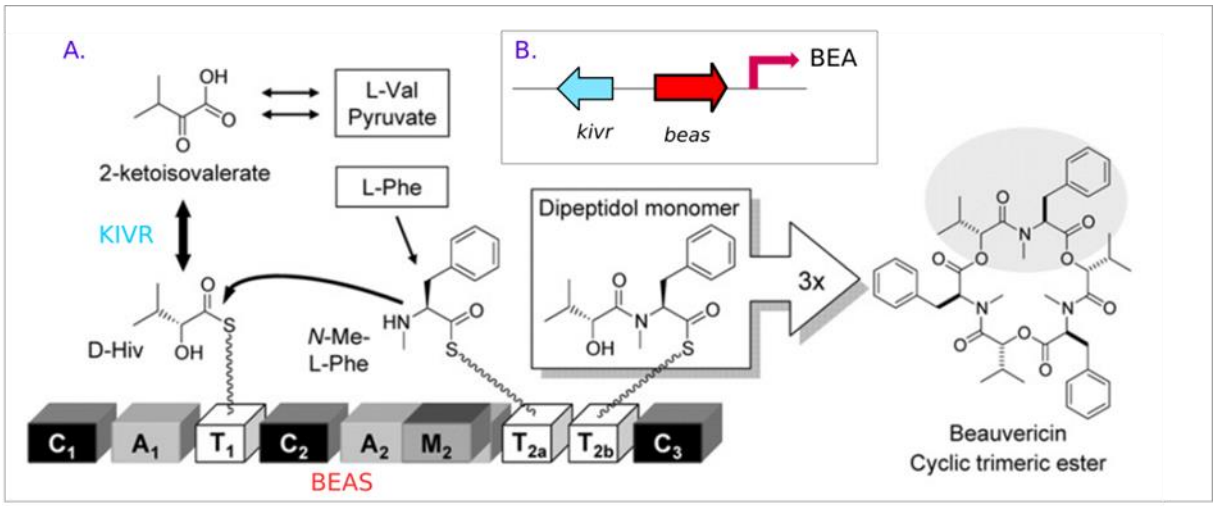


Figure I-16: Beauvericin synthetase locus and proposed biosynthesis of the cyclooligomer depsipeptide beauvericin in *Beauveria bassiana* (from Xu *et al.,* 2008). The modules of beauvericin synthetase enzyme (BEAS) are shown. **A**. First, 2-ketoisovalerate, from valine or pyruvate metabolism, is converted into D-Hiv by KIVR (encoded by *kivr* gene). A1 domain activates D-Hiv and installs it on the T1 domain. In the same way, A2 domain activates L-Phe and loads it on the T2a domain. Dipeptidol formation from D-Hiv and L-Phe is then catalyzed by the condensation domain C2. Finally, C1 and C3 domains are supposed to carry out the cyclooligomerization of the three dipeptidol monomers. **B.** Beauvericin synthetase encoding gene (beas) and ketoisovalerate encoding gene (kivr) are located on the same cluster in *Beauveria bassiana*. A: adenylation domain; T: thiolation domain; C: condensation domain; M: N-methyltransferase domain; KIVR : ketoisovalerate reductase

3.2 Known mechanisms of regulation

It is known that interactions between microorganisms can activate silent gene clusters and co-cultivating microorganisms is a fruitful source of novel secondary metabolites (Sheridan *et al.,* 2015). Therefore, it is reasonable to assume that the chemical communication between *P. variabile* and *F. oxysporum* could interfere with the regulation of secondary metabolites, such as mycotoxin beauvericin in *F. oxysporum*.

The regulatory mechanisms of secondary metabolite gene clusters are poorly understood. However, some recent publications have shed more light on the mechanisms regulating BEA synthesis in *Fusarium* species and possible conclusions are summarized on figure I-17. First, in *Fusarium* species, the BEA cluster organization is similar to the one described in *Beauveria bassiana*. In particular, the bidirectional arrangement of *kivr* and *beas* gene is highly conserved, but an additional *abc3* gene, encoding a putative ABC multidrug transporter was reported in *Fusarium* (Niehaus *et al.,* 2016). Their promoter is involved in the coordinated expression of *abc3*, *kivr* and *beas* gene (Lopez-Berges *et al.,* 2013; Niehaus *et al.,* 2016). Therefore, in the following work, we focused on the transcriptional regulation of *beas* gene only, as we speculated that *kivr* and *abc3* gene would be regulated in the same way. In *F. oxysporum*, BEA synthesis is regulated by the light-sensitive velvet complex and more BEA is produced in the dark (Lopez-Berges *et al.,* 2013). A similar regulation of the mycotoxins sterigmatocystin, fumonisin and trichothecene by the velvet complex has been reported in other fungi (Bok & Keller, 2004; Myung *et al.,* 2012; Merhej *et al.,* 2012). The velvet family proteins are highly conserved across filamentous fungi. VeA, VelB and the methyltransferase LaeA form together the trimeric velvet complex that could be regulating secondary metabolite clusters epigenetically, through modulation of chromatin accessibility (Bayram *et al.,* 2008). Interestingly, in *F. fujikuroi*, the BEA gene cluster is repressed by the histone deacetylase HDA1 and the methyltransferase Kmt6, which both modifiy chromatin structure (Niehaus *et al.,* 2016). It is commonly accepted that acetylated chromatin is relaxed and transcriptionnaly active, in contrast with trimethylated chromatin.

Lopez-Berges *et al.,* (2014) reported a role of nitrogen in BEA regulation, in agreement with the general observation in fungi, that the quantity of nitrogen affects the synthesis of a broad range of their secondary metabolites (Tudzynski, 2014). Finally, Ding *et al.,* (2015), showed the involvement of mitogen activated protein (MAP) kinases in BEA transcriptional regulation in *F. oxysporum f.sp. cubense*. The velvet complex seems to integrate a wide range of external signals, ranging from MAPK signaling, to light receptors (Sarikaya-Bayram *et al.,* 2015). In addition, velvet seems to have a role

in the activation of *Ppo* genes (producing the psi-factor oxylipins) in *Aspergillus nidulans* (Tsitsigiannis *et al.*, 2004).

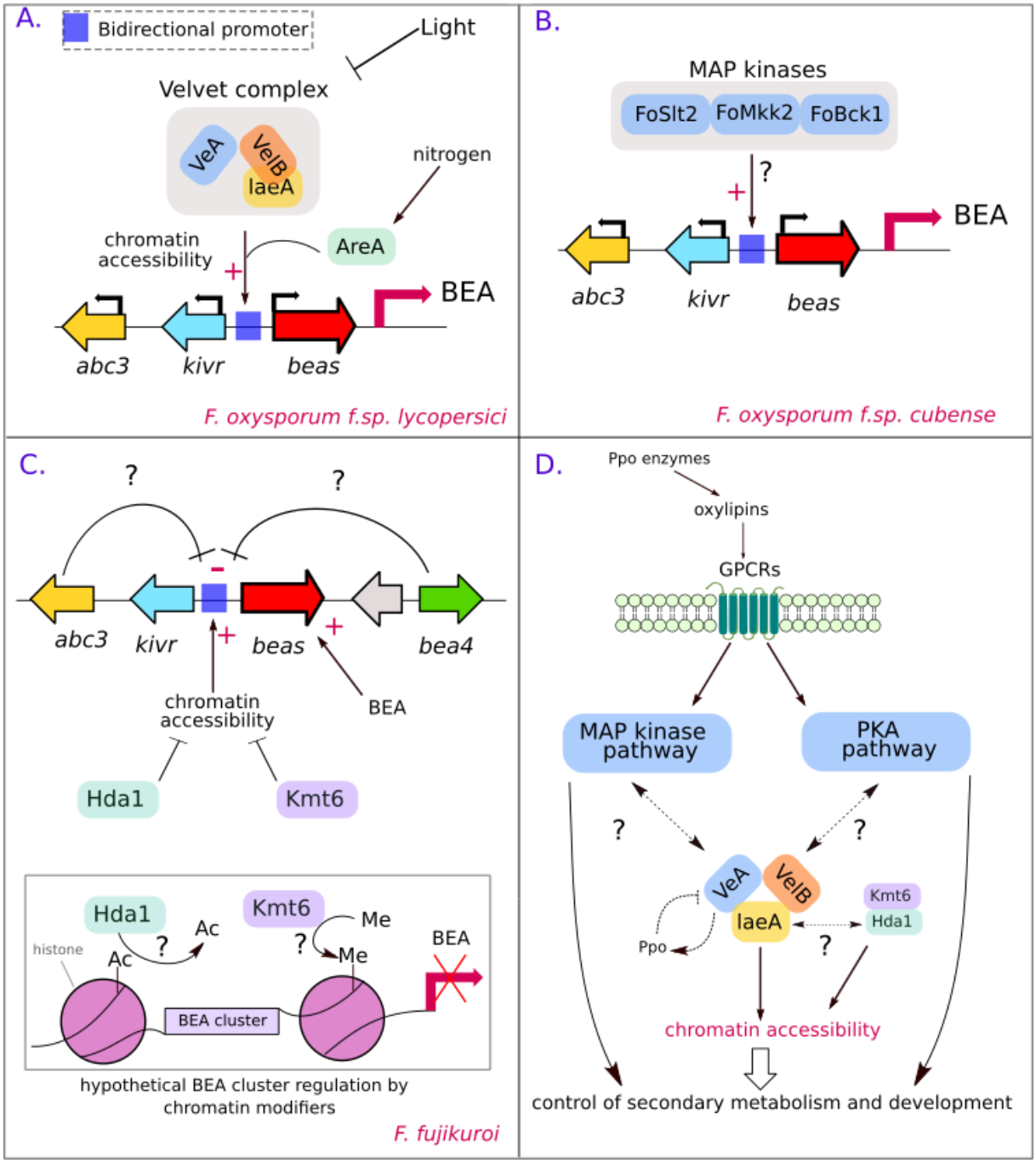


Figure I-17: Synthesis of proposed mechanisms regulating BEA cluster in *Fusarium sp.* **A.** Δvea, Δvelb and ΔlaeA mutants fail to increase chromatin accessibility, leading to lower expression of *beas, kivr* and *abc3*, and lower BEA synthesis. In WT, BEA synthesis is decreased by light (Lopez-Berges *et al.*, 2013). The nitrogen factor *AreA* is also required for activation of *beas* gene, via chromatin remodeling (Lopez-Berges *et al.*, 2014). **B.** Expression levels of *beas*, *kivr* and *abc3* are significantly reduced in MAP kinases mutants, but the exact mechanism is unknown (Ding *et al.*, 2015). **C.** In *F. fujikuroi*, addition of external BEA activates the transcription of *beas* gene, indicating a possible feedback loop. Δ*abc3* and Δ*bea4* mutants produce higher amount of BEA, suggesting that they are transcription factors repressing

the BEA cluster. HDA1 (histone deacetylase) and KMT6 (methyltransferase) knock-down mutants synthetize higher levels of BEA and show increased expression of the three cluster genes *abc3*, *kivr, beas*. HDA1 is supposed to remove the acetyl group from histone residues, and recruits the methyltransferase Kmt6 that methylates the histone in return. Trimethylation of the histone leads to repression of the cluster (Niehaus *et al.,* 2016). **D.** Hypothetical pathways linking secondary metabolite production and fungal development (adapted from Bayram & Braus, 2012). Oxylipins are supposed to be perceived by GPCRs and trigger the PKA pathway (Affeldt *et al.,* 2012). Ppo enzyme (producing oxylipins) and velvet regulation are linked (Tsitsigiannis *et al.,* 2014, 2015). All those pathways regulate secondary metabolite production (including mycotoxins) but the functional connection between them is unknown. **BEA**: beauvericin

Therefore, a complex jigsaw is emerging, explaining the coordination of secondary metabolite production and development in fungi through the velvet complex (Bayram & Braus, 2012), and showing that oxylipins and mycotoxins could share the same regulation pathways. However, the exact molecular connection between all those "protagonists" is still to be elucidated. To our knowledge, no direct link between oxylipin signaling and BEA regulation has been reported in the literature.

Chapter I

I. Establishment of a genetic transformation system in the endophyte *Paraconiothyrium variabile* and construction of oxylipin mutants

Prelude

Protoplast-mediated transformation, a suitable system for *P. variabile*

To date, no genetic tools have been developed for the endophyte *P. variabile* and nothing is known about its genetics. Protoplast-mediated transformation (PMT) is a simple and widely used genetic method for filamentous fungi. An important work on the technical prerequisites was carried out in our laboratory to check if this method was suitable for *P. variabile* (BTS C. Bourgoin, 2014). This technic is based on the efficient transfer of genetic material to protoplasts, cells with their cell wall removed. However, protoplast formation is not always possible in fungi. Preliminary experiments demonstrated that we could obtain protoplasts from fresh mycelium with a mixture of lysing enzymes containing chitinases, cellulases and proteases (Glucanex). The use of young mycelium (2-day-old) seemed critical for this step and 10^5 protoplasts per mg of mycelium could be obtained at best. The species *P. variabile* was also found to be sensitive to hygromycin at 100 $\mu g.ml^{-1}$ on PDA medium. This result indicated that hygromycin was a suitable selective marker for genetic transformation in this endophyte. Prior transformation assays of protoplasts with plasmid pCSN44 (Staben *et al.,* 1989) carrying the hygromycin gene resistance led to the obtention of several transformants. However, those transformants were found to be unstable, indicating the need for further improvements. Altogether, the work of C. Bourgoin validated the use of PMT for *P. variabile*. In this perspective, I constructed two deletion cassettes during my master thesis, to delete *pvlox1* and *pvlox2*. Flanking regions of target genes *pvlox1* and *pvlox2* were cloned on both sides of the drug-resistance marker gene hygromycin B phosphotransferase, conferring resistance to hygromycin. The construction of the two deletion cassettes for genes *pvlox1* and *pvlox2* is given in Appendix 2.

Objectives of the study

- **Establish a robust and efficient genetic transformation system in *P. variabile***
- **Utilize this system design to construct oxylipin mutants in *P. variabile* in order to investigate the role of oxylipin signalling in the interaction between *P. variabile* and *F. oxysporum***

1. Introduction

1.1 Genetic transformation in filamentous fungi

Not all transformation systems are suitable for every fungal species and there is no universal method. Therefore, it is necessary to optimize transformation protocols for every new species intended to be mutated, and to sometimes explore several technics. Various tools have been developed for the genetic engineering of filamentous fungi and figure C1-1 shows the general procedures for undertaking fungal mutagenesis. Transformation is about making DNA enter the cell. In target gene mutations, DNA must integrate at the right locus in the genome and be maintained despite cellular divisions. The advantage of filamentous fungi is that a new colony can grow from almost any fungal tissue. Therefore, it is possible to use mycelium (the vegetative part of any fungus), spores or fruiting bodies for transformation. However, the major barrier of transformation in filamentous fungi is their thick cell wall that preferentially needs to be removed to obtain protoplasts. In parallel, one needs to choose a specific gene targeting strategy in building the vector that will carry the foreign DNA. It is indeed possible to replace a whole gene, insert a mutation or hamper the translation into an active protein by targeting the mRNA. After the transformation process, several kinds of integration events can occur and will be presented. Finally, a screening strategy must be developed to select stable mutants on the basis of their new genetic content and phenotype.

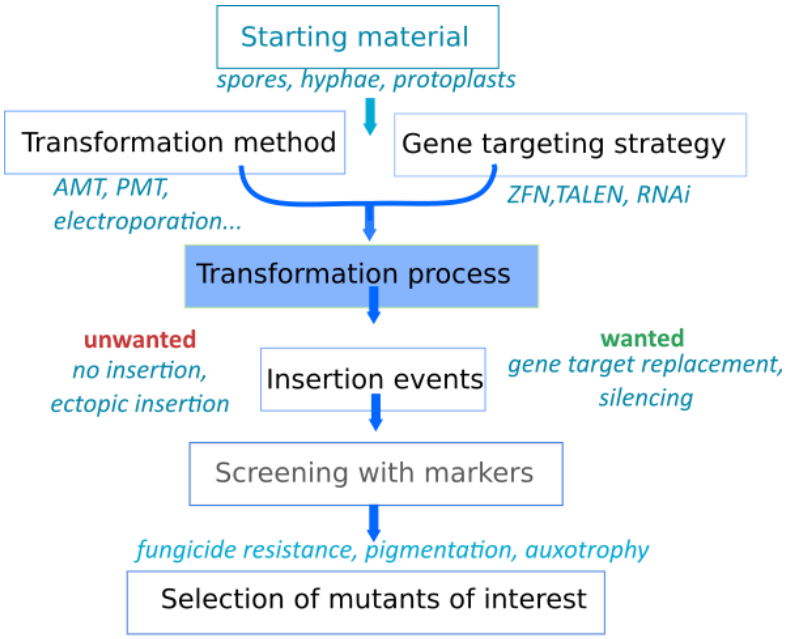


Figure C1-1: General procedure of genetic transformation in filamentous fungi.

1.2 How to make DNA enter the cells?

Agrobacterium-mediated transformation

This technic originates from a bacterial mechanism that was harnessed by scientists to generate mutants in filamentous fungi. *Agrobacterium tumefaciens* is a soil bacteria responsible for gall tumors on plants. This bacteria possesses a tumor-inducing plasmid (called the Ti-plasmid) which triggers tumor growth in plants (Michielse *et al.,* 2005). During plant infection, a fragment of Ti-plasmid is integrated in the plant genome. The idea is to modify the Ti-plasmid to insert any genetic construction inside the fungi genome. The main advantage is that several fungal tissues can be used as starting material and for most species there is no need to prepare protoplasts. Also, single copy integration seems to be favored, which is another advantage (Michielse *et al.,* 2005. A wide range of fungi have been succesfully transformed with *A. tumefaciens* (Jiang *et al.,* 2013) despite transformation frequencies varying from species to species. This technic has been used with success in *Coniothyrium minitans*, a species close to *P. variabile* (Rogers *et al.,* 2004). However, the process is time consuming and the success is dependent on co-culture parameters between *A. tumefaciens* and the studied fungus.

Protoplast mediated transformation

This technic is the most commonly used transformation method (Meyer, 2008) and is the one we selected for *P. variabile*. It relies on the use of lysing enzymes digesting the cell wall and liberating the protoplasts. Chemical reagents such as polyethylene glycol (PEG) are used to generate pores into cell membranes and to allow the entrance of foreign DNA. The main obstacle is the obtention of viable and numerous protoplasts. This technic is illustrated on figure C1-2. Released protoplasts are suspended in an osmotic stabilizer to avoid rupture of cells. Then, protoplasts are incubated with foreign DNA (plasmid or linearized DNA) together with PEG. To ensure good recovery of protoplasts, they are plated on a regeneration medium containing an osmotic stabilizer as well as a source of carbon. Layers of agar and selective compound (hygromycin in our case) are poured on the following days. Transformants able to grow on selective medium are then sub-cultured on a new selective medium. The main disadvantage of this method is the critical step of protoplastisation. However, it is cost-effective and requires few equipments.

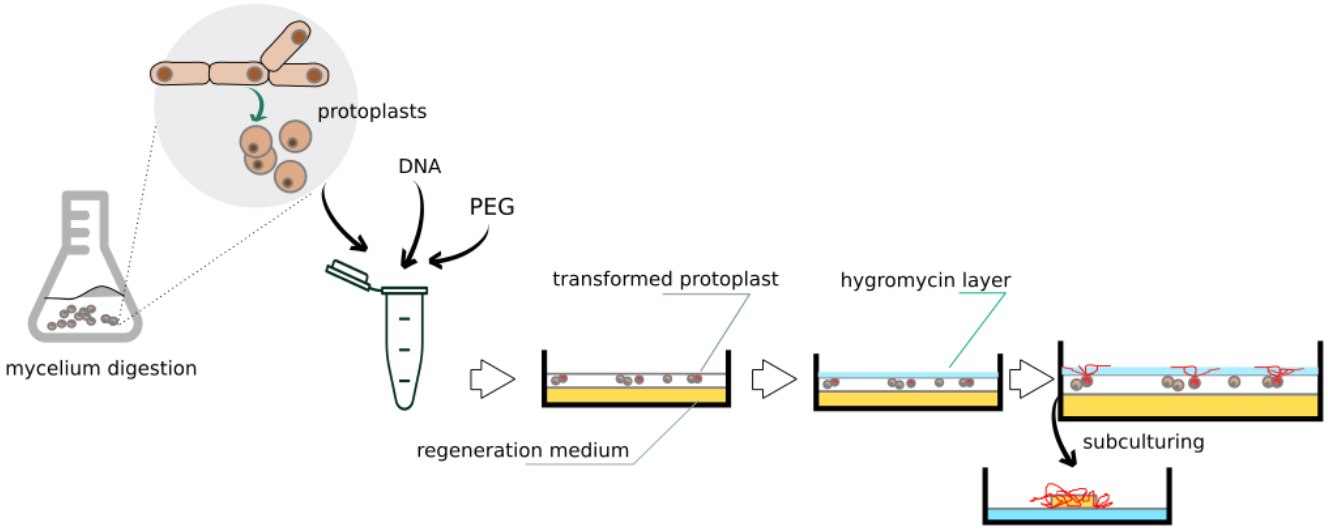


Figure C1-2: Main steps of protoplast-mediated transformation protocol (PMT). Fungal mycelium is digested to release the protoplasts. They are then transformed with polyethylene glycol (PEG) and exogenous DNA and plated on a regeneration medium. The selective pressure is added by pouring overlays containing hygromycin on the following days. Growing transformants are subcultured on hygromycin.

Biolistic method

This technic has been used in few fungi because of its costly equipment. Briefly, DNA is adsorbed on beads made of tungstene or gold, and bombarded on fungal material. In *Aspergillus nidulans*, a comparative study using microprojectile bombardment or protoplast mediated transformation gave similar results, but an increased percentage of mitotically stable transformants were obtained with bombardment (Herzog *et al.,* 1996).

Electroporation

Electroporation was developed for DNA integration in mammals and bacteria. Richey *et al.,* (1989) were the first to report a new system using an electric field to permit DNA integration using protoplasts from *Fusarium solani* and *Aspergillus nidulans*. Rapidly, *Neurospora crassa* was also transformed with electroporation, but using germinated conidia (Chakraborty & Kapoor, 1990). Electroporation is a good alternative for species in which protoplastisation is difficult. The principle is illustrated on figure C1-3. In electroporation, a cell is exposed to an electric field, that induces a voltage between the cellular membranes. This disturbs the phospholipid bilayer of the membrane and results in the formation of reversible pores. The membrane can restore its original structure except if an inappropriate electric field has been applied, leading to cell death. For this reason, electroporation is reported toxic for cells, which can be its major drawback (Li *et al.,* 2017).

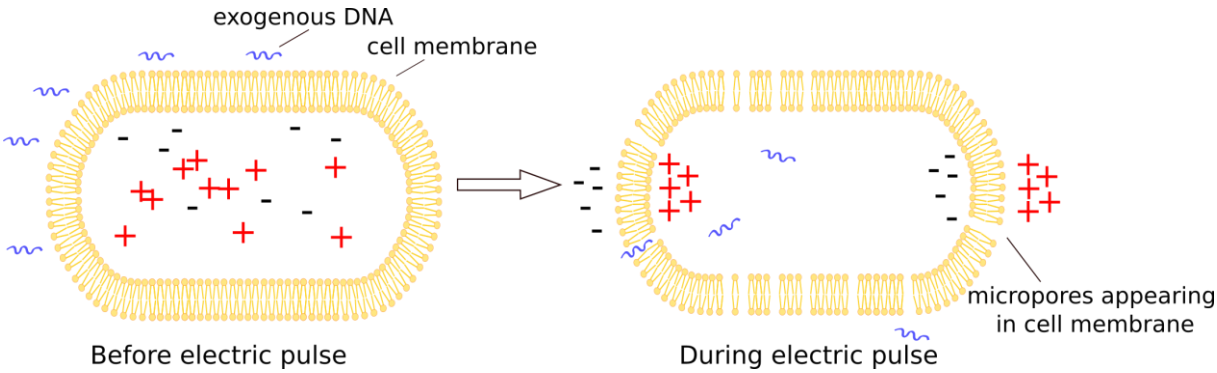


Figure C1-3: Electroporation principle. Exogenous DNA is uptaken by the cells through pores formed by the application of an electric field.

1.3 Gene targeting strategies

There are different ways of studying a gene function: one can perform a complete knockout by replacing the entire target gene or insert single mutations hampering transcription or leading to an inactive enzyme using Transposon Arrayed Gene Knock-out (TAGKO). Finally, one can knock down gene expression with RNAi, preventing mRNA of the target gene from being translated into the final protein. Those three strategies are pictured on figure C1-4.

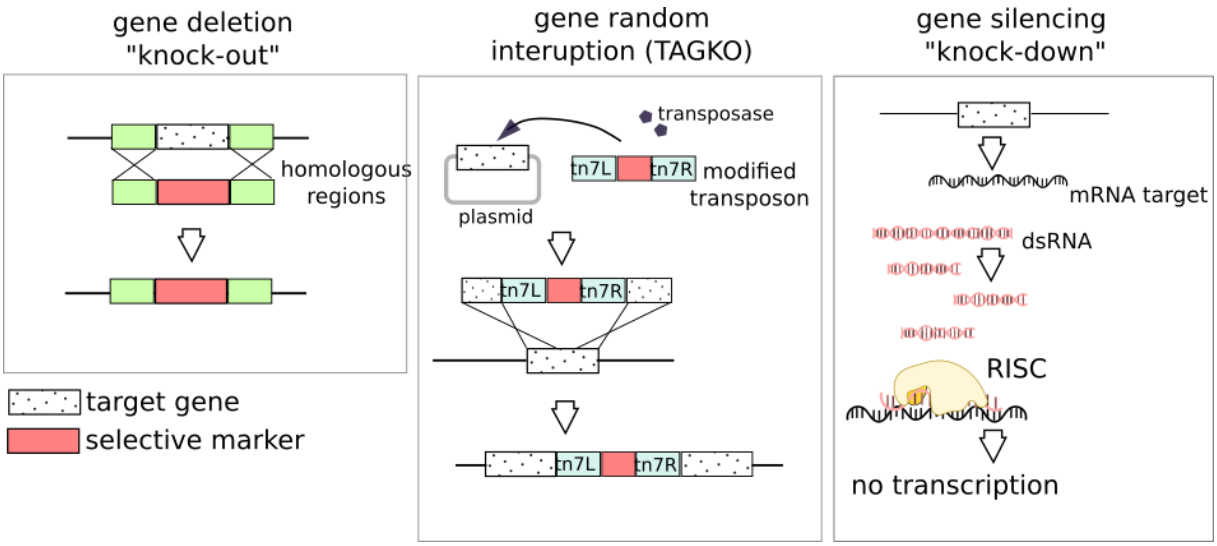


Figure C1-4: Main gene targeting strategies intended at modifying the expression of a specific gene. Gene knockout consists of total removal of target gene using a deletion cassette, which possesses regions of homology (in green). Transposon Arrayed Gene Knock-out (TAGKO) permits the introduction of gene mutations. Tn7L and Tn7R are the "left" and "right" ends of the bacterial Tn7 transposon. Gene silencing or RNAi involves lowering a gene expression by targeting the corresponding mRNA via the RNA-induced silencing complex (RISC).

Deletion cassette to completely remove a target gene

In knockout strategies, the ultimate goal is to inactivate a target gene. For this purpose, the whole gene can be deleted by replacing it with a synthetic DNA construction called "deletion cassette". Thus, the deletion cassette must possess homologous regions with the flanking regions of the target gene. The length of the homologous regions seems to be an important parameter. In *Neurospora crassa* for instance, 30% of transformants from homologous recombination were obtained when using a fragment with 9 kb of homology with chromosomal DNA, whereas shorter homologies yielded no transformants (Asch *et al.,* 1990). However, the main barrier is the synthesis of such large DNA constructions.

TAGKO strategy for introducing mutations

TAGKO stands for "Transposon Arrayed Gene Knock-out". This technic was developed to speed up the process of deletion cassette construction. It is particularly useful when a large collection of mutants is wanted. A fragment of chromosome containing the target gene is cloned into a plasmid. A second construction harbouring a selective marker flanked by two repetitive elements (tnl7 and tnr7) is introduced which corresponds to the transposon. In the presence of transposase, this last construction is cut and inserted randomly in the target gene contained in the plasmid. The resulting plasmid can then be used to transform the fungus. The advantage is that the same transposon can be used to generate any possible deletion cassette for any gene.

Using RNAi

When stable integration of foreign DNA (knockout) can not be achieved, translational repression with RNA interference knock-down (RNAi) can be an option. The mechanistic flow is as follows: a synthetic RNA strand complementary to the mRNA of the target gene is created and introduced in the cells. This synthetic RNA fragment combines with the RNA-induced silencing complex (RISC) and when it matches with its complementary mRNA, the latter is degraded. This technic bypasses the problem of transformation efficiency in fungi. Moreover, as the gene is only « silenced » and part of its activity can remain, partial expression can be studied. It remains the best option for genes that are essential for the fungus's metabolism and cannot be totally inactivated.

1.4 How to obtain a knockout mutant

Once foreign DNA has entered the cell, its journey is far from complete. The fate of DNA in cells is surely the most thrilling part of transformation process in fungi but also the least understood.

Genetic transformation of fungi relies on damaged DNA repair systems. Typically, during mitosis, when fungal cells divide to generate mycelium, double strand breaks (DSBs) can appear in DNA. The cells have developed an ingenious system to repair DNA and prevent aberrant recombination of chromosomes. It is precisely during those repair events that exogenous DNA can be integrated. Two competitive systems exist, triggered by DSBs (Hastings, 1992), known as the non-homologous end-joining pathway (NHEJ) and the homologous recombination (HR). Those two pathways are illustrated on figure C1-5 (A).

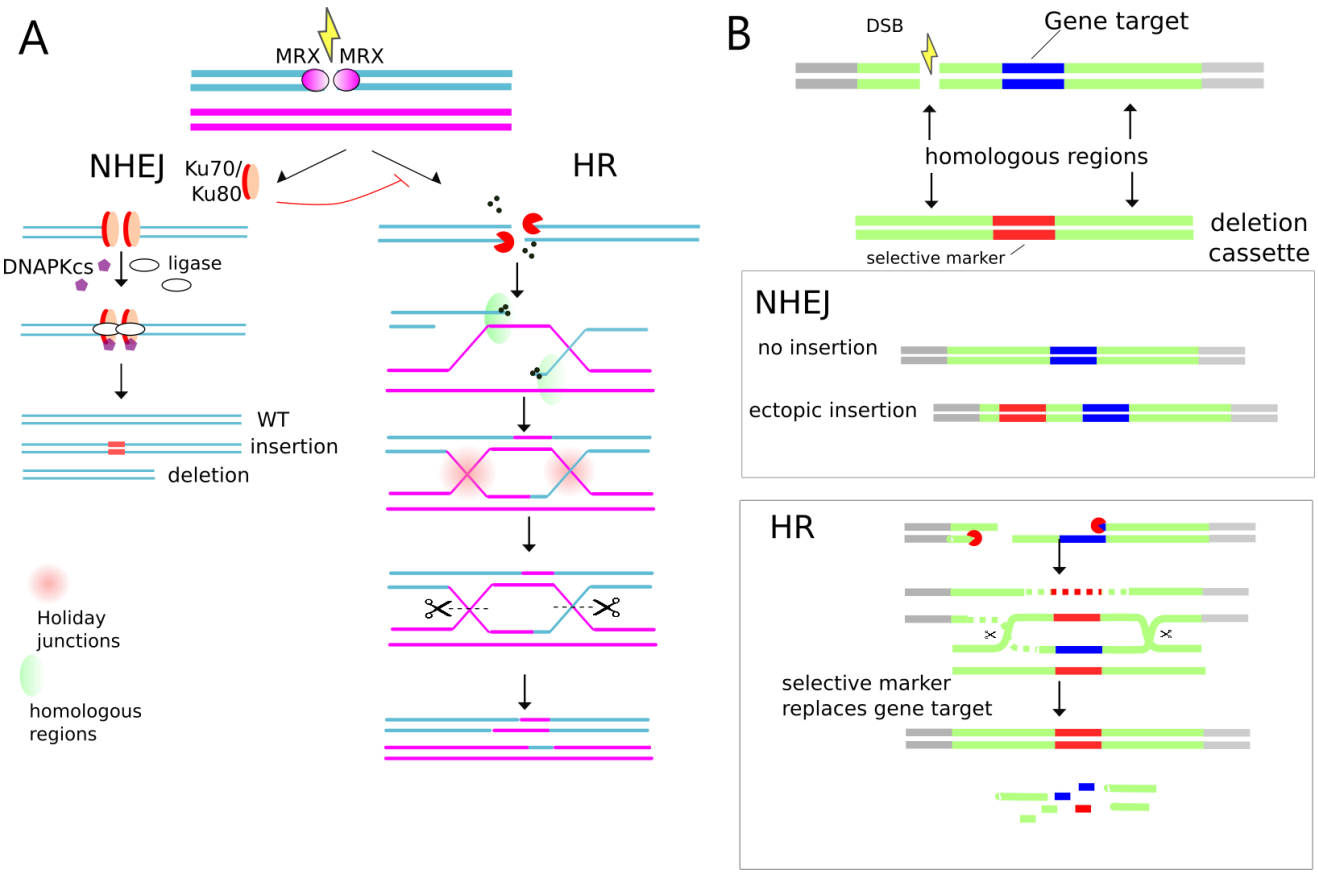


Figure C1-5: Non-Homologous End Joining (NHEJ) and Homologous recombination (HR) mechanisms. A) DNA repair mechanisms occurring during recombination. During NHEJ, initial DNA can be correctly repaired and the wild type (WT) genotype is maintained, or mutations due to insertion or deletion can occur. With HR, the DNA is correctly repaired. **B)** Hypothetical representation of foreign DNA integration during NHEJ or HR events. NHEJ typically leads to ectopic insertion of DNA, or no insertion. Integration of foreign DNA at the target locus only occurs with HR.

The NHEJ and HR pathways

Figure C1-5 (B) illustrates how those repair mechanisms could intervene with the different DNA integration events observed in fungi. In NHEJ as in HR, a protein complex (MRX) is recruited at DNA ends. Then, in NHEJ, Ku70 and Ku80 proteins bind to DNA ends and, in turn, recruit different kinases and ligases to ligate the two DNA fragments (Krappmann, 2007). Fidelity of NHEJ repair is low and consequently it leads to deletions or insertion of mutations at repair junctions (Decottignies, 2013).

When an exogenous DNA fragment is present in the cell, it can be entirely or partly integrated in the host genome by NHEJ process, leading to ectopic integrations. The selective marker may then be integrated at an unwanted locus.

The second repair mechanism, HR, is similar to the recombination event occurring during meiosis and generating genetic diversity through crossover. The HR pathway is repressed by the proteins Ku70 and Ku80, explaining why NHJ and HR are competitive mechanisms. After recruitment of the MRX complex, DNA ends are coated by replication proteins. A loop is formed and the intact strand serves as a template for the synthesis of a new repaired strand. A complex structure involving Holiday junctions is then formed. Holiday junctions can be resolved in multiple ways, only one of them is illustrated on figure C1-5 (A). In the presence of a deletion cassette with fragments homologous to the region where DSBs occurred, the synthetic DNA construct can be integrated by HR as illustrated on figure C1-5 (B).

Transformation is a rather scarce event in fungi. Indeed, NHEJ pathway is hampering transformation efficiency at the target locus since this is the favoured repair system in fungi (Decottignies, 2013). To bypass this problem, several NHEJ deficient strains have been engineered by deleting ku70, ku80 or the ligase4 genes (Jiang *et al.,* 2013). In *Neurospora crassa*, a Ku- recipient strain allowed the obtention of 99 mutants with a transformation efficiency greater than 90% (Colot *et al.,* 2006). Astonishingly, interfering with such an important biological mechanism does not cause undesirable side-effects (Krappmann, 2007), or maybe they are not reported in the literature.

ZFN and TALENs

When performing knockouts, the obtention of the desired mutant entirely relies on the probability of DSBs to occur at the target locus, which is a very scarce event. To circumvent this problem, strategies were developed in the 90s to favour the introduction of DSBs at precise locations. Those technics can be used alone, or combined with a deletion cassette. In the first case, because NHEJ is an error prone system, a mutation or a deletion could be introduced at the target gene and result in the desired mutant (not a knock-out, just a mutated gene). In combination with a deletion cassette, those technics will guide DNA repair by homologous recombination at the target locus and the deletion cassette may eventually replace the target gene (knock-out).

Two of this technics are illustrated in figure C1-6. Zinc-finger nucleases (ZFN) and transcription activator like effector nucleases (TALENs) use proteins to induce site-specific DNA cleavage (He *et al.,* 2016). Both systems rely on an ingenious idea: couple a non-specific DNA-cleaving enzyme (FokI) to a sequence-specific binding protein, just like a "hand with scissors" (Jiang *et al.,* 2013). In ZFN, each unit of 30 amino acids and a zinc atom can bind specifically 3 DNA base pairs (Carroll, 2011). TALEN uses a "one-to-one" correspondence between a module consisting of two amino acids and a DNA base pair. Once these modules have recognized their target, the FokI nuclease cleaves the DNA, causing DSBs and eventually a recombination event in the presence of a deletion cassette. However, those tools are tedious and difficult to build.

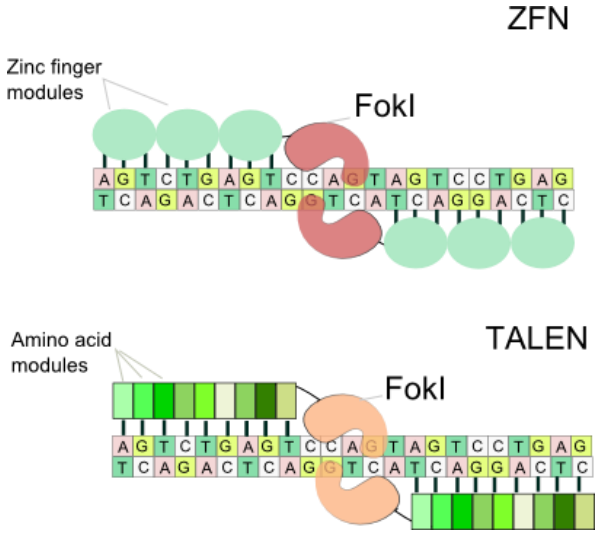


Figure C1-6: Double-strand breaks (DSBs) inducing tools. ZFN (zinc finger nuclease) and TALENs (Transcription Activator Like Nuclease), rely on amino acid modules that can specifically bind to DNA nucleotides. They guide the nuclease FokI that cleaves DNA at a precise location in return.

CRISPR-cas9

Though very promising, ZFN and TALEN technics have been recently supplanted by the emerging CRISPR-Cas9 strategy. This method and its application for fungal transformation in *P. variabile* is discussed with more details in section 3 of this chapter. Briefly, the principle is similar to TALEN and ZFN but instead of relying on synthetic constructions tedious to prepare, CRISPR-cas9 only needs a single bacterial plasmid, containing the nuclease Cas9, and a guiding element, allowing an outstanding flexibility (Nødvig *et al.,* 2015).

1.5 Screening transformants

Genes of resistance

Several selective agents are available for fungi. Among them are the widely known hygromycin and nourseothrycin, interfering with protein synthesis, phleomycin and bleomycin that inhibit DNA synthesis, carboxin that inhibits respiration or benomyl that hinders cell division. The most commonly used marker remains hygromycin because of its effectiveness in most fungal systems (Weld *et al.,* 2006). The hygromycin B resistance gene, *hph*, codes for a hygromycin phosphotransferase, which detoxifies the antibiotic. Disadvantage of these markers are the cost of selective compounds and natural resistance in some species.

Autotrophic markers

An alternative to drug resistance genes is the use of auxotrophic markers, the most commonly used being *pyrG* (orotidine-5'-monophosphate decarboxylase gene) (Jiang *et al.,* 2013). *pyrG* deficient strains require uridine supplementation for growth, enabling the selection of complemented strains. *argB*- and *niaD*- strains are also often used. The first one is auxotroph for arginine and the second one is deficient for nitrate reductase, making it sensitive to chlorate. However, there are few defined auxotrophic markers known for fungi and it requires the upstream isolation of auxotroph strains. As a result, only well-characterized and studied species such as *Aspergillus, Neuropsora* or *Fusarium* possess auxotroph mutants.

2. Results

2.1 Number of nuclei in *P. variabile* protoplasts

Before performing the transformation assays, we wanted to characterize *P. variabile* protoplasts by microscopy, for two reasons. First, protoplastisation can lead in some cases to nuclear loss and protoplast lysis. Second, many fungal species harbour multinucleated cells, but for successful genetic transformation, it is easier to start from mononucleate protoplasts, avoiding the obtention of heterokaryons (cells harboring both transformed and untransformed nuclei). In this purpose, we characterized protoplasts from *P. variabile* by fluorescence microscopy to check their morphology, as well as their cellular and nuclear integrity. Using DAPI staining we investigated the number of nuclei present in *P. variabile* mycelium cells and in protoplasts. 4′, 6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to double stranded DNA, more specifically to the minor grove in DNA helix. It can be excited by a UV source at 358 nm and it emits in the visible light at 461 nm (blue). DAPI can enter live cells and it is therefore not necessary to fix tissues prior to microscopic observation, which could alter cell morphology. In our case, hyphae were incubated with DAPI for 2 h or 4 h and observed under the microscope. Protoplasts from the same mycelium hyphae were prepared by a 2 h digestion and incubated with DAPI for different times.

The figure C8 depicts protoplasts and hyphae from *P. variabile* in bright field or fluorescence microscopy. All protoplasts observed displayed a round shape and harboured a single nucleus with some rare exceptions showing two nuclei (A). On picture B and C, in addition to protoplasts, hyphae being digested just before liberating protoplasts are visible and they also harbour a single nucleus. It should be mentioned that, unexpectedly, DAPI staining was not visible even after 5 hours of incubation with protoplats. Therefore, we waited over night and were able to detect DAPI stained nuclei. When staining hyphae without Glucanex digestion, after 2h of staining, the nuclei were still not visible (D). In contrast, several fluorescence hits were visible inside each cell, probably corresponding to mitochondrial DNA. Septa, dividers between fungal cells, are also visible. After 4h of staining, nuclei became visible and were stained at the same time as the septa (E). On picture F, the apical cell seems to possess several nuclei, which is often the case in filamentous fungi as apical cells are constantly dividing to give new cells. Finally, we could conclude that protoplasts from *P. variabile* were preserved during protoplastisation and were almost exclusively mononucleated, as the hyphal cells from which they originate with the exception of apical cells.

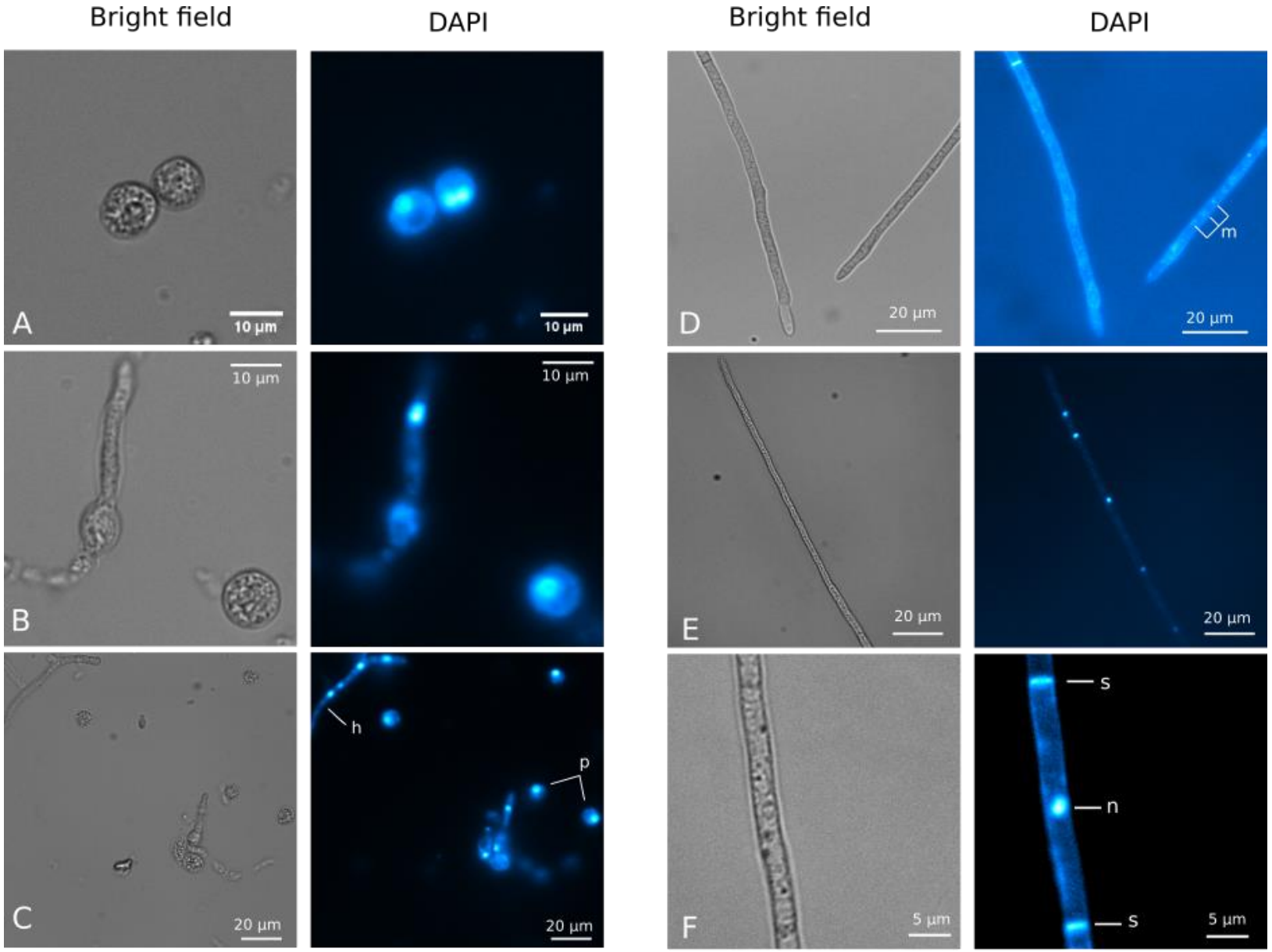


Fig. C8: DAPI stained nuclei of hyphae and protoplasts from *P. variabile*. A,B,C : Protoplasts of *P. variabile* (enzymatic digestion = 2 h, DAPI staining overnight) **D**: Apex of an hyphae from *P. variabile* (DAPI staining 2 h). **E** : Apex after longer DPAI staining (4 h).
F: focus on an hyphal cell (DAPI 4h) **h**: hyphae, **p**: protoplasts, **m:** mitochondria, **n:** nucleus, **s:** septum

2.2 Efficient screening of hygromycin resistant transformants

Preliminary work (BTS C. Bourgoin, 2014) showed that the wild type *P. variabile* is sensitive to hygromycin at 100 μg.ml^{-1} on PDA medium and 50 μg.ml^{-1} on malt agar medium. Unexpectedly, in the first trials to obtain transformants using 100 μg.ml^{-1} overlays (see figure C1-3 for overlay principle) control protoplasts (not having been in contact with transforming DNA) were growing in large number. We experienced several combinations of hygromycin layers by varying their concentration and by adding them earlier or later. Protoplasts need several hours to rebuild their cell wall. Therefore, it is assumed that they are very sensitive to hygromycin during this period and people use to add layers of hygromycin on the following day or several days after the transformation.

Table C1-1 gives the results for the 12 different combinations tested. A combination was considered efficient when no untransformed protoplasts were growing. We concluded that a layer of 300 μg.ml^{-1} added on day 0 was sufficient to eliminate background noise. This corresponded to a final concentration of 70 μg.ml-1 in the plate as we assumed that the hygromycin was diffusing into the lower regeneration medium. Lower concentrations resulted in many untransformed protoplasts regenerating. "Smoother" conditions could also be used by adding hygromycin at 300 μg.ml^{-1} the day after.

Days after transformation			protoplasts growing on control plate
0	1	5	
		200	+++
	100		+++
	200		+++
	100	100	+++
	100	200	+++
100			+++
200			+
100	100	200	++
100	200	200	++
100	300		-
300			-
	300		-

Table C1-1 : Different combinations of overlays tested. Hygromycin concentration is indicated as μg.ml-1.

2.3 Transformation trials to obtain *lox* mutants

Looking for hygromycin resistant transformants

After optimisation of the screening conditions, we attempted to obtain *lox* mutants in *P. variabile*. Plasmid pMB1 (see Appendix 2), containing the deletion cassette for *pvlox1* gene was used in the study. First, we evaluated protoplast viability under standard conditions of preparation. We found that the protoplasts started to germinate on the day following their transformation, as visible on figure C1-9 and regenerated as 4 mm mycelia colonies after 4 days. Protoplast regeneration ranged from 10% to 34%.

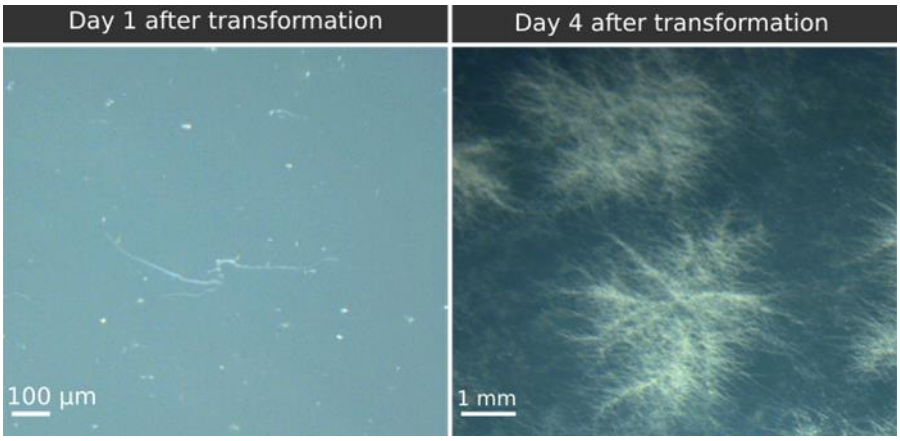


Figure C1-9 : Germinating protoplasts from *P. variabile* and young colonies observed under the stereo microscope. On the left picture, a filament originating from a protoplast is visible. Small dots are non-germinated protoplasts. Those filaments will give colonies 4 days after.

As several studies report that the ratio DNA to protoplasts number strongly influences the success of transformation (Armstrong *et al.,* 1990), we tested different DNA/protoplast ratios. In addition, it has been shown for some fungi, that the success of transformation can depend on the use of the circular or linear plasmid carrying the deletion cassette (Zhang & Wu, 1988). Therefore we used PCR amplified deletion cassettes, or the circular plasmid (pMB1) for transformation experiments. Results are shown in table C1-2.

With the PCR linearized cassette, we obtained 60 potential transformants (assays 3 and 4) that were subcultured on malt with hygromycin (100 µg.ml^{-1}) and 10% of them were developing on selective medium. After their second transfer to a new selective medium, 100% of them maintained their resistant phenotype.

Assay ID	origin of DNA	ratio DNA / protoplasts	potential transformants obtained on hygromycin overlay (300 µg.ml^{-1})	subculturing medium (hygromycin µg.ml^{-1})		potential transformants growing		Transformation efficiency (Tx/p)
				1st subculturing	2nd subculturing	1st subculturing	2nd subculturing	
1	linearized cassette	14 µg/ 7.10^5	0					
2		1,4 µg/ 10^6	0					
3		1,4 µg/10^7	15	Malt (100)		0		
4		1,4 µg/10^7	45	Malt (100)	Malt (100)	6	6	6.10^{-7}
5		1,4 µg/10^7	0					
6	linearized cassette purified	10 ng/10^5	1	Czapek (100)		0		
7		100 ng/5.10^5	6	Czapek (50/100)	Czapek (100)	5	1	2.10^{-6}
8		1 µg/10^7	5	Czapek (50/100)	Czapek (100)	3	1	1.10^{-7}
9		1 µg/10^7	22	Czapek (50/100)	Czapek (100)	11	7	7.10^{-7}
10		1 µg/10^7	0					
11		2 µg/10^7	0					
12		1 µg/10^5	3	Czapek (100)		0		
13		100 ng/10^5	2	Czapek (100)		0		
14		1,8 ng/5.10^4	0					
15	pMB1	1,8 µg /10^6	3	Czapek (100)		0		
16		100 ng/10^5	4	Czapek (100)	Czapek (100)	2	2	2.10^{-5}
17		1,78 µg/2.10^5	0					
18		10 µg/10^6	0					
TOTAL			106			27	9	

Table C1-2 : Results of the different transformation assays of *P. variabile*. The table indicates the number of transformants growing on selective medium after different subculturing events. Transformation efficiency was calculated as the number of transformants obtained per protoplasts (Tx/p).

Transformation trials with the linearized purified cassette, that is, after removal of PCR components, have led us to obtain 39 potential transformants. This time, we subcultured them on a Czapek-Dox medium. In the previous experiments, we observed that 90% of transformed protoplasts germinating in the overlays were unable to recover growth after their transfer on a selective medium. This made us think that our selective medium containing malt agar with the hygromycin could be inappropriate for young colonies development. For this reason we used the synthetic Czapek culture medium because it contains a high amount of saccharose (30 $g.L^{-1}$) as a carbon source, which is metabolized more effectively by fungi than complex sugars present in malt medium. We observed that on Czapek-Dox, 19 out of 39 transformed colonies keep on growing, giving a total of ≈49%, which was higher than on malt medium. However this time only 47% of them maintened their phenotype after a second transfer on selective medium.

Finally, transformation trials with the plasmid pMB1 led us to obtain only 7 transformants and 2 of them could resume growth on selective medium after successive subculturings. All together, we had a transformation efficiency of around 6.10^{-7} stable transformants per protoplast for linearized casettes, 1.10^{-7} to 2.10^{-6} for the purified cassette and up to 2.10^{-5} for pMB1 plasmid. The highest rate was obtained with 100 ng of plasmid for 10^5 protoplasts.

In order to better characterize the transformants, we also measured their radial growth rate on a 20-day period with hygromycin, after the first subculturing event. The results are shown on figure C1-10 for transformation assays 7,8 and 9 .

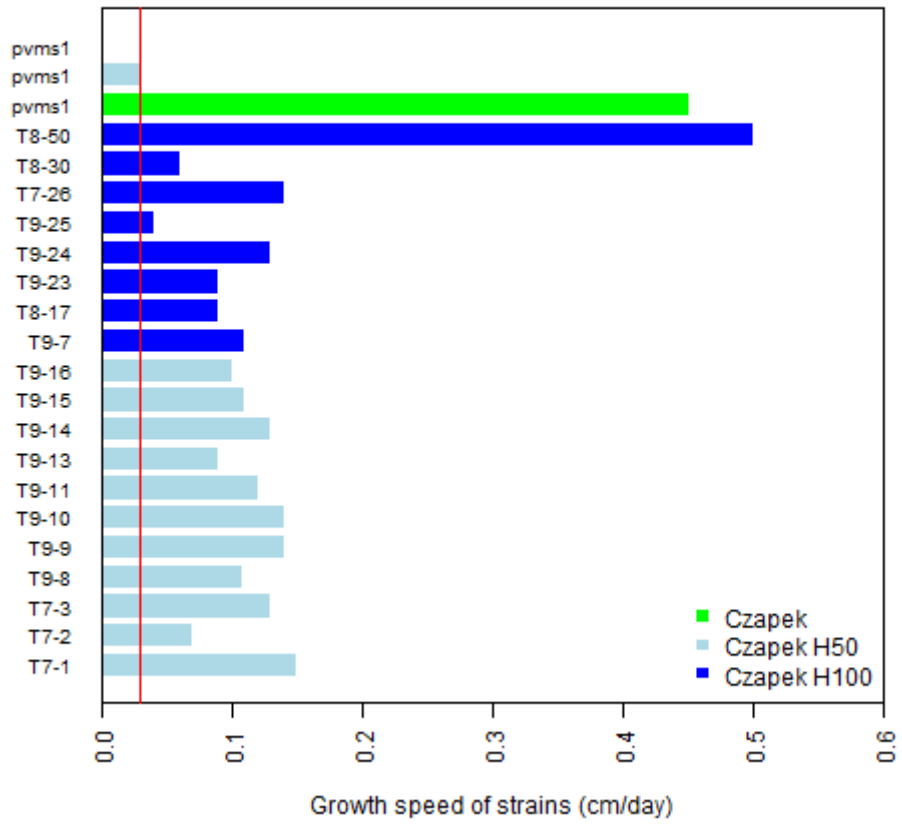


Figure C1-10 : Growth rate of potential transformants. Results shown are from transformation assays 7,8 and 9 on Czapek medium with 50 µg.ml-1 or 100 µg.ml-1 of hygromycin, after a first transfer on selective medium. Pvms1: wild type Monospore strain PVMS1, T = transformant.

The wild type strain (PVMS1) was slowly growing on Czapek with 50 µg.ml^{-1} of hygromycin but was no longer growing at 100 µg.ml^{-1}. In comparison, all transformants characterized grew faster. One of them, T8-50, exhibited the same growth rate of 0.5 cm/day on Czapek H100, as the wild type strain on non-selective medium. All strains were developing a whitish mycelium and never sporulated, as shown for transformant T9-13 (figure C1-11).

Only strain T8-50 exhibited the typical brown-green pigmentation of *P. variabile* and we observed the formation of pycnidia, the typical asexual fruiting body of *P. variabile* containing the spores.

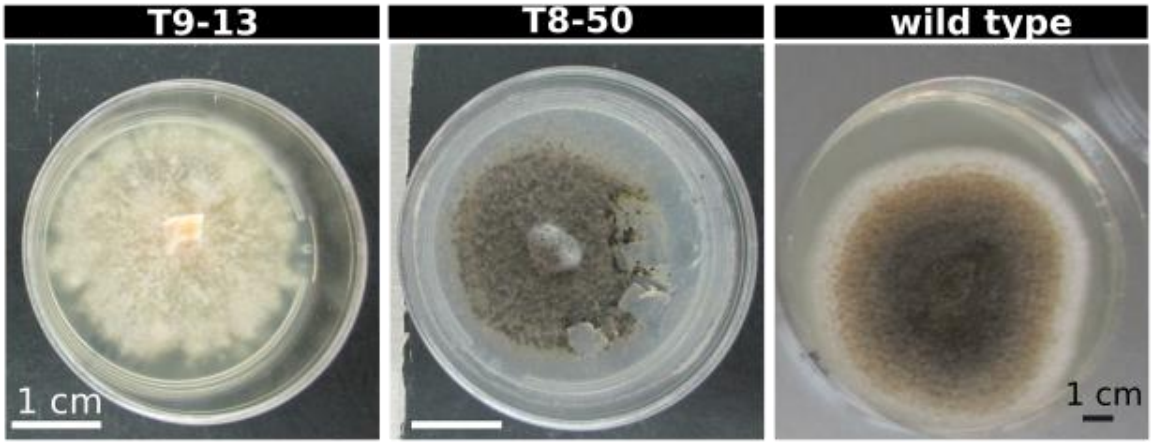


Figure C1-11 : Phenotype of transformant T9-13 and T8-50 on selective medium compared to the WT strain on non-selective medium. Transformants were growing on Czpakek medium with 100 µg.ml^{-1} of hygromycin. The wild type strain was growing on PDA medium.

To study hygromycin resistance stability of the transformant T8-50, we transfered it on a non-selective medium. After 15 days, a piece of mycelium was transferred to Czapek containing 100 µg.ml^{-1} of hygromycin, but the mycelium was unable to develop. In the same perspective, we characterized the phenotype of single-spore cultures originating from transformant T8-50. We plated a suspension of spores on Czapek with hygromycin and transfered 6 growing colonies on a new selective medium. The phenotype of 13-day monospore colonies is illustrated on figure C1-12. We observed that all of them displayed a whitish-flat mycelium, and a lower growth rate (0.2 cm/day) than their parent strain.

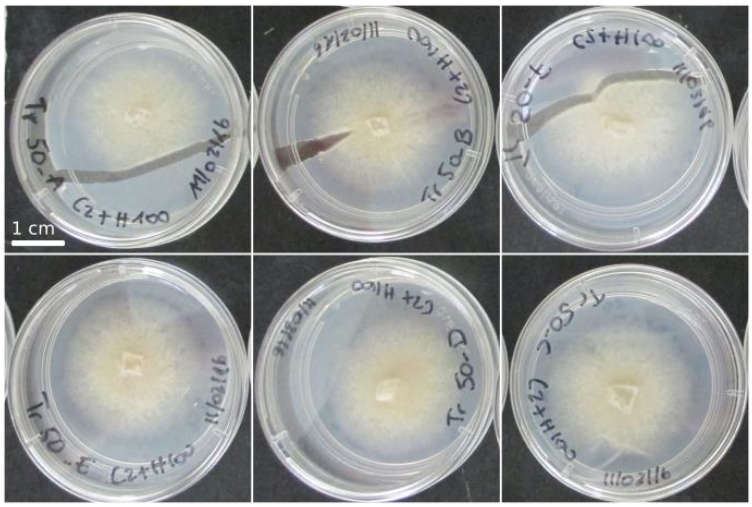


Figure C1-12 : Phenotype of single-spore colonies of transformant T8-50. Strains are growing on Czapek with 100 µg.ml^{-1} of hygromycin.

To conclude, considering all experiments, only 9 out of 106 transformants grew on selective medium after two successive subculturings, giving a rate of 8.4%. All of them except T8-50 displayed a whitish phenotype and were unable to sporulate on selective medium. However, T8-50 seemed to lose its resistance when cultured without selective pressure. In the same way, monospore cultures derived from T8-50 grew slower and were unable to develop pycnidia. Those results led us think that hygromycin resistance acquired by those transformants was somehow transient and lost more or less rapidly by strains. Nevertheless, we decided to go further with those 9 remaining transformants and look for evidences of *hph* gene integration into their genomes.

Stable integration of deletion cassette

We tested genetic integration of the *hph* gene in the nine transformants growing on selective medium after the first subculturing. Loss of hygromycin resistance but stable insertion of the *hph* gene has been demonstated before for a non-pathogenic *Botrytis cinerea* mutant (Kunz *et al.,* 2006). At the same time we analysed our transformants for possible *pvlox1* knock outs and/or ectopic integration of the deletion cassette (Figure C-13, Table C1-3).

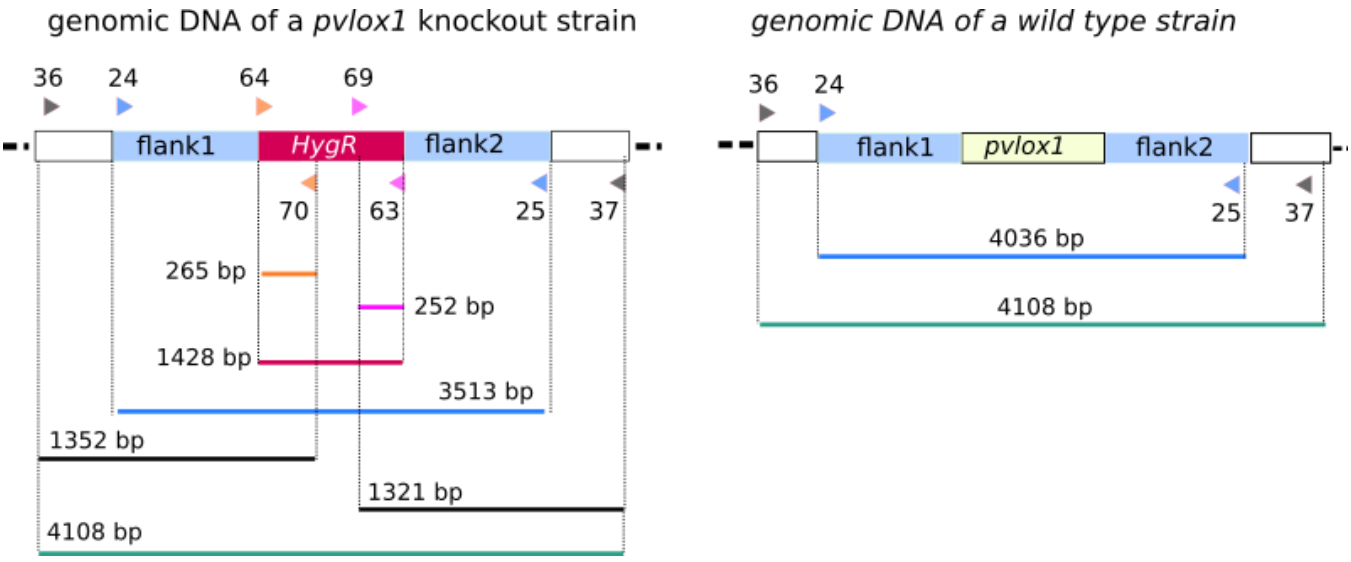


Figure C1-13 : Position of primers used to verify gene *pvlox1* deletion and integration of the *hph* cassette. Diagram shows a transformed chromosome and a wild type chromosome configuration.

primers	WT strain	*pvlox1* knock-out strain	ectopic integration
63/64	x	1428 bp	1428 bp
24/25	4036 bp	3513 bp	4036 bp
36/37	4108 bp	3585 bp	4108 bp
36/70	x	1352 bp	x
37/69	x	1321 bp	x
63/69	x	252 bp	252 bp
64/70	x	265 bp	265 bp

Table C1-3 : Expected length of PCR products. In the case of no integration (WT), replacement of gene *pvlox1* by the deletion cassette or ectopic integration of the deletion cassette.

For all transformants we amplified fragments of 4036 bp with primers 24/25 and 4108 bp with 36/37, indicating that gene *pvlox1* was still present in their genome and no PCR products of 3513 bp and 3518 respectively were detected, indicating the absence of knock-out mutants. PCR with primers 36/70 and 37/69 gave no amplifications confirming this conclusion. However, if *hph* was not integrated at the target locus, it could have been integrated ectopically. Thus, we performed a PCR with primers 63/64 amplifying the whole *hph* gene but obtained no amplification. We then thought that the *hph* gene could have been partly integrated in the genome and performed PCR with primers couple 63/70 and 69/64. Again, no amplification was achieved.

Taken all together, no stable hygromycin resistant mutant was found and neither knockout nor ectopic mutants were obtained.

3. Discussion

3.1 Low efficiency of protoplastisation and consequences for transformation

We can conclude that PEG-mediated protoplast transformation of *P. variabile* using conventional deletion cassettes is not easily achieved. In particular, the number of protoplasts remains a critical factor. In our case, only 7 out of 18 experiments were carried out with 10^7 protoplasts because protoplasts harvest was often insufficient. In yeasts, Aylon and Kupiec (2004) have shown that mitotic recombination is an infrequent event (10^{-7} to 10^{-4} per locus per generation). Therefore, the probability that a double strand break occurs at the right locus and homologous DNA can be inserted via HR is very low, and even lower when few protoplasts are available for transformation. In addition, HR occurs with low frequency in fungi (<5%) and depends on the species (Jiang *et al.*, 2013). In conclusion, as we get few protoplasts and maybe few HR events in *P. variabile*, DNA integration could not be observed.

In our laboratory, the same protocol of protoplastisation applied to the endophyte *Acremonium zeae* led to the collection of around 100 times more protoplasts (S. Mann and C. Kunz, personal communication). Thus, it confirms that protoplastisation efficiency is, above all, a matter of species rather than a matter of enzymatic conditions.

Surprisingly, the highest number of transformants per protoplasts was obtained using the plasmid pMB1 with few DNA (100 ng) and few protoplasts (10^5). However the question remains whether those results truly reflect the effect of DNA to protoplasts ratio or if other parameters could be responsible as they were all independent experiments.

3.2 Transient expression?

Unexpectedly, transformants seemed to loose rapidly their resistance to hygromycin which was reflected in lower growth rates or total loss of growth capacity on selective medium. It suggests that hygromycin resistance was ephemeral and transformants were not mitotically stable as *hph* integration in their genome was not detected. However, we did not check for the presence of the middle part of the *hph* gene. It is, however, unlikely, that such a truncated gene would lead to functional phosphotransferase production. Moreover, as cultures for DNA extraction were prepared in non-selective medium, it is possible that somehow *hph* gene was eliminated by cells during this step. Indeed, transformants seemed to lose their resistance even more rapidly if they were cultured without selective pressure.

As the *hph* gene was not stably integrated in the genome of *P. variabile*, transient hygromycin resistance found in our transformants might have bene due to transient *hph* gene expression.

Mönke & Schäfer, (1993) reported the transient expression of β-glucoronidase (GUS) gene in the maize pathogen *Cochliobolus heterostrophus*. This gene, derived from *E.coli*, participates into complex sugars metabolism and is not essential to the fungus. Therefore, because this gene is selectively neutral, the authors found no stable GUS transformants, although they detected GUS activity even 2 hours after protoplast transformation. However, if fused to the *hph* gene, GUS gene was stably integrated in strains selected on hygromycin. It does not really explain why in our case the gene *hph* is not stably integrated, since it is essential for growth on hygromycin, but it proves that exogenous DNA can be transitory expressed in filamentous fungi. In that respect, it would be interesting for instance to fuse *hph* to a visual marker such as the Green Fluorescent protein (GFP) to track its expression in cells. The mechanisms leading to transient expressions were extensively studied in plants (Sainsbury & Lomonossoff, 2014) but poorly investigated in filamentous fungi.

In some transformation protocols, autoreplicative plasmids have been used to increase transformation efficiency. Those plasmids can replicate in fungal cells. However, in our case, the plasmid pMB1 does not possess autoreplicative sequences and is a bacteria-derived plasmid. Thus, it is excluded that the plasmid could have been replicated during fungal growth, supporting *hph* transient expression in cells.

Methylation of donor DNA is another process that could be involved in unstable expression of *hph* gene. Methylation of DNA is a mechanism intended to regulate gene expression and heavy methylation inhibits gene transcription. In the basidiomycete *Schizophyllum commune*, during transformation foreign DNA was highly methylated, leading to low expression of the *hph* gene. (Mooibroek *et al.,* 1990). In addition, they demonstrated that if donor DNA was flanked by homologous regions, foreign DNA was no longer methylated and *hph* expression was higher. In our case, *hph* fragment is embedded in a large construction with 2 kb homologous regions. Thus, this hypothesis is questionable. However, foreign DNA rearrangements could lead to a transient expression of *hph* gene, prevented by a rapid DNA methylation.

The most likely hypothesis are that either the integrated *hph* cassette is lost during successive mitosis events, leading to abortive transformants, or the *hph* gene remains extrachromosomal and is transiently expressed in fungal cells. As septated hyphae forming mycelium can communicate with each other via septal pores, which allow passage of cytoplasm or macromolecules, it is possible that hygromycin B phosphotransferase is transfered from cell to cell.

Finally, we could think that *pvlox1* mutants are non-viable, explaining why they are never selected. So far, lipoxygenases are shown to be mostly involved in reproduction and secondary metabolite regulation and no vital function has been attributed to these genes. But, we can not exclude this possibility, even though *lox* mutants have been successfully obtained in other filamentous fungi such as *Fusarium verticilloides* (Scala *et al.,* 2014) which displayed similar growth than the wild type strain.

As we did not obtain ectopic mutant, it rather highlights a problem of DNA integration. Taken all together, stable foreign DNA integration in the *P. variabile* genome seems difficult to achieve. Apart from difficulty to obtain numerous protoplasts, some improvements could be advised to favour homologous recombination in our transformation system. First, we could make vary the length of homologous regions and try to increase them. In *Neurospora crassa*, Asch *et al*., (1990) designed a deletion cassette with 9 kb fragments sharing homologies to their target gene and observed that transformation efficiencies were very low for cassettes having less than 5 kb homologous regions. An other strategy would be to exploit genome editing tools intended to increase the probability of homologous recombination (HR).

3.4 CRISPR-Cas9, a tailor-made strategy for filamentous fungi

CRISPR refers to "Clustered Regularly Interspaced Short Palindromic Repeats". They are short repeated sequences found in bacteria in 2006 and proposed to be a part of a more complex bacterial adaptative immune system (Makarova *et al.,* 2006). CRISPR system allows bacteria to protect themselves against invading phages by degrading viral DNA on the basis of their recognition.

In CRISPR-Cas9, an endonuclease cleaves DNA specifically at the target locus, inducing double-strand breaks (DSBs) repair mechanisms. This strategy increases the probability of foreign DNA integration at the right locus by HR. This system consists of two components: the Cas9 endonuclease that will cleave DNA and a single-guide RNA (sgRNA) conducting the endonuclease to its target site. It was used for the first time for filamentous fungi in *Trichoderma resei* (Liu *et al.,* 2015), and in six different *Aspergillus species* (Nodvig *et al.,* 2015). A rapid search in the Pubmed database with the keywords "CRISPR-Cas9" and "filamentous fungi" sends back 34 publications, involving different model or non-model fungi, showing that this system is extending among scientific community. The Dothideomycete *Leptospheria maculans,* a close species to *P. variabile*, was efficiently transformed using this method in 2017 (Idnurm *et al.,* 2017). Beforehand, *L. maculans* could not be genetically transformed using classic PEG-mediated protoplast transformation.

A very complete work was achieved by Nodvig *et al.,* (2015) who developed a versatile CRISPR-Cas9 method using a single plasmid for transformation. This plasmid is efficient for transformation of filamentous fungi and its main features are illustrated on figure C1-14.

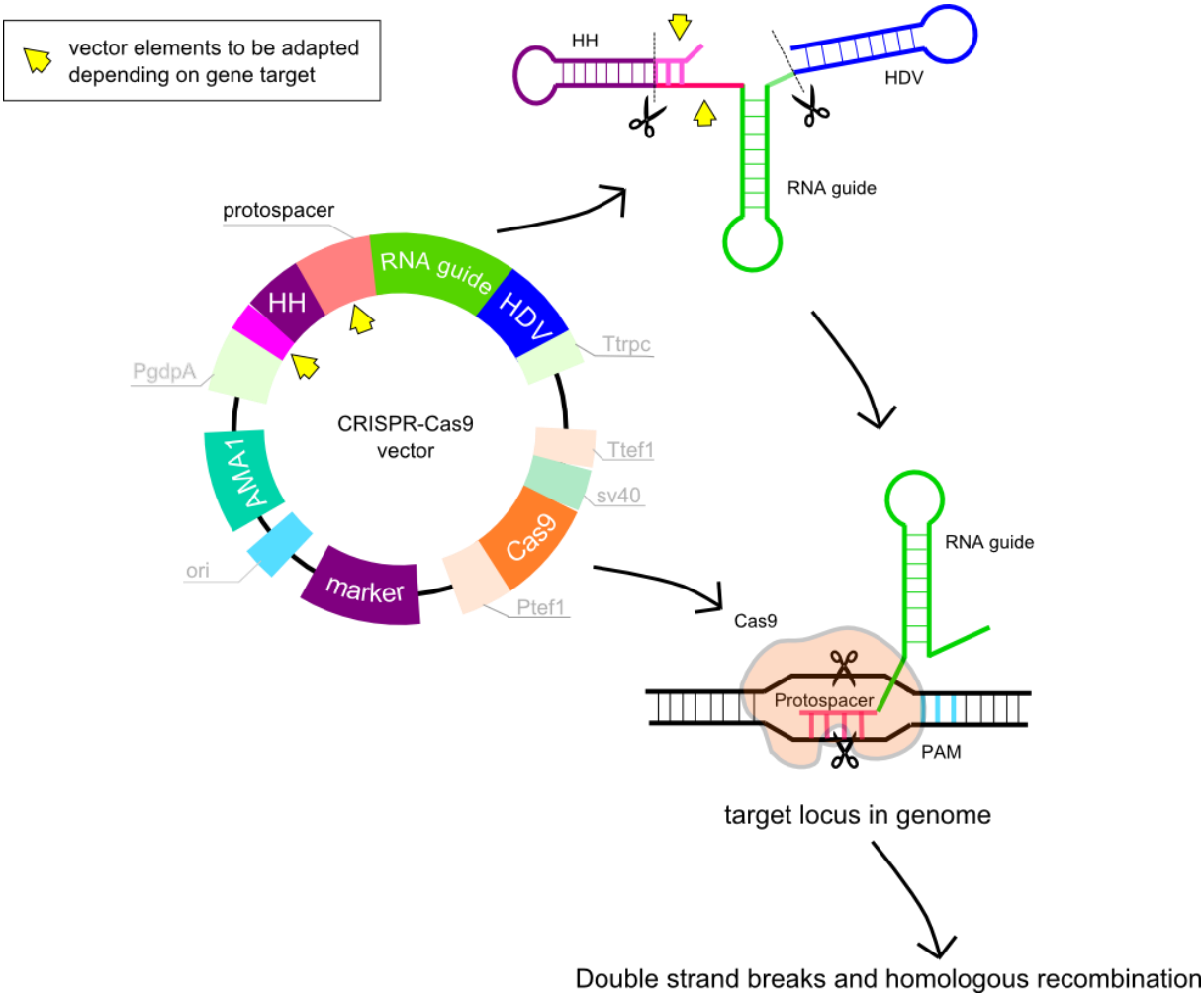


Figure C1-14 : CRISPR-Cas9 plasmid developed for transformation in filamentous fungi (adapted from Nodvig *et al.,* 2015). Cas9 gene is fused to sv40, a nuclear localization signal and strong promoters and terminators of tef1 (elongation factor 1 in *Aspergillus nidulans*). The plasmid was declined in four versions, and fungal markers can be *hph*, *ble* (resistance to bleomycin), *pyrG* (complementation of uridine auxotrophs) or *argB* (complementation of arginine auxotrophs). The sgRNA (or RNA guide) is embedded in a large transcript containing two ribozymes (HH and HDV) that will liberate the sgRNA as illustrated. In addition, the plasmid encompasses the AMA1 sequence, allowing plasmid replication in fungal cells. After the RNA guide has bound to its complementary sequence, Cas9 is recruited and DNA is cleaved.

This plasmid harbours the Cas9 gene and the RNA guide that will lead Cas9 to the target locus. The strength of this system is that only two elements in the plasmid backbone must be adapted from one species to another. Actually, those two sequences indicated by yellow arrows on figure

C1-14, will be forming together the so called "protospacer" in the RNA guide sequence. This region is of high importance as it drives the sgRNA towards the right genomic sequence. It is a 20-nucleotide sequence homologous to a region in the gene of interest. This sequence must be designed taking into account the existence of the PAM element (protospacer adjacent motif). It corresponds to a short 3-nucleotide sequence (5'-NGG-3') in host genome containing at least two guanidines in the end. The Cas9 will cleave DNA approximately 3 bases upstream of this motif. For *P. variabile*, we could therefore adapt this plasmid to target *pvlox1* and *pvlox2* genes. The CRISPR plasmid could then be used together with our deletion cassette to transform the protoplasts.

Of course, this system is not as trivial as widely claimed. It is possible that the Cas9 protein may be inactive in our system. Indeed, Nodvig *et al.,* (2015) codon optimized the sequence of the Cas9. It means they remove "rare" codons and replace them with abundant codons for a more efficient synthesis of this protein in their fungal model *Aspergillus*. However, this will not be possible for *P. variabile* as we do not know the codon usage in this strain. In addition, the design of protospacers is a critical step, and it is important to avoid off-target effects. Sometimes, multiple protospacers must be tested.

Nevertheless, this technic remains very attractive and the construction possibilities offered by this plasmid seem endless. For exemple, in their latest paper, Nodvig *et al.,* 2018 proposed a system in which the fungal marker is only present on the plasmid harboring the Cas9 (see figure C1-15). The plasmid containing the deletion cassette can thus contain only the homologous regions. As a result, the target gene will be simply deleted from the genome, with no insertion of selection marker. Therefore it is not necessary to maintain transformants on selective medium after their transformation has been validated, and also, they can be transformed again, using the same marker. It would be interesting in our case to generate double transformants of *pvlox1* and *pvlox2* in *P. variabile*. Of course, this strategy can be used only if the efficiency of CRISPR in the studied fungus has been demonstrated by upstream work.

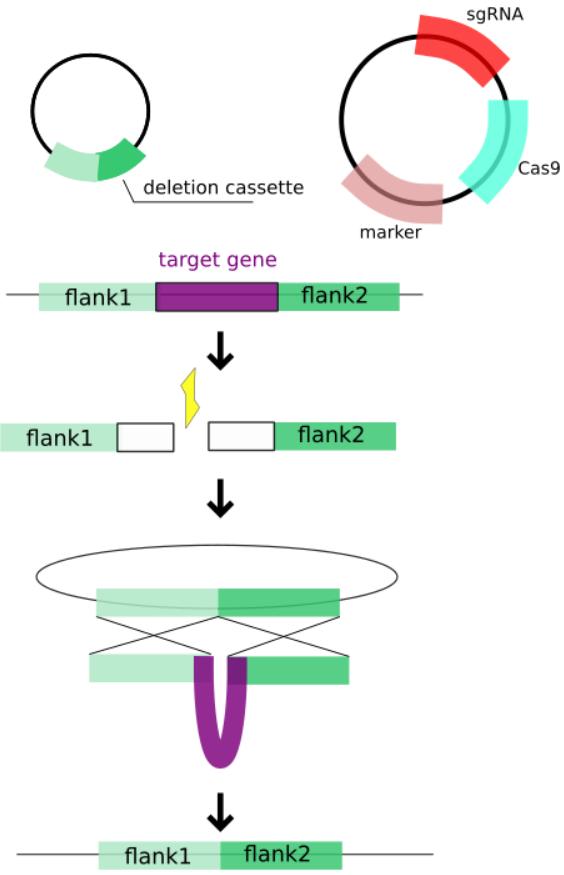


Figure C1-15 : CRISPR-Cas9 strategy to avoid the integration of fungal markers in host genome. A fungus is co-transformed by a plasmid harboring the CRISPR elements (Cas9 and sgRNA) and a plasmid harboring the two flanking regions of the target gene fused to each other (deletion cassette). After transformation, the CRISPR plasmid containing a selective marker is autoreplicated in cells and transformants can be selected. The target gene is deleted by homologous recombination but no selective marker is integrated in the genome, because it is carried by the CRISPR plasmid. The same marker can be used again to perform multiple transformations in the same species.

4. Experimental procedures

4.1 Strains and plasmids

We used a monospore strain MS1 derived from the endophyte *Paraconiothyrium variabile*, isolated from the conifer tree *Cephalotaxus harringtonia* by Combes *et al.,* (2012). This strain is conserved in the fungal culture collection of the Muséum National d'Histoire Naturelle under the identifier LCP5644. The strain MS1 was conserved in our laboratory in glycerol 20% at -80°C. Cultures were maintained on PDA solid medium (Potato Dextrose Agar Difco, CONDA: potato starch 4 $g.L^{-1}$, dextrose 20 $g.L^{-1}$, agar 15 $g.L^{-1}$, pH 5.6).

The plasmid pMB1 (see figure C1-16) obtained during my Master thesis according to the protocol from Colot *et al.,* (2006) was derived from the *E.coli* shuttle vector pUC18. It contains the dominant drug-resistance marker gene hygromycin B phosphotransferase (*hph*) from *E. coli*, flanked by the promoter of the *trpC* gene from *Aspergillus nidulans*. This gene lacks the trpC terminator and is designed to be inserted in the antisense direction relative to the target gene ORF. This plasmid also carries the ampicillin resistance gene (*Amp*R). The *hph* gene is flanked by approximately 1 kb regions identified as flank1 and flank2 on the plasmid map. They match respectively with the 5' and 3' DNA sequences flanking the target gene ORF *pvlox1*.

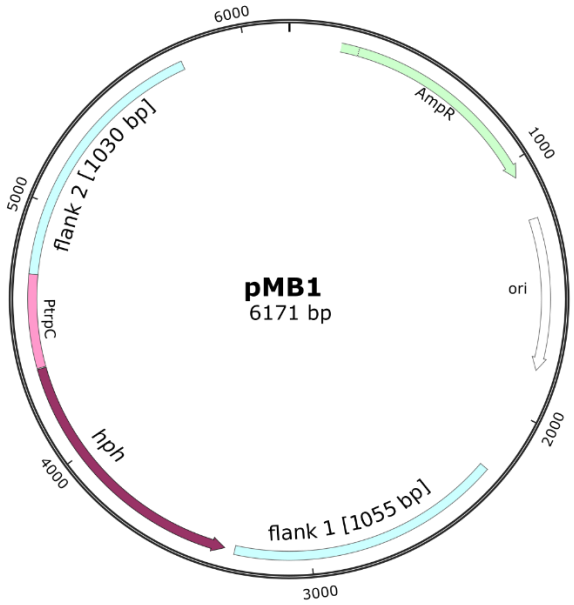


Figure C1-16 : Map of plasmid pMB1 containing the cassette for genetic deletion of *pvlox1* in *P. variabile*.

4.2 Isolation of protoplasts

Spores of *P. variabile* were collected by grinding a piece of mycelium (1 cm^2) from a 20-day-old culture in 1 ml of glycerol 25%, after elimination of the gelose. Spores were filtered on bolting clothes (100 µm and 25 µm) and the suspension was adjusted by counting spores in a haemocytometer cell-counting chamber. 1 ml of each suspension was inoculated into 50 ml of Malt medium (Malt Extract from CONDA, 10 $g.L^{-1}$) to reach a final concentration of 10^5 $spores.ml^{-1}$. We prepared 4 flasks and incubated them at 25°C on a rotary shaker (100 rpm) for 40 hours. Young mycelium grown in 500 ml flasks was collected by filtration on a bolting clothes (100 µm) and centrifuged in 50 ml of distillate water during 5 minutes, at 6000 g and 4°C, to remove excess of culture medium. Mycelium was then incubated in 50 ml of osmotic stabiliser (KCl 0.6 M, KH2PO4 25 mM, pH= 5.75) for 10 minutes. After filtration on bolting clothe (100 µm), the mycelium was weighted and incubated for 2 h to 5 h at 25°C on a rotary shaker (150 rpm), in a solution of Glucanex (Novozymes). Glucanex was prepared by diluting the powder in the osmotic stabilizer to reach a concentration of 0.6 mg/mg of fresh mycelium, and filter-sterilized at 20 µm. The protoplasts were then separated from mycelium debris by successive filtrations on bolting clothes (25 µm and 10 µm) and pelleted by centrifugation at 2000 g, for 15 minutes, at 4°C. Pellet was resuspended in 1 ml of KCa solution (KCl 0.6 M, CaCl2 50 mM) and protoplasts concentration was estimated using a haemocytometer.

4.3 Fluorescence cell imaging

A suspension of protoplasts was prepared as detailed in section 4.2 after 2 h of digestion with 7 mg of Glucanex/mg of fresh mycelium. Protoplasts were resuspended in a solution of DAPI and KCa (1.5 ml of KCa, 3 µl of DAPI (1000X)). We performed several mounts between slide and cover slip and observed them at different time points. The same mycelium as the one used for protoplastisation was also stained with DAPI for 2 h to 4 h. Control slides were prepared with unstained protoplasts and mycelium. Slides were observed under epifluorescence by using a NIKON ECLIPSE TE 3000 inverted microscope at the Museum microscopy platform (CEMIM), with the help of C. Willig. Images were acquired with Plan-Apochromat 60X and 40X oil immersion lens. Images were captured with a Photometrics Coolsnap HQ camera device and files were acquired with Metamorph (7.8.3.0) software. We used ImageJ (1.52) as the image processing software.

4.4 Preparation of deletion cassette

Plasmid pMB1

An overnight culture (170 rpm, 37°C) of *E. coli* TOP10 strain containing the pMB1 plasmid was prepared in 5 ml of LB medium (Tryptone 10 $g.L^{-1}$, yeast extract 5 $g.L^{-1}$, NaCl 10 $g.L^{-1}$) and ampicillin at 100 $\mu g.ml^{-1}$. 2 ml of bacterial culture were centrifuged at 8000 rpm for 2 minutes and plasmid pMB1 was extracted from the pellet with a GeneJET plasmid miniprep kit (Thermo Scientific #K0502), following the manufacturer's instructions with the following modification: the elution buffer was pre-heated to 70°C. DNA concentration was estimated with a spectrophotometer (Nanovue Plus CE Healthcore).

PCR-linearized cassette

The pMB1 plasmid was amplified by PCR from 11.5 ng of plasmidic DNA, using 1.4 µl of Phusion high-fidelity DNA polymerase, 28 µl of HF buffer 5X (Thermo Fisher Scientific #F-530S), 11.2 µl of dNTPs at 2.5 mM (Thermo Fischer Scientific #R0191), 7 µl of primers at 10 µM in a final volume of 100 µl. The deletion cassette was amplified with the primers 24 and 25 given in table C1-5 p.74. PCR amplification was performed with the following program: preliminary step at 98°C for 30 seconds, then 35 cycles including 1 minute at 98°C, 1 mn 45 at 72°C and a final step at 72°C for 10 minutes. PCR products were purified with the Nucleospin ® PCR clean-up kit from Macherey-Nagel according to the manufacturer's instructions.

4.5 Transformation of protoplasts

10^4 to 10^7 protoplasts in 100 µl of KCa were used in each transformation. 20 µl of DNA (linearized cassette ranging from 10 ng to 14 µg or pMB1 plasmid, ranging from 100 ng to 10 µg) in TE-CaCl2 (Tris-HCl 10 mM, CaCl2 10 mM, EDTA 1 mM, pH 7.5) were added with 50 µl of PEG. After a 20-minute incubation at room temperature, 170 µl of PEG were added. After a new 10-minute incubation at room temperature, the reaction was stopped by adjusting the final volume to 1 ml with KCa. A control without DNA was prepared with 20 µl of TE-CaCl2. Protoplasts were then solubilized in 9 ml of cooled-melted SH medium (noble agar 0.6%, HEPES 5 mM, saccharose 0.6 M, pH 7) and 3.3 ml were poured on solidified SH medium. Hygromycin B from *Streptomyces hygroscopicus* (Sigma-Aldrich H3274) at different concentration was added in the SH layer. Control plates without hygromycin were used to estimate protoplasts regeneration on the following day.

Plates were incubated at room temperature. In some experiments, additional layers of noble agar (0.6%) and hygromycin B at different concentrations were poured on the following days. This protocol was adapted from Hamada *et al.,* (1994).

4.6 Estimation of protoplasts regeneration

Regeneration rate was calculated as the ratio "germinated protoplasts/total protoplasts" in the absence of selective pressure. Protoplasts were counted along a vertical line drawn at the bottom of the Petri dish, with a stereo microscope (Olympus SZX12). This enabled us to estimate regeneration rate over more than 500 protoplasts.

4.7 Selection of transformants

Mycelium forming colonies on selective medium were cut out from medium and subsultured on malt agar medium (Malt Extract from CONDA at10 $g.L^{-1}$, agar from Carl Roth at 6 $g.L^{-1}$) with 100 $\mu g.ml^{-1}$ of hygromycin or Czapek-DOX medium (composition is given in table C1-4) containing 50 or 100 $\mu g.ml^{-1}$ hygromycin.

Table C1-4 : Czapek-DOX medium composition

Product	Final concentration ($g.L^{-1}$)
$NaNO_3$	0.8
KCl	0.2
$MgSO_4$	0.2
$FeSO_4$	$1.6.10^{-3}$
KH_2PO_4	1
$ZnSO_4$	$3.2.10^{-4}$
$CuSO_4$	$1.6.10^{-4}$
saccharose	30
agar	15

4.8 DNA extraction and PCR screening

Genomic DNA extraction

Flasks with 50 ml of liquid malt were inoculated with spores from fungal strains or small pieces of mycelium if the strain formed no spores. After incubation at 25°C on a rotary shaker (160 rpm) during 2 days, we collected the mycelium on the sides of the flasks or from the gelose pieces. The

mycelium was washed in 1 ml of distilled water. Around 0.2 g of mycelium was grinded using lysing beads (MP Biochemicals lysing Y) in 1 ml of Sarcosyl buffer (Tris-HCl 50 mM, EDTA 50 mM, sarcosyl 0.2%, NaCl 150 mM), in a FastPrep homogenizer (FastPrep-24 from MP Biochemicals). The beads were shaken two times for 30 seconds at 6 movements per seconds with a middle step on ice for 5 minutes. Cellular debris were removed by centrifugation (5 minutes at 4°C and 4 g). 700 µl of supernatant were mixed with 700 µl of phenol and incubated for 3 hours on ice. Samples were then centrifuged for 15 minutes (13000 rpm, 4°C) and the supernatant was precipitated with an equal volume of isopropanol. DNA was pelleted by centrifugation for 15 minutes at 13000 rpm and washed with ethanol 70%. The pellet was dried at 37°C and resuspended in 50 µl of TE buffer (Tris-HCl 10 mM, EDTA 1mM, pH 8). Around 20 ng of genomic DNA was introduced in a PCR mix containing 0.1 µl of DreamTAq DNA polymerase, 2 µl of buffer (Thermo Fisher Scientific), 1.6 µl of dNTPs at 2.5 mM (Thermo Fischer Scientific #R0191), 0.4 µl of each primer at 10 µM in a final volume of 20 µl. Details of primers are given in table C1-5 and thermocycler programs are given in table C1-6.

Primer n°	Primer sequences (5'→3')
24	AATGAGCGTACCAGCGAACATAAC
25	ACTCCTTGTTGTTGGTGCCATTC
36	ATGTGGCTGTCCTCCTAGTCG
37	TTAGGCTCCTCCGTAGTCGC
63	GACAGAAGATGATATTGAAGGAGC
64	GATTTCAGTAACGTTAAGTGGAT
69	CAAGTTATCGTGCACCAAGCAG
70	CCGCATTGGTCTTGACCAAC

Table C1-5 : PCR primers used for amplification of deletion cassette or discriminant analysis of transformants.

PCR step	Number of cycles	24/25	63/64	36/37	36/70 and 37/69
Initial denaturation	1	3 min, 95°C			
Denaturation	30	30 sec, 95°C			
Annealing		1 mn, 54°C	1 min, 46.3°C	1 min, 57°C	1min, 53°C
Elongation		4 min, 72°C	1 min, 72°C	4 min, 72°C	1 min, 72°C
Final elongation	1	10 min, 72°C			

Table C1-6 : PCR programs used for discriminant analysis of transformants

Chapter II

II. Heterologous expression of PVLOX1 and PVLOX2 lipoxygenases from *Paraconiothyrium variabile*

1. Introduction

1.1 Lipoxygenases are « juggling enzymes »

During the formation of hydroperoxides by fungal lipoxygenases (LOXs), oxygen can be inserted at the [+2]-position or [-2]-position on the fatty acid substrate, yielding 13-hydroperoxides or 9-hydroperoxides (it is referred to as "regiospecificty"). In addition, the oxygen insertion can yield *S* or *R*-hydroperoxides. In this case, one refers to "stereospecificity" (Figure C2-1).

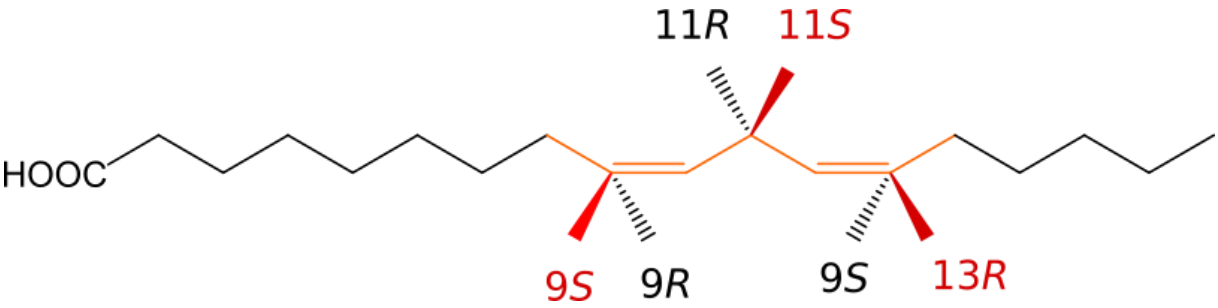


Figure C2-1: Overview of possible lipoxygenation positions of linoleic acid. The pentadiene is highlighted in orange (From Wennman & Oliw, 2013)

Regio and stereospecificity are key steps for the enzymatic cascade leading to the formation of hydroperoxides. In plants for example, the positional specificity is of high importance, as only (13S)-hydroperoxy octadecatrienoic acid (13-HPOTE) but not 9-HPOTE is the precursor for the plant hormone jasmonic acid that is crucial for defense responses (Creelman *et al.,* 1995). Up to now, despite numerous papers addressing the structure-function relation of LOXs, no unifying model has succeeded in fully explaining how this specificity is controlled in LOXs.

Regiospecificity

Several important amino acids, termed "determinants", have been proposed to explain why LOXs catalyses the insertion of oxygen on a specific carbon. In the human 15-LOX, a methionine at position 418 is responsible for the positioning of the fatty acid (arachidonic acid) substrate in the active site (Sloane *et al.,* 1991). Replacement of Met418 by a less "space-filling" amino acid such as valine, shifted oxygen insertion from C-15 to C-12 (see figure C2-2)..

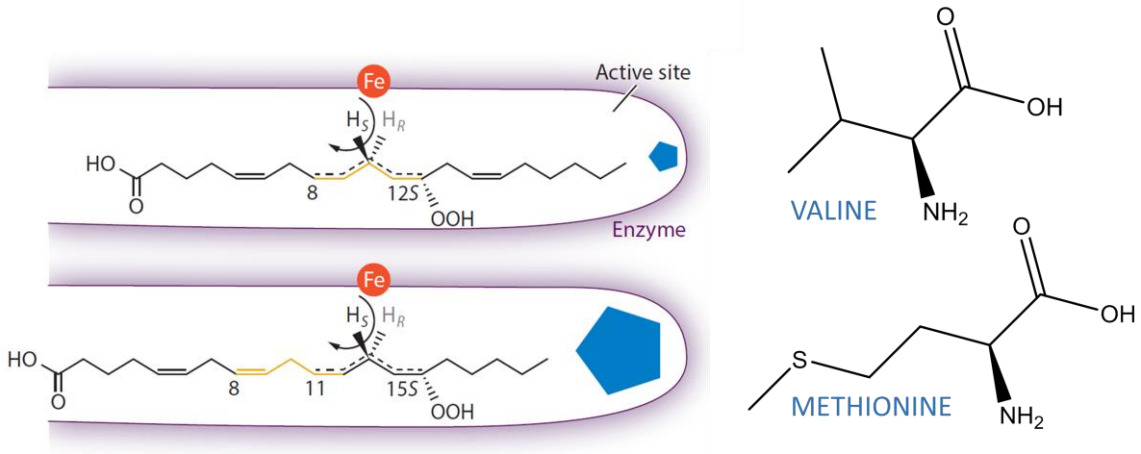


Figure C2-2: Sloane model explaining the regiospecificity of human 15-LOX (Adapted from Wasternack & Feussner, 2018. The pentadiene is highlighted in yellow and the blue pentagons symbolize the different volumes of different amino acid chains, such as valine and methionine. The arrow indicates hydrogen abstraction. In the case of amino acid with a smaller chain, arachidonic acid can go deeper in the active site, and is oxygenated on C-12.

In plant LOXs, a conserved arginine residue, which carries a positive charge, is found at the bottom of the active site. In 13-LOX from cucumber, an histidine was found at the position described by Sloane (Hornung, 1999). Replacement of this amino acid by a less-space-filling valine altered the positional specificity of this linoleate in favor of 9-lipoxygenation (Figure C2-3). It was proposed that this specific amino acid alters the orientation of the substrate, by masking or not the positive charge from the conserved arginine. In the case of cucumber 13-LOX, the entrance of the substrate with its methyl end first is favoured, whereas in 9-LOX, the arginine is unobstructed and the fatty acid can enter with its carboxyl end first. In accordance with these results, most plant 13-LOXs possess a cumbersome amino acid such as phenylalanine at the position described by Hornung, whereas 9-LOX usually harbor a small valine (Feussner & Wasternack, 2002). However, fatty acids were shown to bind with their carboxyl end first in a 13-LOX from soybean, indicating that this model is not universal (Skrzypczak-Jankun *et al.,* 2001).

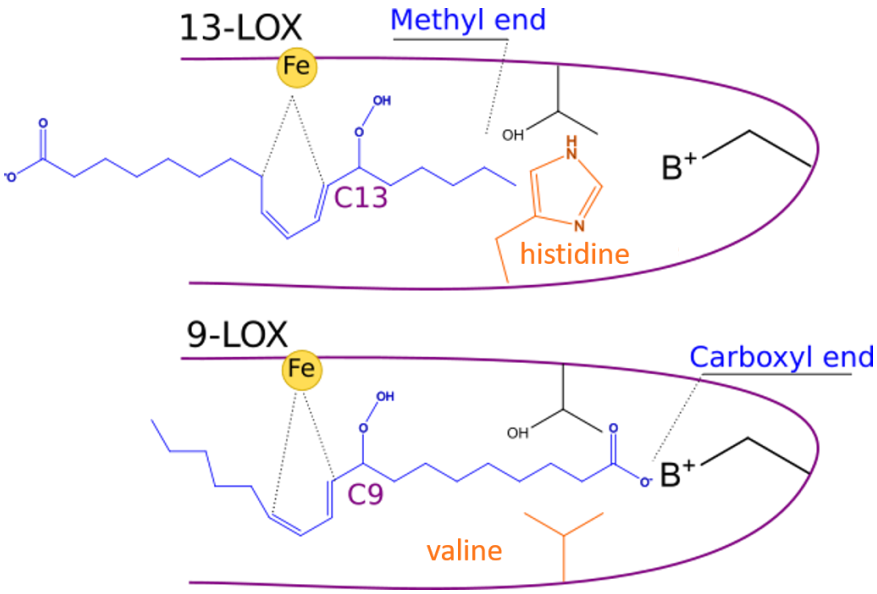


Figure C2-3: Model of "reversed orientation" of the substrate (Adapted from Hornung *et al.,* 1999). In the case of 13-LOX, a cumbersome phenylalanine blocks the interaction of the carboxyl end of linoleic acid with the positively charged residu at the bottom of the active site. Oxygenation thus occurs on the C-13. In 9-LOX, a smaller amino acid allows the fatty acid substrate to enter with its carboxyl end first, changing the site of oxygenation from C-13 to C-9.

Recently, the characterization of a cyanobacterial LOX suggested a new model in which substrate may enter with its methyl end first in both cases, but the positioning of molecular oxygen depends on the space available around the pentadiene system (Newie *et al.,* 2017). The presence of space filling amino acids would shift the dioxygen insertion from C-9 to C-13 whereas if more space is available, oxygen is inseted at the C-9 (Figure C2-4).

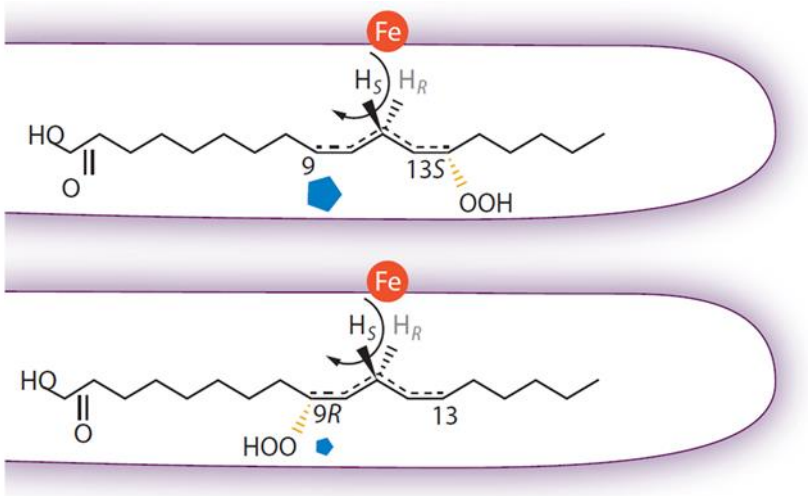


Figure C2-4: Model explaining the selectivity of O_2 insertion (From Wasternack & Feussner, 2018). This model was deduced from a cyanobacteria LOX in which amino acids occupying different volumes and close to the site of oxygen insertion might prevent oxygen insertion at C-9 and favour C-13.

Stereospecificity

The oxygen insertion by LOXs can yield *S* or *R*-hydroperoxides. Coffa & Brash (2004) highlighted a specific amino acid which was further called the "Coffa-Brash" determinant, explaining stereospecificty in mammalian LOXs. *S*-LOXs are characterized by the presence of a conserved alanine at this position, whereas *R*-LOX possess a glycine. After hydrogen abstraction, the cumbersome methyl group from alanine forces oxygen insertion on the opposite carbon, whereas the small glycin chain allows insertion at the carbon facing it. The Coffa-Brash residue is conserved in all described LOXs, even fungal LOXs. However, several enzymes do not follow this rule, indicating more complex mechanisms.

1.2 What do we know about fungal LOXs ?

Apart from the well-described 13*R*-MnLOX from *G. graminis*, no other fungal LOX have been studied in detail. Table C2-1 summarizes the state of our knowledge concerning all characterized fungal LOX forming 13-HPODE by 2018. 13-LOX activities were also reported for *Thermomyces lanuginosus* (Li *et al.,* 2001b), and the truffle *Terfezia claveryi* (Perez-Gilabert *et al.,* 2005) but the enzymes are not available in public databases. Seven fungal LOXs producing 13-hydroperoxides have been characterized so far and could be extracted from NCBI database. Among them, five manganese (Mn)LOX were identified in *Gaeummanomyces graminis*, *Magnaporthe salvinii*, *Magnaporthe oryzae*, *Aspergillus fumigatus* and *Fusarim oxysporum*, showing that MnLOX might be more prevalent than previously thought. In *Fusarium oxysporum* and *Pleurotus ostreatus*, two Fe-LOX similar to plant LOXs, have been reported. All those LOXs form both 13-HPODE and 9-HPODE, and sometimes 11-HPODE. They differ in their preference to oxygenate a certain carbon and are named accordingly: 13-LOX mainly forms 13-hydroperoxydes, 9-LOX has 9-hydroperoxides as major products. Moreover, it was observed that LOXs producing 13*R*-HPODE always form the 9S-HPODE and inversely.

Taxon	*Gaeummanomyces graminis*	*Magnaporthe salvinii*	*Magnaporthe oryzae*	*Aspergillus fumigatus*	*Fusarium oxysporum*
Enzyme	13R-MnLOX	9S-MnLOX	9S-MnLOX	13-MnLOX	Fo-MnLOX
ACCN	Q8X150.2	CAD1974.4	G4NAP4.2	Q4WA38.1	F9FRH4.1
Reference	Oliw *et al.*, 1998	Wennman *et al.*, 2013	Wennman *et al.*, 2015,2016	Heshof *et al.*, 2014	Wennman *et al.*, 2015
linoleic acid	13R-HPODE (71%), 11S-HPODE (29%)	9S-HPODE, 11S-HPODE, 13R-HPODE	9S-HPODE, 11S-HPODE (intermediate), 13R-HPODE	13-HPODE, 9-HPODE	11R-HPODE, 9(R/S)-HPODE, 13S-HPODE
α-linolenic acid (18:3n-3)	13-HPOTrE	9/11/13-HPOTrE	9-HPOTrE	nd	11S-HPOTrE
γ-lnolenic acid (18:3n-3)	13-HOTrE, 11-HOTrE	9-HOTrE	nd	nd	nd

Taxon	*Fusarium oxysporum*	*Pleurotus ostreatus*
Enzyme	FoxLOX	Po-13LOX
ACCN	KNB01601.1	BAI99788.1
Reference	Brodhun *et al.*, 2013	Kuribayashi *et al.*, 2002
linoleic acid	13S-HPODE, 9R-HPODE	13-HPODE, 9-HPODE (9:1)
α-linolenic acid (18:3n-3)	13-HPOTrE	nd
γ-lnolenic acid (18:3n-3)	13/9/6-HOTrE	nd

Table C2-1: Fungal LOXs producing 13-hydroperoxides purified and characterized by 2018. Information on MnLOX (Top) and FeLOX (bottom) are provided together with their NCBI accession number (ACCN) . Products forme from oxygenation of linoleic acid, α-linoleic acid, and γ-linoleic acid are indicated.

The comparison between the active site of the 13*R*-MnLOX from *G. graminis* and the iron sLOX-1 from *Glycine max* (soybean), highlighted the presence of a cumbersome phenylalanine in position 337 in the fungal LOX which could explain its stereospecificity (figure C2-5). This phenylalanine seems well conserved among fungal MnLOX (see figure C2-11).

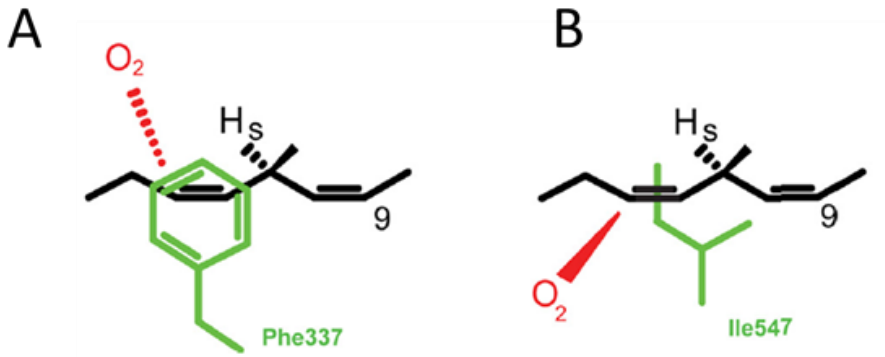


Figure C2-5: Comparison of the position of linoleic acid in the active site of fungal 13*R*-LOX and plant 13*S*-LOX (from Wennman *et al.,* 2012). In the fungal 13*R*-MnLOX from *G. graminis*, a cumbersome phenylalanine blocks the antarafacial oxygen insertion at C-13, yelding 13*R*-HPODE (A), whereas the soybean LOX-1 produces 13*S*-HPODE (B).

The Sloane position, first decribed in mammalian LOXs (Sloane *et al.,* 1991) seems also an important determinant for the regiospecificity of LOXs. Wenman *et al.,* (2015) later confirmed the importance of this single amino acid in a fungal LOX. Indeed, at this position, a serine (348) is found in the *Fusarium* MnLOX, producing mainly 11R-HPODE, 9S-HPODE and 13S-HPODE. The single replacement of this serine by a phenylananine in Fo-MnLOX resulted in the production of 13R-HPODE and 9S-HPODE alike the other MnLOXs.

Although these models have helped considerably the deciphering of protein structure – protein function relationships, at the moment, the exact activity of a LOX can not be easely deduced from its amino acid sequence. Further biochemical characterisations of LOXs with known amino acid sequences will, however, shed new knowledge on this interesting subject. Moreover, oxylipins, through their immense variety in terms of location and stereo specificity of the oxidation as well as the length and saturation of the PUFA substrate, are often referred to as "words", used in intra- and interspecies cross-talk. Elucidating their exact biosynthesis seems, therefore, an exciting research topic to me. For these reasons and in order to understand the provenance of the 13-HPODE oxylipin found in the interaction zone between *P. variabile* and *F. oxysyporum,* we proceeded to purification and characterisation of the two LOXs in *P. variabile*.

1.3 *E. coli* expression system for heterologous expression of eukaryotic genes

Protein expression in E. coli

In order to characterize the putative LOX from *P. variabile*, we decided to express them in a bacterial system, to finally study their catalytical activity. Although *E. coli*, as a bacteria, does not possess the entire cellular machinery needed for maturation of eukaryotic proteins (no glycosylation, no phosphorylation), it is the favoured heterologous expression system even for eukaryotic genes, because bacteria are easy to transform, they are growing fast, and they can yield a high amount of recombinant protein. Moreover, the *F. oxysporum* 13*S*-LOX was readily expressed and purified in *E. coli* by Brodhun *et al.,* (2013). However, since bacteria do not posses intronic regions in genes, that is regions that are removed from RNA during RNA maturation, we had to clone the cDNA of *pvlox1* and *pvlox2* genes, corresponding to the already mature RNA (see figure C2-7 p. 86).

Recombinant proteins can be expressed either in the cytosol, the periplasm (the inner space between the two membranes of *E. coli*) or the extracellular medium. In the absence of specific signal peptides, the recombinant proteins are mainly expressed and refolded in the cytosol, with many other proteins from the host strain. In order to purify the candidate protein, it is possible to attach "tags" to it and use methods such as affinity chromatography to isolate and purify it. We selected the histidine tag, containing 6 histidines showing high affinity to nickel matrix. When His-tagged proteins are applied to the matrix, they specifically bind to the resin, while most untagged proteins do not. Finally, the choice of bacterial expression strain and plasmid is crucial.

The pET system

In our case, we used the widely known system called "pET expression system", illustrated on figure C2-6. In this system, the target gene is cloned in a pET plasmid, under control of a strong bacteriophage T7 promoter. This promoter is only recognised by the T7 RNA polymerase, thus the plasmid must be cloned in an *E. coli* strain genetically modified to express the T7 RNA polymerase, such as the BL21 strains. T7 polymerase is very selective, and when induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) or lactose, almost all the cell resources are directed towards the target gene expression. The recombinant protein can represent up to 50% of the total cell protein content (Rosano *et al.,* 2014). To avoid "protein leakage", that is, expression of recombinant protein in the absence of induction, we chose *E. coli* strains that possess a T7 lysozyme, which removes basal level of T7 RNA polymerase.

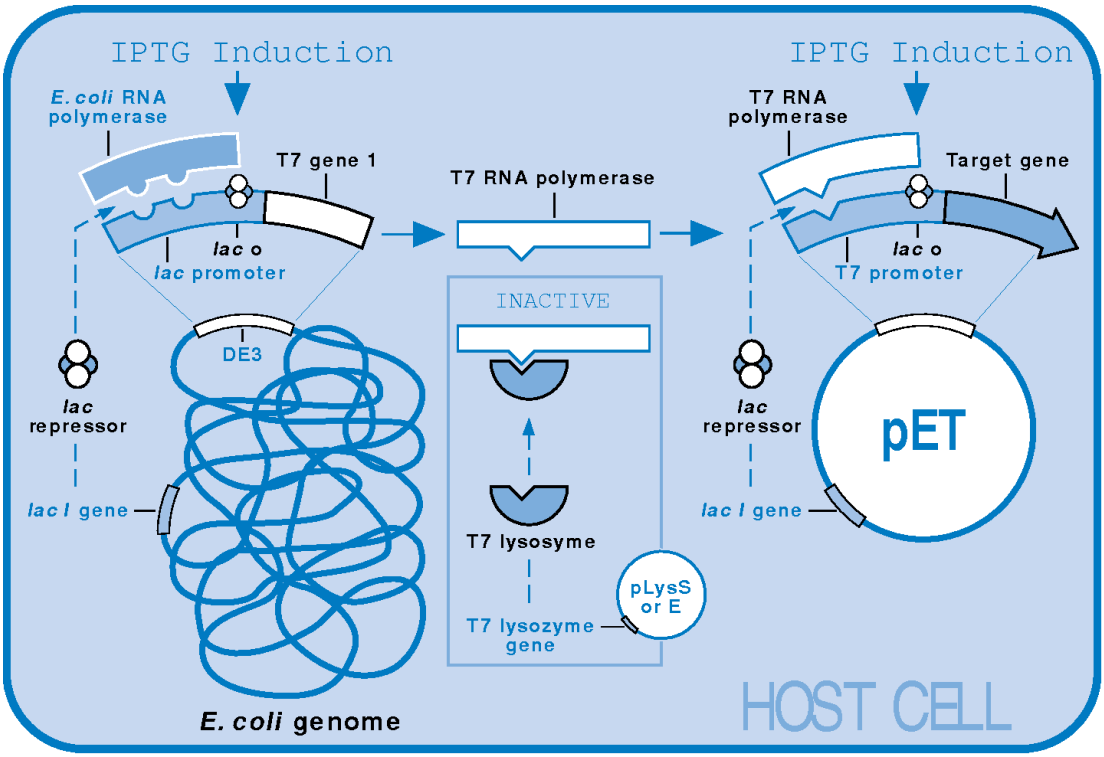


Figure C2-6 : The pET expression system (from Novagen catalog, Madison, 1995). Under IPTG induction, IPTG binds the lac repressor and allows the expression of the T7 RNA polymerase. In turn, the polymerase specifically induces the expression of the target gene, upon recognition of the T7 promoter.

1.4 Objectives of the study

In order to study the role of lipoxygenases (LOX) in the chemical communication between the endophyte *P. variabile* and *F. oxysporum*, we wanted to characterize the two putative LOX enzymes that had been identified previously in the endophyte. It is a necessary step to confirm that *P. variabile* could produce the 13-hydroperoxides found in the interaction.

The objectives of this work were the followings:

- construct expression vectors for genes *pvlox1* and *pvlox2*
- express and purify recombinant proteins PVLOX1 and PVLOX2 in *Escherichia coli*
- develop a rapid method to confirm LOX activity in recombinant proteins
- characterize recombinant PVLOX1 and PVLOX2 catalytic activity in presence of diverse fatty acid substrates to confirm/infirm the synthesis of 13-hydroperoxides

2. Results

2.1 Construction of vectors for the heterologous expression of PVLOX1 and PVLOX2

cDNA isolation and cloning strategy

At the time we decided to study the heterologous expression of *lox* genes from *P. variabile*, nothing was known about the regulation of *lox* genes in this fungus. The only hint we had, came from the study of Combès *et al.*, (2012) who showed that 13-HPODE was found in higher amounts in co-culture with *F. oxysporum*, indicating a possible upregulation of *lox* genes in the interaction. For this reason, we chose to extract RNA from *P. variabile* grown in co-culture.

In the same way, we ignored the presence of intronic regions or their localization. To get a clearer idea, we used FEGENESH software to predict possible intronic regions. This software uses different algorithms to identify intronic regions, based on a model organism. To predict introns in *pvlox1* and *pvlox2,* we chose 8 available fungi belonging to Dothideomycetes like *P. variabile* (*Baudoinia compniacensis, Pyrenophora, Oidendron maius, Leptosheria maculans, Macrophamina phaseolina, Neofusicoccum parvum and Stagonospora nodorum).* For each *lox* gene, the software predicted the presence of two short introns, more or less similar depending on the model organism (<80 pb).

Concerning the cloning strategy, we decided to clone *pvlox1* and *pvlox2* cDNA into the pET28a vector by homologous recombination. The overall process is shown on figure C2-7. Briefly, we extracted the mRNA from *P. variabile*, already spliced (that is without intronic regions), and we retro-transcripted them into a mono-strand cDNA. Then, the cDNAs were amplified with primers specific to *pvlox1* and *pvlox2*. The PCR products were cloned in *E. coli* TOP10, into the pET28a vector by homologous recombination, giving the final expression vector. This vector was then cloned into strains designed for heterologous expressions such as BL21 derivatives. The recombinant protein should finally be expressed and purified thanks to its His-tag motif. The cloning step was done in *E. coli* TOP10 because we do not know if homologous recombination can be done directly into BL21 strains.

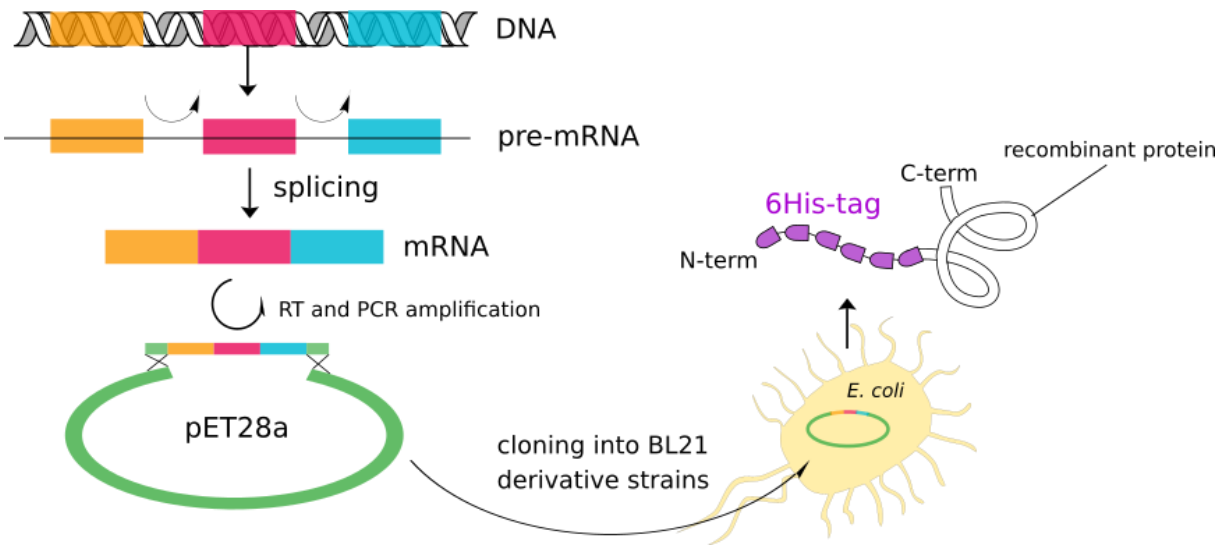


Figure C2 -7: Overview of heterologous expression of genes *pvlox1* and *pvlox2*. The diagram illustrates the strategy from RNA extraction to final recombinant protein production

The cloning site of pET28a is detailed in figure C2-8. This vector is designed especially for heterologous expression of proteins in *E. coli*. It contains His-tag motifs with six histidines, that can be added either at the N-terminal position or at the C-terminal position. In our case, the last amino acid at the C-terminal position in lipoxygenases is involved in the catalytic center. Therefore, we found it more appropriated to add the His-tag at the N-terminal position, to avoid misfolding of recombinant proteins at the active site. The plasmid provides a stop codon but we decided to remove it to keep the stop codon already present in *pvlox1* and *pvlox2*. Figure C2-7 shows in blue the region deleted by PCR amplification of pET28a. This region will be replaced by the cDNA of *pvlox1* or *pvlox2*. We also kept the thrombin site in case we needed to remove the His-tag motif.

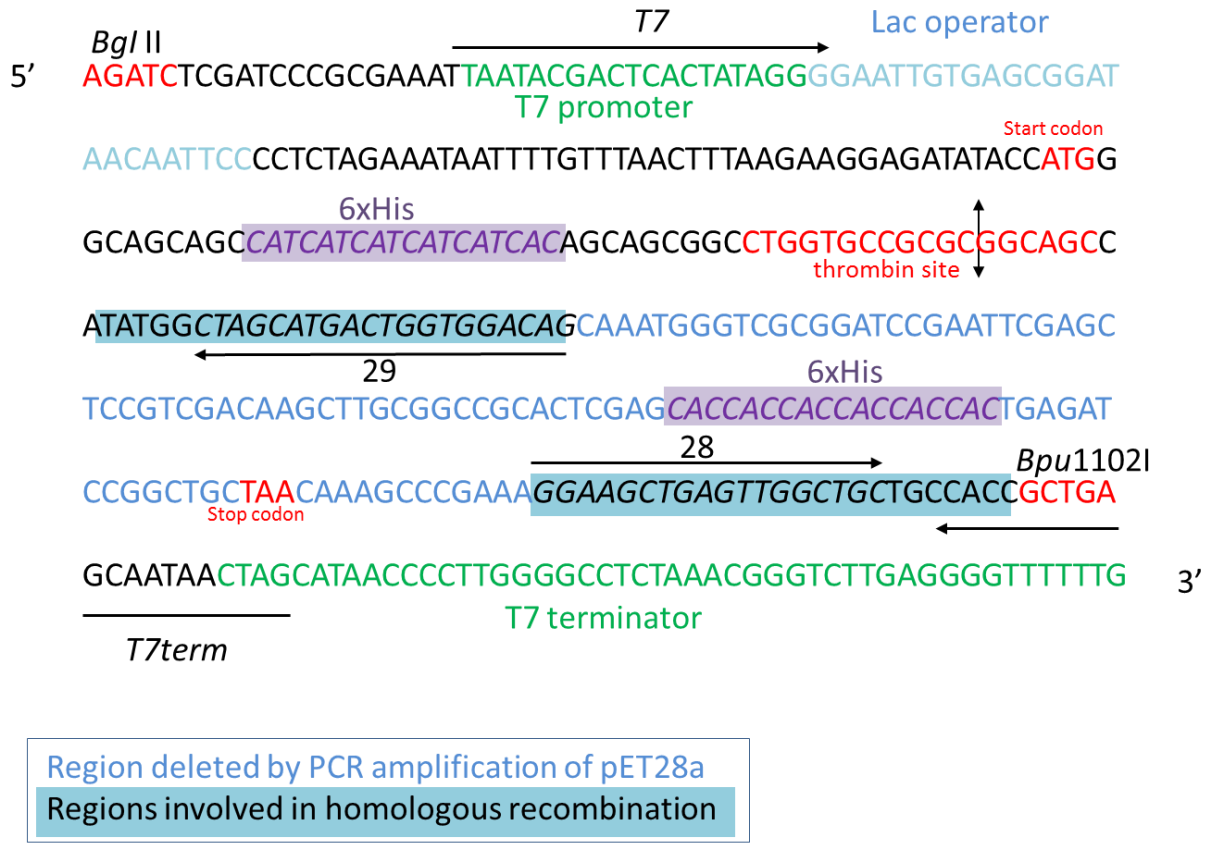


Figure C2-8: Details of the cloning site of the pET28a+ plasmid. Plasmid pET28a is linearized with primers 29 and 28. The cDNA of *pvlox1* and *pvlox2* is then integrated in between.

Finally, we adopted two different cloning strategies illustrated on figure C2-9, to construct the expression vectors, based on homologous recombination. This technic implies that the DNA fragment to be cloned into a plasmid needs to share homologous regions with the plasmid. Those homologous regions can be added either on the plasmid or the DNA fragment to be cloned.

First, we decided to add homologous regions to the cDNA sequence. For this purpose, we designed primers 26 and 27, that amplify *pvlox1* cDNA and we added tails at the 5' and 3' ends homologous to pET28a. With this strategy, though we obtained the desired plasmid (pMB4), the amplification of cDNA was difficult. We thought it might be due to the presence of 20 bp tails at the 3' end of the primers and we moved on to a second strategy. Thus, for *pvlox2* cloning, we added the homologous tails on the pET28a vector and finally obtained the pMB3 plasmid more easily.

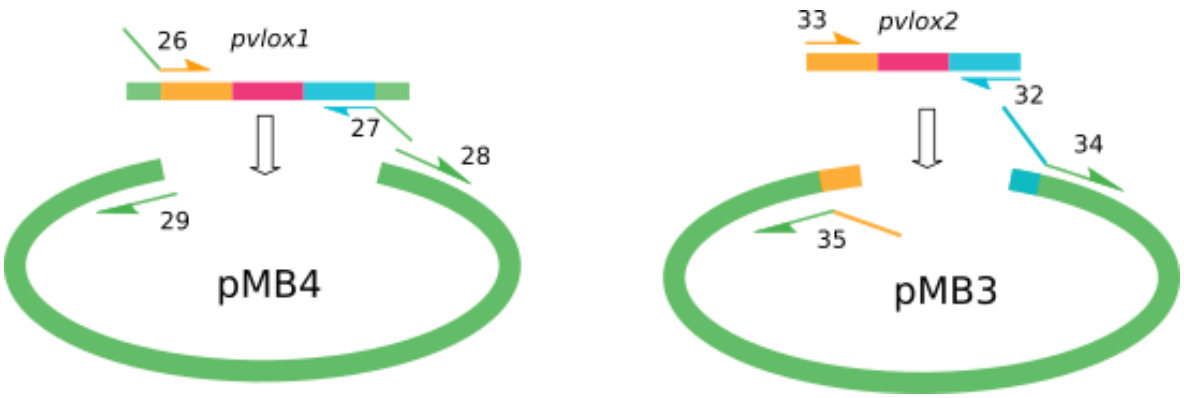


Figure C2-9 : Illustration of two different strategies used to obtain plasmids pMB3 and pMB4. The diagrams show the primers used for the plasmid linearization and the amplification of cDNAs from *pvlox1* and *pvlox2*. For pMB4, tails are added to the pvlox1 PCR product, whereas they are added to the pET28a plasmid for the construction of pMB3.

cDNA analysis and protein predictions

The plasmids obtained previously were sequenced with primers *T7* and *T7 term* (see figure C2-7), flanking the cloning site. We confirmed that the two plasmids were harboring the cDNA sequences from *pvlox1* and *pvlox2*, in frame with the N-terminal His-tag motif. We identified two intronic regions in both sequences (see figure C2-10) that matched exactly with the prediction using *Leptospheria maculans* as a model organism. The two identified intronic regions were short and flanked by 5'GU and AG 3' sequences, as reported for filamentous fungi (Kupfer *et al.,* 2004).

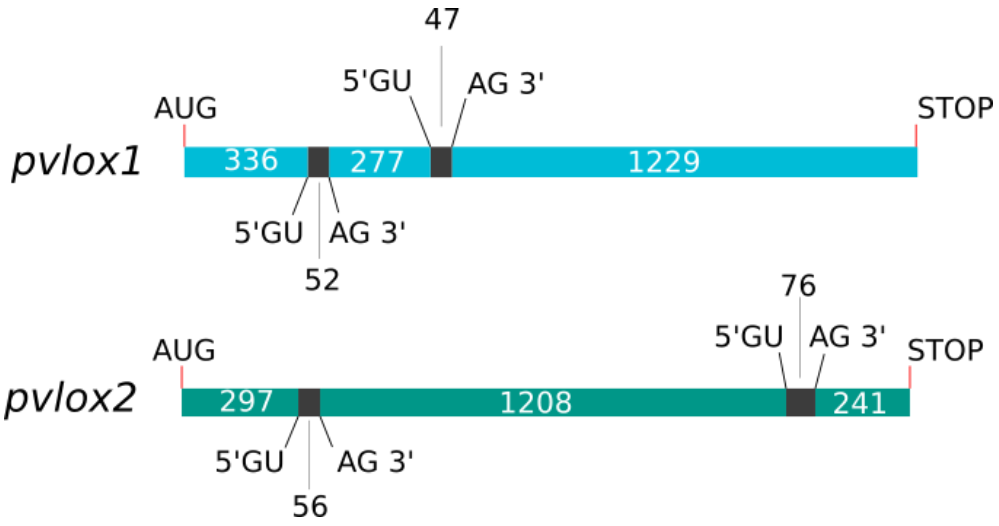
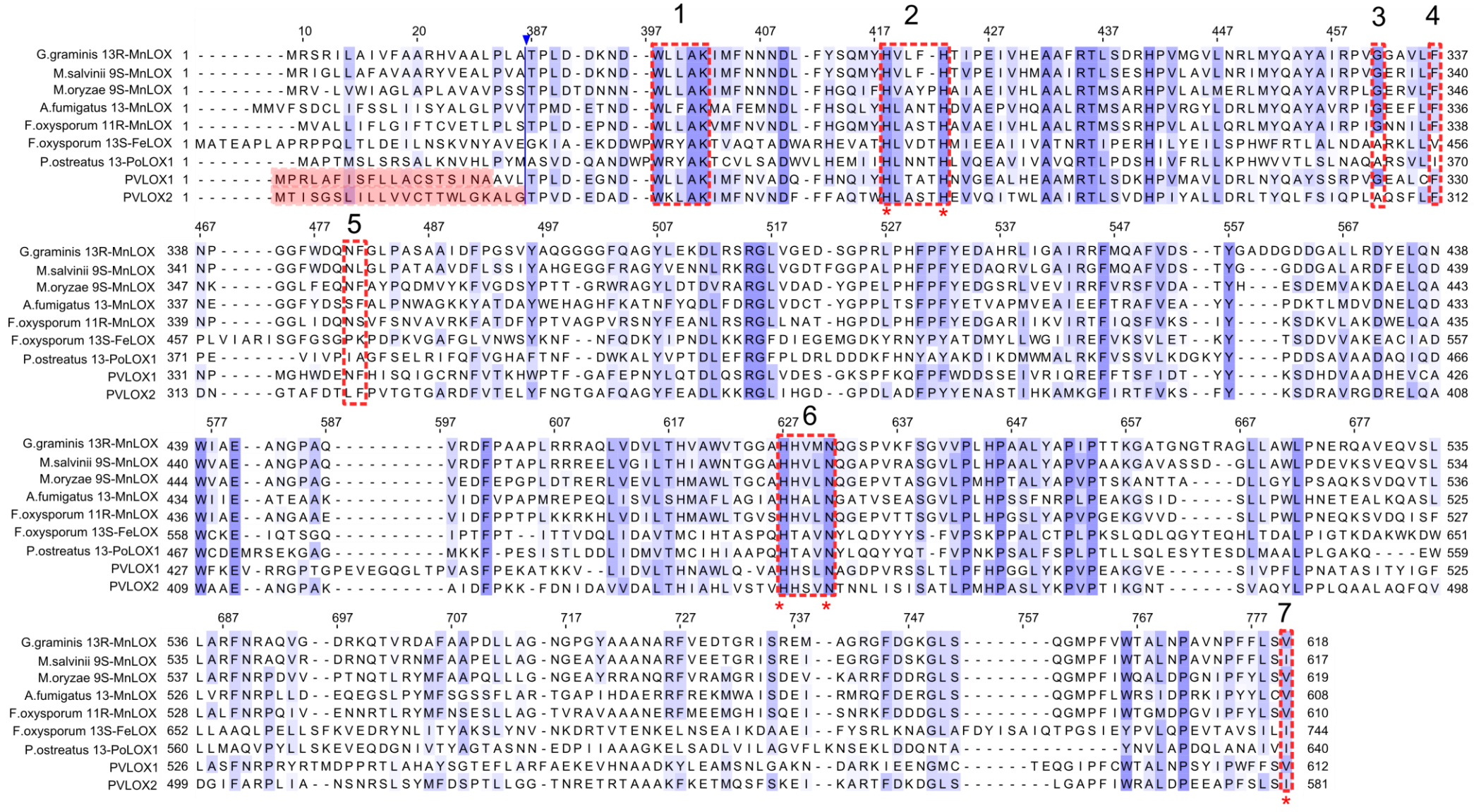


Figure C2-10: Schematic representation of genes *pvlox1* and *pvlox2* indicating the localization of intronic regions (in black). Length of DNA sequences are given in bp.

We then deduced the exact amino acid sequences of proteins PVLOX1 and PVLOX2 using FEGENESH software and were able to identify all the typical motifs described for LOXs (The complete sequences of PVLOX1 and PVLOX2 can be found in Appendix 3) . The C-terminal part of the two sequences were aligned with the 7 fungal LOXs presented in the introduction, and known to produce 13-HPODE. The alignment is shown on figure C2-11. Comparison with the already described 13R-MnLOX from *G. graminis* (Q8X150.2) suggested the presence of the typical W-X_2-AK motif as well as the two catalytic sites H-X_4-H and H-X_3-N and the five amino acids binding the metal cofactor (H, H, H, N, I/V). At the Coffa-Brash position we identified an alanine for PVLOX2 and a glycine for PVLOX1, indicating that their main products could be *S* and *R*-hydroperoxides, respectively. At the Sloane position, we identified a conserved Phenylalanine in both proteins, which make them close to the *G. graminis* 13*R*-MnLOX, the *A. fumigatus* 13-MnLOX and the *M. oryzae* 9-MnLOX. Interestingly, at the position of phenylalanine 337 described to be responsible for the formation of 13*R*-HPODE instead of 13*S*-HPODE in *G. graminis* (Wennman *et al.,* 2012) we also found a phenylalanine in both endophytic lipoxygenases and in every MnLOX.

A more detailed comparative analysis can be found in Chapter III, where we aligned PVLOX1 and PVLOX2 with 99 lipoxygenases from fungal origin. We demonstrated that they clustered with the other described fungal MnLOX and therefore it is very likely that they can form 13-hydroperoxides.

Legend of figure C2-11 (next page): Alignment of the C-terminal catalytic domain of PVLOX1 and PVLOX2 with 7 characterized fungal LOXs. The signal peptide of PVLOX1 and PVLOX2 is highlighted in red. **1**: W-X-AK motif, **2**: H-$X_{3/4}$-H, **3**: Coffa-Brash site residue, **4**: amino acids corresponding to Phe337 in *G. graminis*, **5**: Sloane position, **6**: H-X_3-N, **7**: C-terminal position. Asterisks indicate the position of the five presumed ligands binding the metal cofactor. The blue arrow indicates the position at which the alignment is shortened. Sequences were aligned with MUSCLE, and colored with a BLOSUM matrix, the darker amino acids being the most conserved. GenBank accession numbers from top to bottom: *Gaeumannomyces graminis* [Q8X150.2], *Magnaporthe salvinii* [CAD61974.4], *Magnaporthe oryzae* [G4NAP4.2], *Aspergillus fumigatus* [Q4WA38.1], *Fusarium oxysporum* Fo-MnLOX [F9FRH4.1], *Fusarium oxysporum* FoxLOX [KNB0160.1], *Pleurotus ostreatus* [BAI99788.1]


1
2
3
4
5
6
7
G.graminis 13R-MnLOX
M.salvinii 9S-MnLOX
M.oryzae 9S-MnLOX
A.fumigatus 13-MnLOX
F.oxysporum 11R-MnLOX
F.oxysporum 13S-FeLOX
P.ostreatus 13-PoLOX1
PVLOX1
PVLOX2

Finally, we identified a secretion peptide of 19 and 22 amino acids in PVLOX1 and PVLOX2 similar to the one found in *G. graminis*, suggesting that these proteins might be secreted outside the cell. We know little about where fungal LOXs are usually addressed to, in which organs and tissues they are expressed. Ppo enzymes in *A. nidulans* fused to a GFP reporter were found to be expressed in reproductive tissues and were targeted towards the lipid bodies inside the cytoplasm (Fischer & Keller, 2016). Hesof *et al.,* (2014) identified signal peptides in most fungal LOXs suggesting that they could be secreted out of the cell. The software prediction used for PVLOX1 and PVLOX2 sequences predicted a highly probable secretion scenario, but also indicated that these proteins could be directed to the endoplasmic reticulum, were lipid metabolism takes place.
The signal peptide of an eukaryotic protein will not be processed by the cellular machinery of the bacteria *E. coli*. Therefore, it can be left or removed in the recombinant protein. The recombinant FoxLox protein from *F. oxysporum* was cloned in pET28a and expressed with success in *E. coli* (Brodhun *et al.,* 2013) without signal peptide removal. In the same way, we decided to keep the signal peptides in both recombinant proteins.

Knowing the sequences of PVLOX1 and PVLOX2, we were able to make a few predictions on the proteins, summarized in table C2-2. We predicted that they would have a size of 68 and 64 kDa, that they would be probably extracellular and will not contain di-sulfure bridges, which makes their expression easier. Because the reducing environment of bacterial cytosol inhibits the formation of disulfide bonds, proteins with such structures might be more difficult to properly refold. The predicted size for recombinant proteins was a bit higher due to the presence of the his-tag motif and the thrombin site. An important consideration for heterologous expression is the presence of rare codons. Indeed, the frequency of codons varies between eukaryotes and bacteria. "Rare codons" refer to codons appearing with low frequencies in *E. coli*. Therefore, the bacteria synthetizes less corresponding tRNAs (Transfer RNAs carrying the amino acids during protein synthesis) for those codons. Consequently, *E. coli* may inefficiently express various genes. Moreover, rare codons can cause misincorporation of amino acids. We found 26 and 24 rare codons in *pvlox1* and *pvlox2* sequences. *E. coli* expression strains have been specifically engineered to circumvent this problem. Those strains harbor plasmids encoding additional tRNAs. For this reason, we decided to express PVLOX1 and PVLOX2 in the strain BL21 (DE3) CodonPLus, which encodes the rare codons AGA, AGG, AUA, CCC and CUA. The two recombinant proteins were expected to be of 68.6 kDa and 68 kDa with an isoelectric point of 7.21 and 8.46, and were predicted to be secreted in the periplasmic space of *E. coli*.

	PVLOX1	PVLOX2	6HIS-PVLOX1	6HIS-PVLOX2	software
Size (Da)	68 kD	64 kD	68.6 kD	68 kD	Expasy
pI	5.78	5.78	7.21	8.46	Expasy
Subcellular localization	Extracellular, ER	Extracellular, ER	-	-	PSORT (fungi)
	-	-	periplasmic	periplasmic	PSORT (*E. coli*)
Disulfure bridges	No	No	-	-	Disulfind
Signal peptide	Yes	Yes	-	-	Signal IP4.1
Rare codon	18 (CCC, CGA, CUA, AUA, GGA)	29 (CUA, CGA, GGA, CCC, AGG, AGA)	-	-	RACC (http://nihserver.mbi.ucla.edu/RACC)

Table C2-2: Some predicted features of recombinant and native proteins PVLOX1 and PVLOX2. ER: endoplasmic reticulum

2.2 Expression of recombinant lipoxygenases 6His-PVLOX1 and 6His-PVLOX2

Overview of experimental procedure

Our goal was to express recombinant proteins in order to purify them and to characterize their catalytic activities. For this purpose, recombinant proteins have to be soluble and properly refolded. They are usually produced in the cytoplasm of *E. coli* and can be easily recovered by cell lysis. Figure C2-12 describes the main step of protein recovery from induction to isolation of the soluble fraction. It also provides the terminology that will be used in this section to identify the different protein fractions.

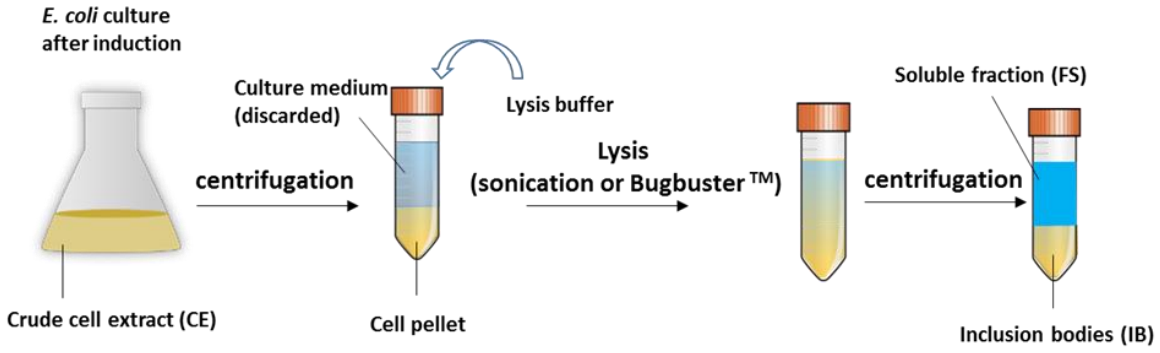


Figure C2-12. Overview of cell lysis after heterologous protein induction to collect recombinant proteins.

After induction, the cells are pelleted and a fraction called "crude extract" is put aside. The pellet is lysed mechanically (sonication) or chemically (BugBuster™) in a buffer containing detergents and the supernatant called "soluble fraction (FS)" is separated by centrifugation from the pellet containing cellular debris and misfolded proteins aggregated in "inclusion bodies (IB)".

LOX Expression with IPTG induction in BL21 (DE3) CodonPLus

Expression at 37°C

It is not possible to predict the optimal conditions for protein expression; therefore it is usual to test a range of temperatures and incubation times, starting at 37°C, which is the optimal temperature for the growth of *E. coli*. We induced expression of 6His-PVLOX proteins at 37°C with 0.1 mM of IPTG for two hours. A control flask was treated the same way but without IPTG induction. The resulting SDS-PAGE gel is visible on figure C2-13. We observed in the crude extracts and inclusion bodies of PVLOX1 and PVLOX2 expressing *E. coli* the apparition of a new band in the presence of IPTG around 70 kDa, suggesting they were PVLOX1 and PVLOX2. To confirm it, we performed a Western Blot with an antibody targeting the His-tag. Indeed, the two overproduced proteins around 70 kDa reacted with the antibody confirming that recombinant 6His-PVLOX were efficiently expressed in our system, and fused to the His-tag as expected. Nevertheless, no his-tagged protein was detected in the soluble fraction. We could also observe that a certain amount of recombinant protein was degraded (lower band at 20 kDa on the blot), probably during lysis.

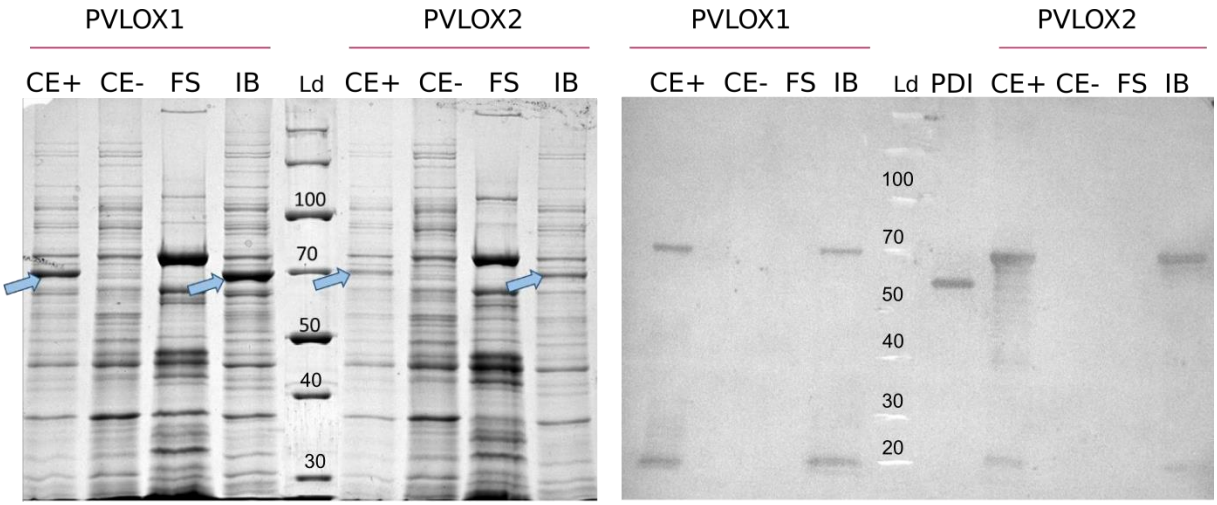


Figure C2-13: Expression of recombinant PVLOX1 and PVLOX2 at 37°C in BL21 (DE3) CodonPlus. SDS-PAGE gel (left) and Western Blot immunoassay (right) on samples collected after induction at 37°C for two hours. **CE+**: cell crude extract after IPTG induction, **CE**: cell crude extract without induction, **FS:** soluble fraction, **IB**: inclusion bodies, **Ld:** Protein Ladder, **PDI**: protein containing a his-tag, used as a positive control. The blue arrows indicate the position of expected recombinant proteins.

Expression at different temperatures

Inclusion bodies are supposed to be formed when a protein is overexpressed and the cell machinery cannot achieve its proper folding (Sorensen & Mortensen, 2005). Thus, it is suggested to decrease protein expression by lowering the temperature. We tried expression at different incuabation times and at different temperatures such as 30°C, 25°C and 16°C. These temperatures were succesfully used by Brodhun *et al.,* (2013) for *F. oxysporum* FoxLox expression in *E. coli* and are shown in Table C2-3. However, recombinant proteins were always found in inclusion bodies and at 16°C, they were not expressed at all. In some cases, adding a co-factor can help proteins to refold properly. Thus, based on sequence prediction, PVLOX1 and PVLOX2 should contain a Manganese co-factor at the active site but they might also contain iron. We then performed induction assays at 37°C, 30°C and 25°C, adding MnCl2 and FeCl3 at 2 µM in the culture medium. However, the recombinant proteins were found misfolded in inclusion bodies (table C2-3). We concluded that the addition of metal co-factors was not improving the solubilization of recombinant proteins.

Temperature	Incubation time	Presence of recombinant protein in crude extract	Presence in soluble fraction or inclusion bodies
6His-PVLOX1			
37°C	2 h	Yes	Inclusion bodies
30°C	4 h	Yes	Inclusion bodies
25°C	2 h	Yes	Inclusion bodies
16°C	21 h	No	-
6His-PVLOX2			
37°C	2 h	Yes	Inclusion bodies
30°C	4 h	Yes	Inclusion bodies
16°C	21 h	No	-
6His-PVLOX1 + $MnCl_2$/$FeCl_3$			
37°C	2 h	Yes	Inclusion bodies
30°C	2 h	Yes	Inclusion bodies
25°C	2 h	Yes	Inclusion bodies
6His-PVLOX2 + $MnCl_2$/$FeCl_3$			
37°C	2 h	Yes	Inclusion bodies
30°C	2 h	Yes	Inclusion bodies
25°C	2 h	Yes	Inclusion bodies

Table C2-3: Different combinations of temperature and incubation time tested for the expression of recombinant PVLOX1 and PVLOX2 in BL21(DE3) CodonPLus strain with or without metal cofactors.

Protein production by auto-induction in BL21 (DE3) CodonPlus

We then decided to try an alternative induction protocol proposed by Studier (2005). In the following experiments, we focused on recombinant 6His-PVLOX1. The protocol from Studier relies on the progressive depletion of glucose in culture medium, which forces cells to use lactose instead, triggering gene expression under control of the *lac* promoter. Auto-induction by lactose spreads protein expression over a longer period and favors the proper refolding of proteins. We tested this protocol at 37°C and 25°C for 16, 24 or 42 hours. We observed the production of PVLOX1 at 37°C and at 25°C, after 16 hours and 24 hours (Figure C2-14). At 42 hours, the recombinant protein was no longer detected, suggesting it was degraded. Each time the protein was found in inclusion bodies but after induction at 37°C for 16h, it seemed that a small band that could correspond to PVLOX1 was present in the soluble fraction (indicated by a red arrow).

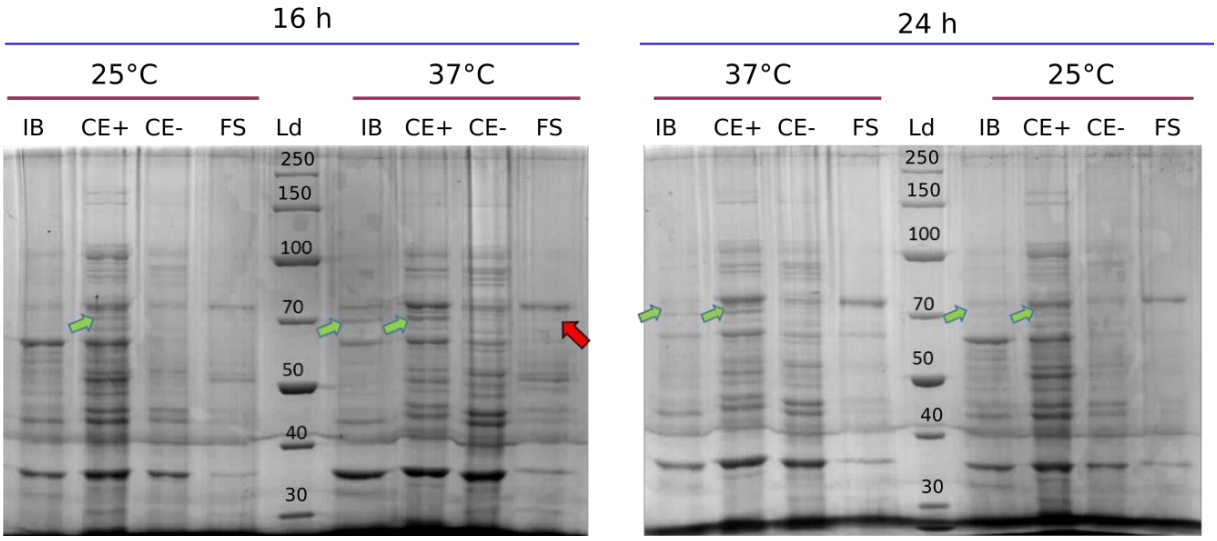


Figure C2-14: SDS-PAGE after Coomassie blue staining showing the expression of recombinant PVLOX1 after auto-induction at 25°C or 37°C, for 16h or 24h. IB: inclusion bodies, **CE+:** crude extract after auto-induction, **CE-:** negative control without protein induction, **FS:** soluble fraction, **Ld:** protein ladder. The green arrows indicate the protein band corresponding to 6His-PVLOX1. A small band seems to be present at 37°C indicated by a red arrow.

To check the presence of a small amount of recombinant protein in the soluble fraction, we decided to concentrate it five times, to purify it by affinity chromatography using nickel-grafted beads binding His-tagged proteins (figure C2-15), and detect the presence of the recombinant protein by immunoassay.

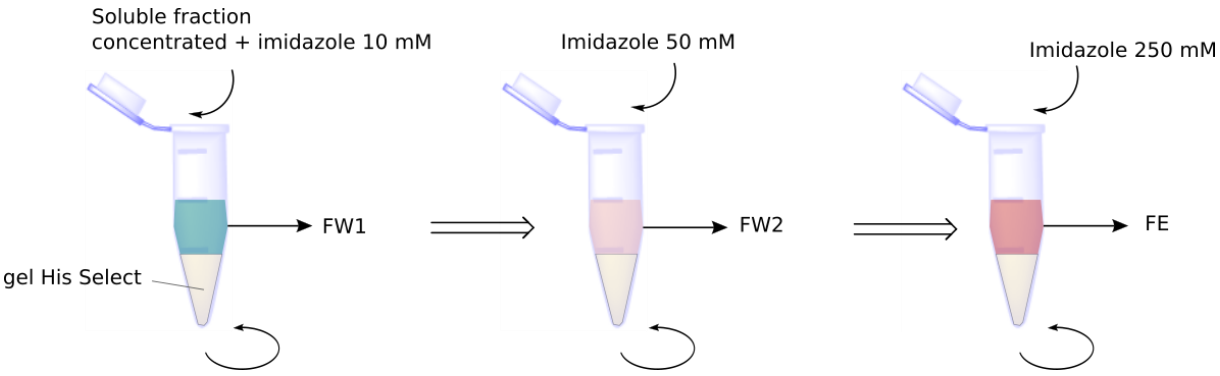


Figure C2-15 : Overview of His-tagged protein purification using affinity chromatography. Proteins containing a histidine motif are retained on a gel containing nickel-grafted beads. Imidazole competes with histidines for binding the beads and proteins with low affinity are eluted at 10 mM and 50 mM. His-tagged proteins are usually eluted with 100-250 mM of imidazole. The collected fractions are called FW1, FW2 for wash flow-througs and finally the fraction of elution is called FE.

The first wash fractions (FW1 and FW2), and the fraction of elution (FE) were run on SDS PAGE gel and revealed on a Western-Blot.The resulting immunoassay is illustrated on figure C2-16.

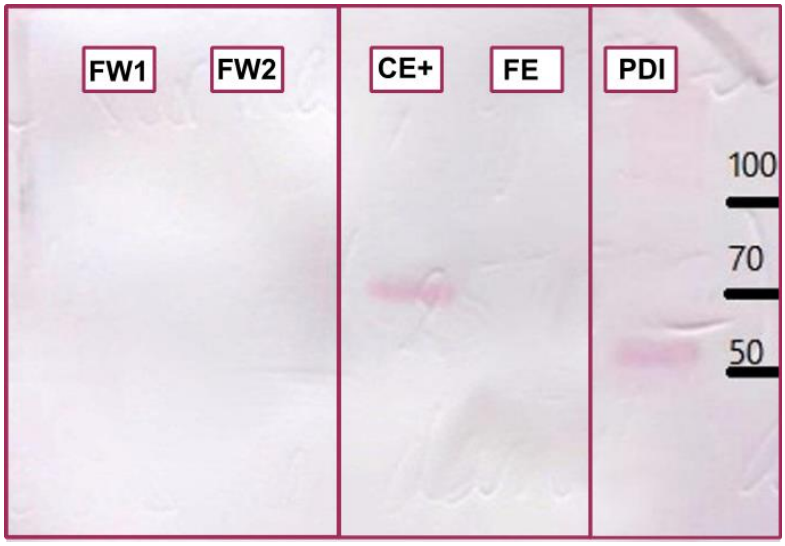


Figure C2-16: Immunoassay on the different fractions collected after purification of the soluble fraction by affinity chromatography. Fraction FW1 and FW2 were eluted with 10 and 50 mM imidazole respectively. **CE+:** crude extract containing the recombinant PVLOX1**, FE**: fraction eluted with 250 mM imidazole, PDI: protein containing a his-tag, used here as a positive control. Numbers on the right indicate the protein size in kDa.

We detected the His-tag protein in the crude extract as expected, but found no recombinant protein in any of the purified fractions, not even when eluated with 250 mM imidazole. This meant that the slightly visible band around 70 kDa seen in the soluble fraction FS (figure C2-17) was not the recombinant PVLOX1 protein.

Expression with IPTG induction in Rosetta™ (DE3) pLysS

All expression trials in BL21 (DE3) CodonPlus led to insoluble proteins in inclusion bodies, even when trying a soft lactose induction. Thus, we considered moving on to another strain and chose Rosetta™ (DE3) pLysS. This strain is very similar to CodonPlus but it encodes an additional rare codon (GGA). The plasmid pMB4 containing the cDNA of *pvlox1* was cloned into Rosetta. We tested different conditions of induction with IPTG. The recombinant 6His-PVLOX1 was detected at 37°C and to a lower extent at 25°C. The soluble fractions were concentrated 5 times and analyzed through immunoassay. This time, we also analyzed extracellular proteins in the culture medium, as PVLOX1 might have been secreted. Culture medium was concentrated 10 times and assayed in the Western Blot. The results at 37°C are visible on figure C2-17. The histidin-tagged protein was detected from the crude extract but neither in the soluble fraction nor the culture medium. The results were similar at 25°C (not shown).

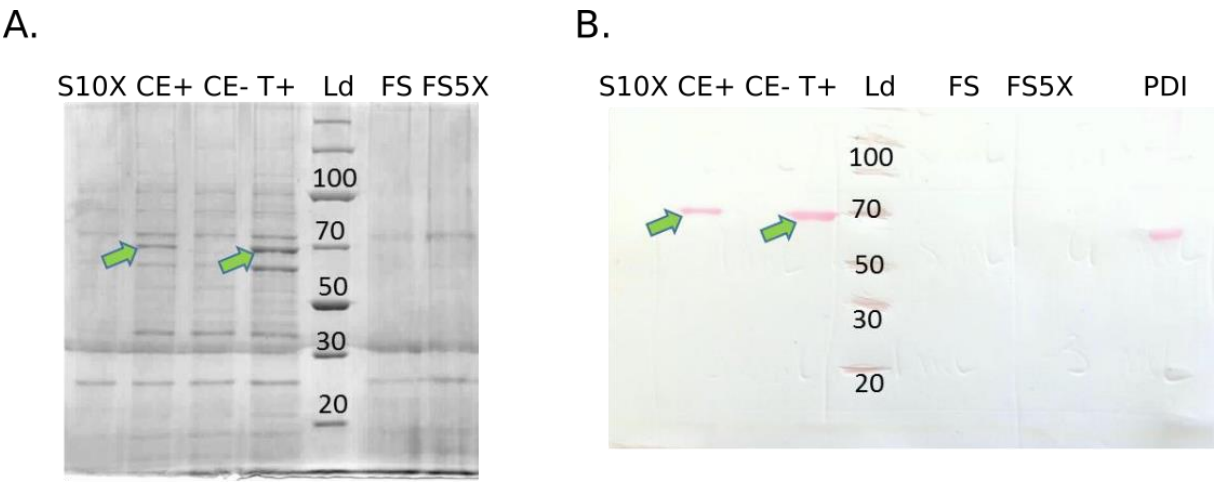


Figure C2-17: Expression of recombinant PVLOX1 and PVLOX2 in Rosetta (De3) pLysS. A. SDS-PAGE gel showing the expression of recombinant PVLOX1 after induction with IPTG at 37°C for two hours in Rosetta strain**. B.** Imunoassay performed on the same samples to detect the His-tagged recombinant protein. **S10X**: culture medium concentrated 10 times**. CE+:** crude extract after induction, **CE-:** crude extract without induction, **T+:** recombinant PVLOX1 from inclusion bodies, **FS**: soluble fraction, **FS5x:** soluble fraction concentrated 5 times, **Ld:** protein ladder (kDa).

2.3 Development of a rapid test to detect lipoxygenase activity "in gel"

Inclusion bodies result from an unbalanced equilibrium between protein precipitation and refolding (Carrio *et a*l., 1998). It led us think that a fraction of the protein in the inclusion bodies might be properly refolded and active. To test this idea, but also to have a rapid protocol to detect lipoxygenase activities, we set up an assay detecting the formation of hydroperoxides directly in protein gels. We adapted a test from Heinisch *et al.,* (1996), who proposed a staining method for

lipoxygenases directly in electrophoretic gels. A native gel is run to keep proteins fully folded and then incubated with the substrate. We chose linoleic acid and linolenic acid because they are both substrates for known fungal lipoxygenases. After incubation with the substrates, the gel is incubated with N,N-dimethyl-p-phenylenediamine (DMPD). In the presence of an oxidant, a colored DMPD radical cation is formed (DMPD•+), absorbing at 510 nm (it appears pink to the eye). Indeed, during oxidation of fatty acids by lipoxygenases, hydroperoxides are formed, that attack the DMPD to form the colored quinone. The reaction is illustrated on figure C2-18. This test is not specific to LOX as it can in fact, detect any oxidizing molecule. LOX activity is detected indirectly through the formation of hydroperoxides.

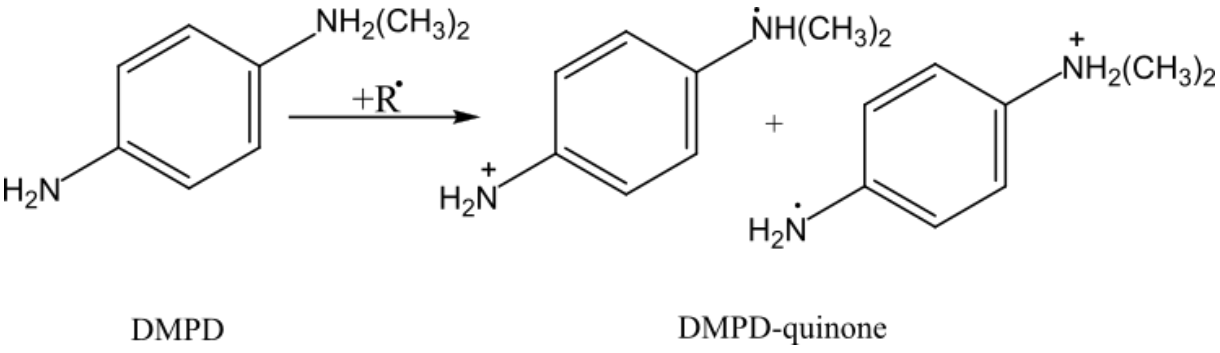
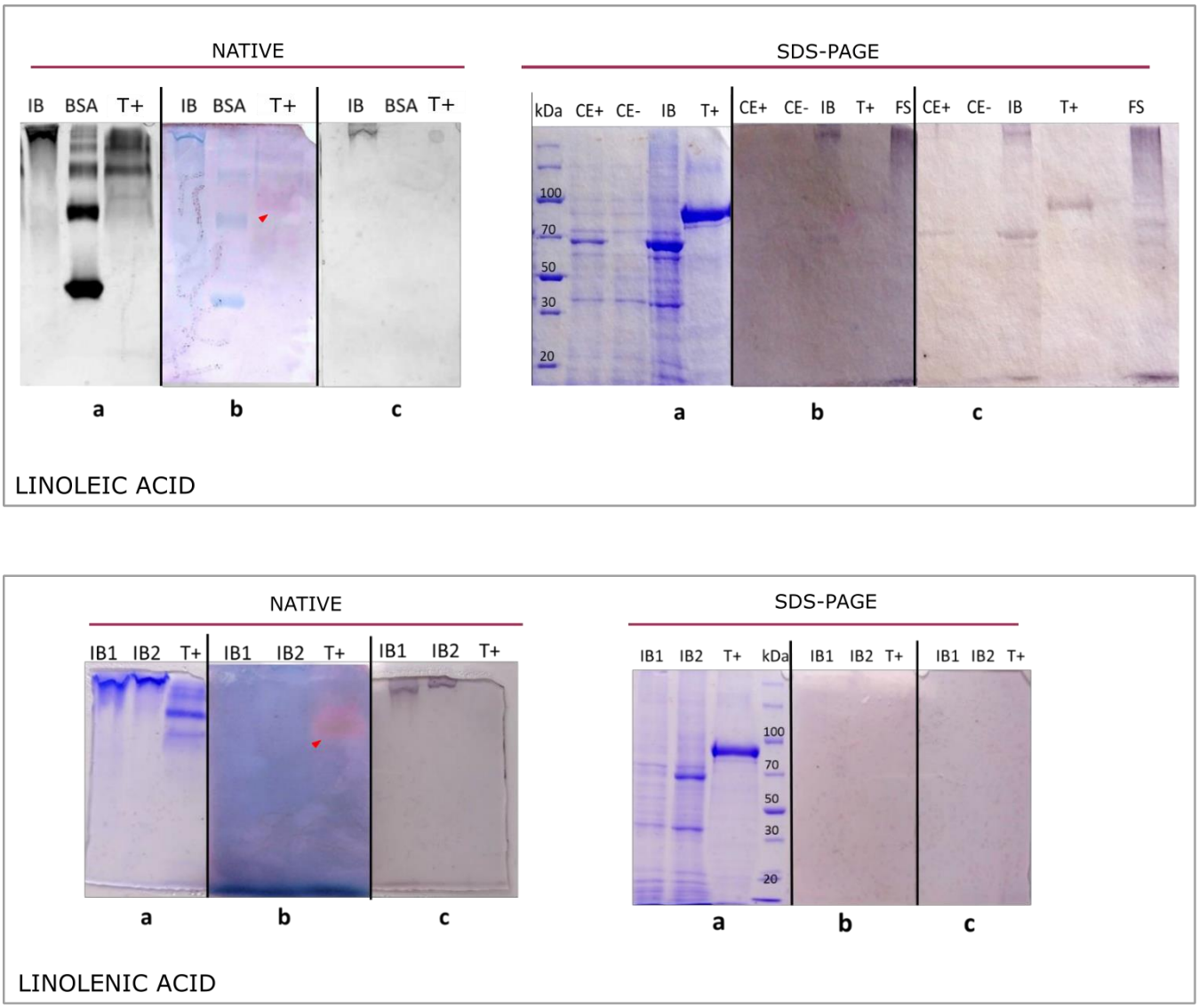


Figure C2-18: Production of pink-coloured cationic DMPD-quinone radicals by oxidative conversion of DMPD

To test this method, we used a commercial soybean LOX, oxidizing linoleic and linolenic acid to hydroperoxides (Fuller, 2001). At the same time we performed the LOX assay on inclusion bodies from a BL21 (DE3) CodonPlus culture induced for 2 hours at 37°C. Each sample (inclusion bodies, commercial soybean LOX, crude extract) were run on the same electrophoresis gel, in non denaturing conditions. The latter was then split into three gels. The first one was stained with Coomassie blue to check the presence of proteins, the second was incubated with the substrate and the DMPD solution. Finally, to ensure that the pink colour observed was specifically induced by the oxidation of fatty acids by lipoxygenases, we incubated the third gel without the substrate. We also executed the same tests on SDS-PAGE gels in which proteins are denaturated. Hereby, the boiling step of the samples was omitted and after electrophoresis, SDS was removed from polyacrylamide gels by washing them in a phosphate buffer (Zienkiewicz *et al.,* 2014). The results are shown on figure C2-19.



a: Coomassie stained gel
b: with substrate
c: control without substrate

Figure C2-19 : LOX detection assays « in gel » with linoleic acid or linolenic acid as the substrate, in non-denaturing (native) or denaturing (SDS-PAGE) conditions. On native gels incubated with the substrate, a red arrow indicates the presence of a pink coloration, corresponding to LOX activity. For top panel - IB: inclusion bodies, BSA: Bovin Serum Albumin used here as a negative control, T+: soybean LOX, used here as a positive control. CE+: crude extract after IPTG induction, CE-: crude extract without induction, FS: soluble fraction. For bottom panel -IB1: inclusion bodies from expression of PVLOX1, IB2: inclusion bodies from expression of PVLOX2.

With linoleic acid, we observed a faint pink band in native gels corresponding to the soybean LOX. No pink coloration was visible for inclusion bodies containing the recombinant 6His-PVLOX1 nor 6His-PVLOX2 (data not shown). No pink coloration was observed in the absence of the substrate or in denaturing conditions, confirming the specificity of this colorimetric test.

With linolenic acid as the substrate, a stronger pink band was observed for the soybean LOX but again we detected no lipoxygenase activity in inclusion bodies for both recombinant LOXs. The commercial soybean LOX seems to prefer the linolenic acid as a substrate. The commercial mixture of soybean LOX might contain several isoenzymes with different substrate preference, explaining why a stronger activity is detected with linolenic acid rather than linoleic acid. We did not succeed to visualize LOX activity after SDS-PAGE for the soybean LOX as has been reported by Zienkiewicz *et al.,* (2014).

We now have at our hands an assay to verify LOX activity of isolated potential LOX enzymes directly in gel. This will be of interest for further investigations trying to obtain heterologous PVLOX enzymes, as the assay is easy and can be performed at the same time as analyzing samples for histidine tags on Western blots.

3. Discussion

We have cloned and sequenced the cDNA of the two putative endophytic lipoxygenases PVLOX1 and PVLOX2, which allowed the identification of two intronic regions in both genes. Their deduced protein sequences contain 613 amino acids for PVLOX1 and 581 amino acids for PVLOX2. They both have a molecular weight around 68 kDa, which is very similar to other described fungal LOXs. They also aligned with 37.2% and 39.3% with the well described 13*R*-MnLOX from *G. graminis*.

Alignment of PVLOX1 and PVLOX2 with other described fungal LOXs confirmed that they could have a lipoxygenase activity as they possess the five highly conserved amino acids, that bind the metal cofactor and that are involved in the formation of the catalytic center. Their sequences also suggest that they could catalyze the formation of 13*R*-HPODE as they both contain a phenylalanine residue in the substrate pocket, such as the five other described fungal MnLOXs in contrary to *Fusarium* and *Pleurotus* FeLOXs, which contain less space filling amino acids at this location and form *S*-HPODE. In contradiction with those predictions, if we follow the theory of Brash (Coffa & Brash, 2004) concerning mammalian LOXs, PVLOX1 possesses a glycine at the Coffa-Brash site residue, like the 5 other fungal MnLOXs, predicting it to be an *R*-LOX, whereas PVLOX2 contain an alanine, like the two FeLOXs predicting it to be an *S*-LOX. Therefore, we need to purify and determine the precise enzymatic activity of PVLOX1 and PVLOX2, ie we need to identifiy R- or S-stereospecificity of the 13- hydroperoxides formed by these enzymes. This will shed new light on the importance of each site residues in LOX stereospecificity.

In regard to the heterologous expression of PVLOX1 and PVLOX2, both recombinant proteins were correctly expressed in our bacterial system, fused to the His-tag. Nevertheless, we failed to obtain soluble proteins properly refolded, which is an essential prerequisite to the characterization of their enzymatic activity.

Indeed, recombinant proteins were found in the form of inclusion bodies, which result from aggregation of poorly folded proteins. This problem is usually faced with recombinant expression in bacteria and figure C2-20 illustrates major solutions proposed to obtain soluble proteins. So far, we only modified strategies to enhance soluble expression of proteins (1), but did not try to solubilize and refold proteins from inclusion bodies (2). Another possibility is to express the proteins in yeasts instead of bacteria (3), which possess the appropriate eukaryotic cellular machinery for protein refolding.

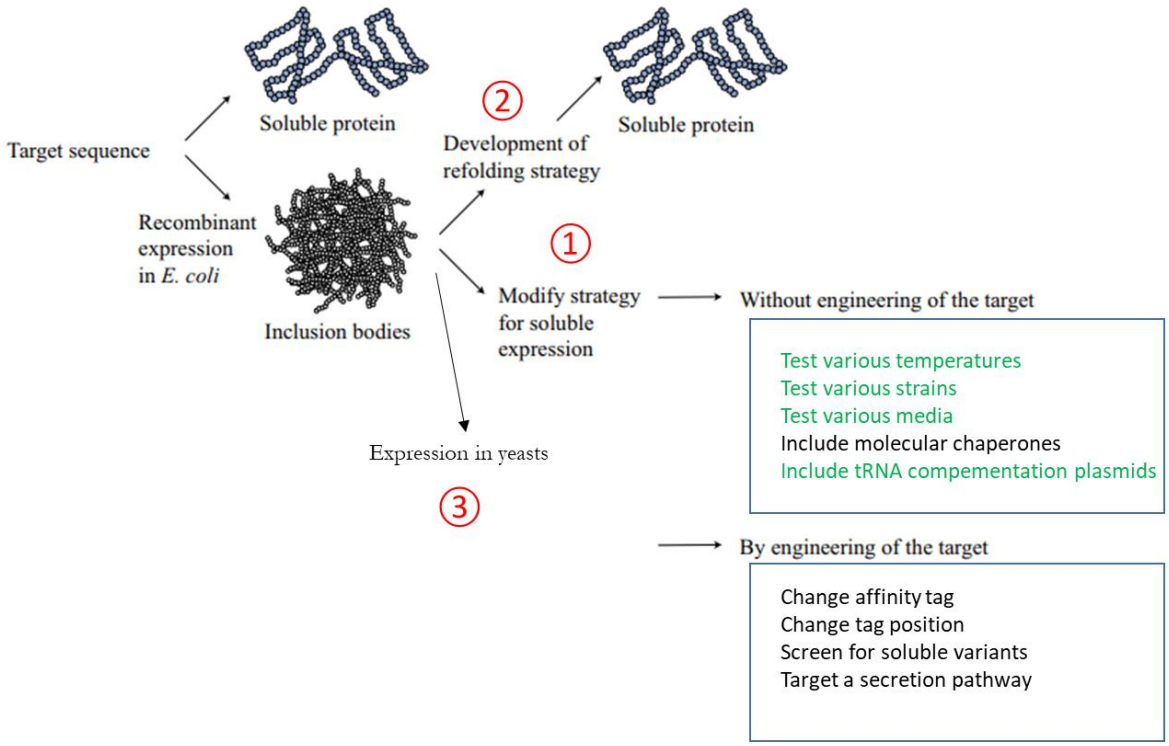


Figure C2-20: Downstream applications employed to obtain soluble proteins from *E. coli* (adapted from Sorensen *et a.l,* 2005). The solutions we have tested are highlighted in green.

So far, all our trials, including various temperatures, strong induction conditions with IPTG or auto-induction with lactose, as well as testing of two different bacterial strains encoding rare tRNAs have failed. No recombinant proteins were detected neither at 16°C, the most widely used technic to avoid the formation of inclusion bodies, as proteins are formed slowly. A synthesis rate too much reduced, and an incorrect functioning of chaperones at low temperatures could explain this. To circumvent this problem, we could have tested production in strains especially engineered for expression at very low temperatures, like the ArticExpress ™ strain from Stratagene. This strain possesses chaperones working at cold temperatures down to 4°C (Ferrer *et al.,* 2004). Moreover, we could also have included helper plasmids encoding for additional chaperones and foldases. Many commercial plasmids are available. Among them we can cite the widely used chaperones DnaK and GroEL. Those enzymes mediate the disaggregation of inclusion bodies (Sorensen & Mortensen, 2005).

Building of new expression constructs can form totally different strategies to obtain the desired proteins. For instance, it has been shown that the affinity tag (His-tag in our case) could be changed in terms of position or nature. For instance, expressing the affinity tag at the C-terminal position instead of the N-terminal can improved protein refolding. Furthermore, other tags like the maltose-

binding protein (MBP) can be fused to the target protein and has been shown to increase protein solubility, although the reasons are still unclear (Rosano *et al.,* 2014). However, as those solubilizing partners have important sizes they usually need to be removed to perform enzymatic assays, and protein solubility after their removal is not guaranteed. Many different solubilizing partners exist and to select the most appropriate, it is possible to use the Green Fluorescent Protein (GFP) reporter system. With this strategy, one can construct different plasmids containing the desired protein fused to GFP and fused to different solubilizing partners: fluorescing strains indicate the proper refolding of the target protein.

Two major pathways of secretion for proteins exist in *E. coli*: the Tat pathway for Twin Arginine translocation pathway and the Sec pathway for Secretion pathway (Baneyx, 1999; Freudl*,* 2018). Tat leads to transportation of already folded proteins whereas Sec allows folding in the periplasm after translocation, where chaperones can be found. Eukaryotic proteins lack the signals needed for secretion in prokaryotes and if no bacterial peptide signal is added to the recombinant protein, *E. coli* will mainly express this latter in the cytoplasm. In some cases, it can be relevant to direct protein expression to the periplasm (Sec pathway), a more oxidative environment, especially if there are disulfide bonds, or to the extracellular medium. However, in our case, LOXs do not contain di-sulfure bonds and this strategy might not change their solubility. In addition, this strategy might be time consuming as it is usually advised to screen a large diversity of signal peptides to find the optimal one.

Another set of strategies involves the recovery of proteins from inclusion bodies, their denaturation using diverse agents like urea or guanidine, and their re-folding (Baneyx & Mujacic, 2004). However, the process of protein denaturation and re-folding into native and biologically active protein is hazardous. In addition, working with unfolded proteins is difficult because they are more sensitive to degradation by proteases.

Finally, the ultimate solution is to radically change the host expression system and try expression in eukaryotic organisms such as the yeasts *Saccharomyces cerevisiae* or *Pichia pastoris*. This system has been underestimated for a long time because much more tools were available for *E. coli* and also because the protein yield is usually very low with yeast compared to bacteria. However, in our case low protein yield is not a major problem since a few amount of enzyme is sufficient to perform enzymatic assays. In this perspective, the project of expressing PVLOX1 and PVLOX2 has been taken over by two master students who cloned the *lox* genes in a pESC shuttle vector compatible with expression in *S. cerevisae.* The two recombinant proteins were readily expressed, purified and shown to be present in the soluble fraction.

4. Experimental procedure

4.1 Strains and plasmids

Escherichia coli strains

The strain JM109 was used for the extraction of pET28a plasmid. This strain has the following genotype: endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14- [F' traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^- m_K^+$).

The strain *E.coli* TOP10 was used for the construction of the expression vectors. Its genotype is the following: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$

Two additional *E.coli* strains were used for the heterologous expression of genes *pvlox1* and *pvlox2*.

- Strain BL21 CodonPlus (DE3) RIPL has the following genotype: F$^–$ *ompT gal dcm lon hsdS*($rB^- mB^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S) pLysS[*T7p20* araU ileX proL leuW *ori*p15A](Cm^R). This strain contains a plasmid encoding 4 tRNAs (argU, ileY, proL, leuW) corresponding to 5 rare codons of *E. coli* (AGA, AGG, AUA, CCC, CUA).
- Strain Rosetta™ (DE3) pLysS is derived from BL21 strain and has the following genotype: F$^–$ *ompT gal dcm lon hsdSB*($rB^- mB^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K-12}$(λ^S) pLysSRARE[*T7p20 ileX argU thrU tyrU glyT thrT argW metT leuW proL ori*p15A](Cm^R). This strain is also designed for expression of proteins needing the use of rare codons and supplies tRNAs for 6 rare codons (AGA, AGG, AUA, CUA, GGA, CCC).

Both strains contain the pLysS plasmid, which expresses T7 lysozyme and suppresses basal expression of T7 RNA polymerase in the absence of induction. They are both resistant to chloramphenicol and are depleted for Lon and OMpT proteases, reducing the degradation of recombinant proteins.

All strains were cultured in liquid LB medium (Lysogeny Broth : trypton 10 g.L^{-1}, yeast extract 5 g.L^{-1}, NaCl 10 g.L^{-1}, pH 7) or plated on LB agar (same composition as LB medium with addition of 15 g.L^{-1} of agar) with appropriated antibiotics (Kanamycine [50 μg.ml^{-1}], ampicilline [100 μg.ml^{-1}], chloramphenicol [35 μg.ml^{-1}]).

Plasmid pET28a

For all expression constructs, we used the plasmid pET28a. Its map is given in figure C2-21. The cloning region carries an N-terminal or C-terminal His-Tag downstream a T7 promoter, allowing the overexpression of His-tagged recombinant proteins. This plasmid harbors the *kanR* gene, supplying resistance to kanamycin.

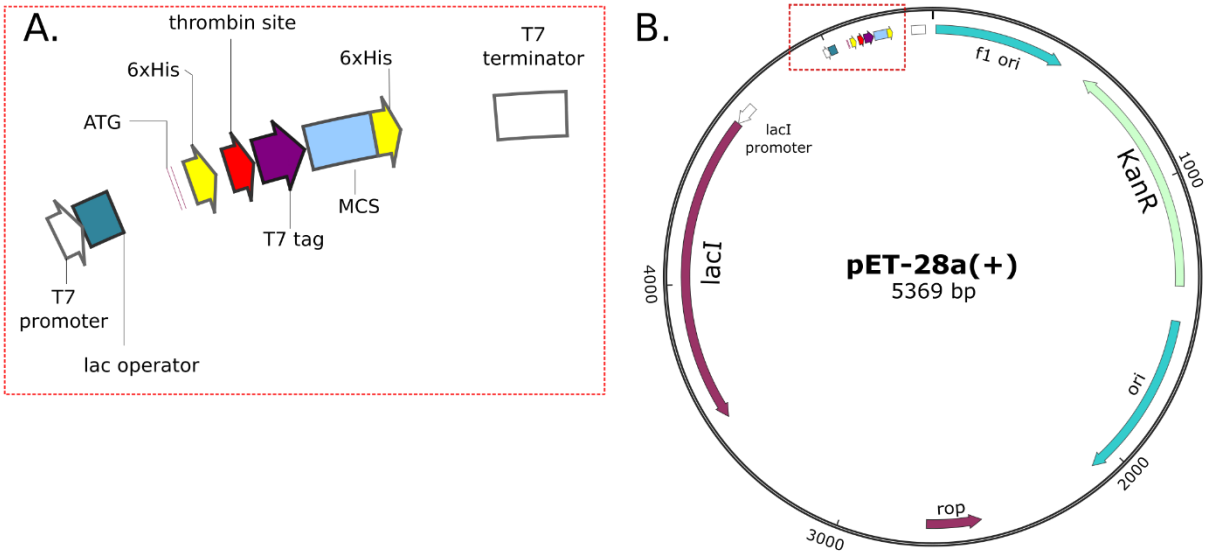


Figure C2-21: pET28a vector map. **A.** Focus on the pET28a cloning/expression region. His-Tag sequences can be added at the C-term or N-term position of the recombinant protein. A thrombin site allows further removal of His-Tag. MCS (multi-cloning site) is embedded by T7 RNA polymerase regulatory sequences. **B.** Map of the full plasmid showing position of the MCS. The sequence is visualized with Snapgene software.

4.2 Plasmid extraction

Overnight cultures of 5 ml of cells containing the desired plasmid were pelleted and plasmids were extracted with the GeneJET plasmid miniprep kit (Thermo Scientific #K0502), following the manufacturer's instructions.

4.3 Preparation of competent cells

E.coli TOP10, BL21 CodonPlus and Rosetta™ (DE3) pLysS competent cells were prepared from a culture of 0.5 DO. The pellet from 10 ml of culture was re-suspended on ice in 100 µl of cold TSS ($MgSO_4$ 20 mM, DMSO 5%, PEG3350 10% in LB liquid) and kept for up to one year at -80°C in glycerol 20%.

4.4 Transformation of *E.coli* strains

90 µl of cold KCM 5X (KCl 0.5 M, $CaCl_2$ 0.15 M, $MgCl_2$ 0.25 M) were added to 90 µl of competent cells on ice with transformant DNA (either plasmid or a mix of PCR products for expression construct). Cells were successively incubated on ice for 10 minutes, at 42°C for 90 seconds and again on ice for 20 minutes. Cells were resuspended in 100 µl of SOC medium (Tryptone 2%, yeast extract 0.5%, NaCl 10mM, KCl 2.5 mM, MgCl2 10 mM, $MgSo_4$ 1M, glucose 20%) and incubated at 37°C for 1h before being plated. They were kept at 37°C until the apparition of bacterial colonies.

4.5 Expression constructs

RNA extraction

Mycelium was collected as described for RT-qPCR experiments in Chapter III. Because we hypothesized that *pvlox1* and *pvlox2* genes would be upregulated in co-culture with *F. oxysporum*, we used mycelium of *P. variabile* grown in co-culture with the phytopathogen as starting material. Around 100 mg of mycelium was grinded in liquid nitrogen and transferred in 1 ml of TRIZOL ® (Life Technologies, 15596-026). Cellular debris were removed by centrifugation 10 minutes, at 12 *g* and 4°C. The upper phase was incubated with 200 µl of chloroform for 3 minutes before a new 15 minutes centrifugation step. RNA was precipitated with 500 µl of isopropanol, incubated for 10 minutes, then centrifugated (10 mn). The pellet was washed with 1 ml of ethanol 70% RNAse-free and let to dry at room temperature. Final RNA was resuspended in 20 µl of DEPC treated-water and heated for 15 minutes at 55°C. DNAse treatment was performed as described in Chapter III.

cDNA synthesis

5 µl of RNA were reverse transcripted as described in chapter 3 (p. 136-137) with slight modifications. We used 2 µl of oligo(dT) at 50 µM, 4 µl of reaction buffer, 0.5 µl of Ribolock RNAse inhibitor, 2 µl of DNTPs. We also removed the final step at 70°C to avoid degradation of long cDNA. Oligo(dT) were used instead of hexamers because they allow the amplification of full length cDNA.

Intron prediction and 3'UTR-5'UTR location in pvlox1 and pvlox2

Localisation of introns in nucleic acid sequences of *pvlox1* and *pvlox2* was predicted using FGENESH (www.softberry.com), an online gene finder program (Solovyev *et al.,* 2006). As *P. variabile* is a Dothideomycete, we chose 8 available Dothideomycetes as models for gene-finding parameters (*Baudoinia compniacensis, Pyrenophora, Oidendron maius, Leptosheria maculans, Macrophamina phaseolina, Neofusicoccum parvum and Stagonospora nodorum*). This same software was used to identify the 5'-UTR and 3'-UTR (untranslated regions) in *pvlox1* and *pvlox2*.

PCR amplification

Pvlox1 and *pvlox2* cDNA were amplified from 1 µl of undiluted cDNA with the HighFidelity Phusion polymerase (ThermoFischer #F530-S) in a final volume of 20 µl. For *pvlox1* we used primers 26 and 27. *Pvlox2* cDNA was amplified with primers 32 and 33. Primer sequences, details of PCR mix and programs are available in table C2-5. pET28a was amplified with Phusion polymerase from 2.5 ng of extracted plasmid with primers 34/35 for *pvlox2* construction or primers 28/29 for *pvlox1* construction, in a final volume of 100 µl. Primers 34 and 35 contain tails homologous with *pvlox2* sequence. PCR programs and details of primers are listed in table C2-6. All PCR products were purified with the Nucleospin ® PCR clean-up kit from Macherey-Nagel according to the manufacturer's instructions.

Multi-cloning in E.coli

To obtain PVLOX1 recombinant protein, E.coli TOP10 competent cells were transformed with 250 ng of *pvlox1* PCR product and 24 ng of pET28a amplified by primers 28/29. The final plasmid was called pMB4. For PVLOX2 protein, cells were transformed with 250 ng of *pvlox2* PCR product and 48 ng of pET28a amplified by primers 34/35 and the final plasmid called pMB3.

Sequencing

All constructs were confirmed by sequencing at Eurofins (Cologne) with standard primers T7 and T7-term given in table C2-4. These primers flank the cloning site and allow the verification of proper insert integration. For *pvlox1* construction, T7 and T7-term amplified sequences were not overlapping and we used the internal primer 12 to sequence the missing part. Sequences were analysed with BioEdit software and Chromas 2.6.

Primers	Sequence (5'→3')
12	TGTACAAGGCGACTGACAAG
T7	TAA TAC GAC TCA CTA TAG GG
T7 term	CTA GTT ATT GCT CAG CGG T
26	**TATGGCTAGCATGACTGGTGGACAG**CCAGCCGCCATGCCTC[1]
27	**GTGGCAGCAGCCAACTCAGCTTCC**TTGCTATGCATGCCAGAGAATG[1]
28	GGAAGCTGAGTTGGCTGC
29	CTGTCCACCAGTCATGCTAG
32	CGCTTGGTGCAACGAATCTG
33	TAGCTTGCCTTCGTATCGCCAG
34	CGTGAAGTACCTGGCGATACGAAGGCA**CTGTCCACCAGTCATGCTAG**[1]
35	TCGATCTGATCAGATTCGTTGCACCAA**GGAAGCTGAGTTGGCTGC**[1]

Table C2-4: Details of primer sequences for expression constructs and their sequencing.

[1] For primers containing tails, nucleic acids in bold are annealing with plasmid pET28a, in blue with *pvlox1* and in green with *pvlox2*.

Reagents	Volume for 20 µl final mix
H2O	11.2
Buffer 5X	4
dNTPS [2.5 mM]	1.6
Primer (reverse)	1
Primer (fwd)	1
Phusion HF	0.2

Table C2-5: Detail of PCR mix. All products were from ThermoScientific, except primers that were synthetized by Eurofins.

PCR step	26/27		28/29		32/33		34/35	
Initial step	98°C-3 mn		98°C-30 s		98°C-3 mn		98°C-30 s	
Denaturation	98°C-10 s	40 c.	98°C-10 s	35 c.	98°C-10 s	38 c.	98°C-10 s	35 c.
Annealing	65°C-30 s		72°C-2 mn		68°C-30 s		61.5°C-30 s	
Elongation	72°C-80 s				72°C-80 s		72°C-90 s	
Final step	72°C-10 mn		72°C-10 mn		72°C-10 mn		72°C-10 mn	

Table C2-6 : Details of PCR program for the construction of expression vectors (c. is for number of cycles)

4.6 Transformation of *E.coli* BL21 and Rosetta strains

BL21 CodonPlus competent cells were transformed with 60 ng of plasmid pMB3 and pMB4 for the expression of PVLOX1 and PVLOX2 recombinant proteins. Rosetta competent cells were transformed with 30 ng of plasmid pMB4.

4.7 Expression of PVLOX1 and PVLOX2 recombinant proteins with IPTG

For each following experiments, we prepared overnight cultures of *E.coli* cells in LB medium with chloramphenicol (35 $\mu g.ml^{-1}$) and kanamycin (50 $\mu g.ml^{-1}$).

High scale production of proteins

A pre-culture of *E. coli* cells was diluted 1:100 in 1L of fresh LB with antibiotics and incubated at 37°C until we reached an OD of 0.5. A sample of 5 ml was put aside for a no-induction control. Isopropyl β-D-1-thiagalactopyranoside (IPTG) was added at a final concentration of 0.1 M to the remaining culture. In some experiments, we also added $MnCl_2$ at 2 µM. Induction was performed during 2 hours at 37°C, 30°C or 25°C. Cultures were centifugated and pellets (≈2 g) were kept at -

20°C before cell lysis. 500 µl of control and induced cultures were put aside to check the presence or absence of recombinant proteins in total cell extracts.

Small scale development of protein expression

To test several different induction conditions and for all preliminary experiments, we tested the protein induction in small culture volumes of 10 ml, after a 1:100 dilution of a pre-culture of *E.coli* cells.

4.8 Lactose induction trials

The protocol was adapted from Studier *et al.*, (2005). An overnight culture of *E. coli* cells was prepared in MDG medium (Na2HPO4 25mM, KH2PO4 25 mM, NH4Cl 50 mM, Na2So4 5 mM, MgSO4 2 mM, $MnCl_2$ 2 µM, $FeCl_3$ 10 µM, glucose 0.5%, aspartate 0.25%, pH=7) with chloramphenicol (35 $\mu g.ml^{-1}$) and kanamycin (50 $\mu g.ml^{-1}$). This culture was diluted 1:1000 in ZYM5052 medium (same composition as MDG medium without aspartate and with lactose 0.2 %, glycerol 0.5 %, tryptone 1 %, yeast extract 0.5 %). Cultures were incubated at 37°C until we reached OD 0.6, and incubated for different times (16h, 24h, 48h) at 37°C or 25°C. Cultures were pelleted and kept at -20°C.

4.9 Cell lysis

Sonication

Cell pellets were resuspended in 10 ml of lysis buffer (NaCl 0.3M, Na3PO4 50 mM, imidazole 10 mM, glycerol 5%) and sonicated with a Digital Sonifier (Branson), 60% vibration amplitude, with 4 cycles of sonication (pulse on, 20 seconds) and rest on ice (pulse off, 1 minute). Samples were then incubated on ice with 1 ml of Triton-X100 (10%) for 10 minutes before a centrifugation step (15000 rpm, 4°C, 10 minutes). We then collected the supernatant, containing the soluble proteins and called "soluble fraction". Pellets, containing cellular debris and inclusion bodies (IB) were resuspended in 10 ml of lysis buffer with 1 ml of Triton-X100 (10%) and we repeated the incubation and centrifugation step. This step was repeated a third time on the remaining pellet and we finally collected a pellet containing the washed inclusion bodies. Those washing steps are important to remove cellular components like cell wall and outer membrane material. Triton-X100 does not solubilize inclusion body proteins but is rather used to extract lipid and membrane-associated proteins.

Protein extraction with Bugbuster TM

In some cases, it was useful to prepare a small extract of proteins with this rapid technic to check the presence or absence of the recombinant proteins in the soluble fraction before going further with proteins purification. After induction, we sampled 1 ml of culture and resuspended the pellet in 10 µl of BugBuster™ 10X (Novagen, 70921-4), 2 µl of lysozyme (50 mg/ml), 1 µl of DNAse in a final volume of 100 µl. Pellets were incubated for 30 minutes at room temperature before three successive "freeze-thaw" cycles in liquid nitrogen. Samples were then centifugated 20 minutes at 13000 rpm and 4°C to separate the soluble fraction from cellular debris and inclusion bodies. The pellet was resuspended in 100 µl of MilliQ water before SDS-PAGE analysis.

4.10 SDS-PAGE and Native PAGE

All gels were prepared with 10 % of acrylamide and 8% of bis-acrylamide (Sigma, A3574). The molecular weight of recombinant proteins could be estimated with SDS PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Since the mobility of a substance in a gel is influenced by both charge and size, in SDS PAGE samples are treated in order to have a uniform charge, so electrophoretic mobility in SDS PAGE depends mainly on size. 10 µl of samples were resuspended in a same volume of Laemmli buffer 2X (glycerol 20%, SDS 0.2%, stacking buffer 50%, EDTA 0.01M, bromophenol blue 1 $g.L^{-1}$) with Dithiotreitol at 0.1 M (DTT) as a reducing agent and heated at 95°C for 2 minutes. They were shortly centrifugated (2 minutes, 13000 rpm) before running the gel. SDS PAGE was carried out at 150 V for 1 hour using a Bio-rad PowerPac™ Basic supply, in the presence of an anionic detergent sodium dodecyl sulfate (SDS, 0.1%), Tris-base (3.02 $g.L^{-1}$) and glycine (14.4 $g.L^{-1}$). pH buffer was adjusted to 8.3. For native PAGE electrophoresis, proteins were prepared in a non-reducing and non-denaturing sample buffer (neither SDS nor DTT in the sample buffer and in the gel, and no heating step), to maintain both the proteins' secondary structure and native charge density. We used Page Ruler Broad (Thermoscientific #26630) as size standard in protein electrophoresis. Proteins were revealed with Coomassie blue (Brillant blue 1 $g.L^{-1}$, isopropanol % (v/v), acetic acid % (v/v)). Gels were destained in acetic acid (10%).

4.11 Batch purification of recombinant proteins

In order to verify the presence of the recombinant proteins PVLOX1 or PVLOX2 in the soluble fraction, proteins were concentrated 5 times with a Spin-X® UF concentrator (Corning). The resulting fraction was transferred on an His Select HF Nickel affinity gel (Sigma H0537), designed to bind His-tagged proteins thanks to metal ion, in 200 µl of equilibration buffer (NaCl 0.5M, Tris 50 mM, imidazole 10 mM). We prepared a gradient of imidazole by mixing buffer A (NaCl 0.5M,

Tris 50 mM) and B (NaCl 0.5M, Tris 50 mM, imidazole 500 mM). The gel was first washed several times with 10 mM imidazole until all proteins without affinity were eluted. For this purpose, a Bradford assay was performed in parallel with 10 µl of each flow through. We then performed successive elution steps with 50 mM, 100 mM, 250 mM of imidazole. The gel was washed for further use with 500 mM imidazole. Imidazole competes with the His-tag for binding to the metal-charged resin and His-tagged proteins should be eluted with 100-200 mM of imidazole.

4.12 Western blot

For immunoassays, proteins separated by SDS-PAGE were transferred on a membrane made of polyvinylidene fluoride (PVDF, GE Healthcare) for 2h at 400 mA with a Bio-rad PowerPac™ Basic supply. In order to identify recombinant proteins on the basis of their His-tag motif, we used a Monoclonal Anti-polyHistidine antibody (Sigma H1029). The secondary antibody was an IgG antibody (Sigma Fab Goat anti-mouse IgG, BETHYL A92-238), coupled to the alkaline phosphatase enzyme. Alkaline phosphatase activity was detected by the presence of a red end product in presence of substrate Naphtol AS-MX phosphate (Sigma N5000) and Fast Red TR (Sigma F-2768). The reaction was stopped with the addition of HCl 1N.

4.13 "In gel" detection of lipoxygenase activity

We adapted an existing protocol designed to detect lipoxygenase activity from proteins directly in the electrophoresis gel (Heinisch *et al.,* 1996). A native PAGE gel was incubated for 30 minutes in 20 ml of borate buffer at 0.2M (pH 9) with 20 µl of linolenic or linoleic acid (Sigma L2376 and L1376) diluted in 20 µl of ethanol. The soybean LOX (Sigma L7395) was used as positive control and 500 U were run on the gel. A control gel was run and treated in the same way without the addition of fatty acid substrates. To reveal the presence of hydroperoxides formed by lipoxygenase activity, we used the N,N-dimethyl-p-phenylediamine (DMPD) (Sigma T-8133). The revelation solution was prepared with 250 mg of DMPD, 2.25 ml of methanol, 0.25 ml of acetic acid in a final volume of 25 ml. In the presence of hydroperoxides, a pink band should be observed after about 2 minutes.

Chapter III

III. Molecular crosstalk between *P. variabile* and *F. oxysporum* *(article)*

Molecular crosstalk between the endophyte *Paraconiothyrium variabile* and the phytopathogen *Fusarium oxysporum* – modulation and degradation of beauvericin production involving oxylipin signaling

(in preparation)

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Abstract

Foliar endophytes are beneficial microorganisms providing plant protection against pathogens. However, the mechanisms involved in their molecular crosstalk with phytopathogens remain elusive. Here we analyse the communication between the endophyte *P. variabile* and the phytopathogen *F. oxysporum* involving oxylipin signaling. Oxylipins are fatty acid derivatives produced by lipoxygenase enzymes. We identified two novel lipoxygenases (PVLOX1 and PVLOX2) in the foliar endophyte *P. variabile*, with *pvlox2* being specifically upregulated in the interaction, and confirmed the synthesis of 13-HPODE by the endophyte. To test whether 13-HPODE was involved in beauvericin regulation in *F. oxysporum*, we analyzed the expression pattern of the beauvericin synthetase (*beas*) encoding gene upon competition with *P. variabile* and in the presence of 13-HPODE by RT-qPCR, and assessed beauvericin production during the interaction. The *beas* gene was induced in the presence of *P. variabile* or 13-HPODE and higher levels of beauvericin were detected in the mycelium of *F. oxysporum* and in the culture medium. We then show that beauvericin inhibits the growth of the endophyte, which in turn counteracts and degrades the mycotoxin. Some hypothetical propositions are given on how the outcome of the interaction between the antagonists could be advantageous for the plant and its microbiota.

Keywords: oxylipins, lipoxygenases, beauvericin, endophyte, phytopathogen, quantitative PCR, metabolites, *Fusarium*

Introduction

Communication is vital in all aspects of life processes and oxygenated polyunsaturated fatty acids (oxylipins) act as chemical mediators in plants, animals and fungi. It has become credible that fungi not only produce oxylipins to coordinate their developmental program, but to remodel plant and mammalian host responses (Fisher & Keller, 2016). The following work treats oxylipin communication between an endophyte and a plant pathogen, precisely the fungal *Paraconiothyrium variabile* – *Fusarium oxysporum* interplay.

Endophytes are microorganisms that live internally in the host plant and remain asymptomatic for at least part of their life cycle (Petrini, 1991; Wilson, 1995; Partida-Martinez & Heil, 2011). Indeed, they colonize the plant interior and contribute to the growth, development, fitness, and adaptation of the host plant (Rodriguez *et al.,* 2008; Partida-Martinez & Heil, 2011; Hardoim *et al.*, 2015; Vandenkoornhuyse *et al.* 2015; Rho *et al.,* 2018). Whereas a fair amount of studies exist on plant microbiota associated with belowground plant organs (Lundberg *et al.,* 2012, Bulgarelli *et al.,* 2012, 2013) less is known about aboveground compartment endophytes, especially for fungal endophytes. However, evidence is accumulating to suggest substantial abundance and diversity of microbial endophytes residing inside aerial tissues of plants (Penuelas & Terradas, 2014). In 2013, our team (Langenfeld *et al.,* 2013) isolated foliar and stem endophytes of the conifer *Cephalotaxus harringtonia* (Japanese Plum Yew), which were then further analysed for their functional diversity (Combès *et al.,* 2012). Hereby, we found that the Dothideomycetes *Paraconiothyrium variabile* shows inhibitory effect on the *in vitro* growth of various phytopathogens including *Fusarium oxysporum sp. medicaginis*. Furthermore, the fungal endophyte specifically metabolizes the host-plant metabolome for its own benefit (Tian *et al.*, 2014), produces anti-bacterial, original eremophilanes (Amand *et al.,* 2017) and hydrolyzes surfactins of co-isolated *Bacillus subtilis* bacteria (Vallet *et al.,* 2017). Combès *et al* (2012) further investigated the interaction of *P. variabile* with the phytopathogen *F. oxysporum,* which causes vascular wilt disease in over 100 different plant species (Dean et *al.,* 2012; Michielse & Rep, 2009). We investigated competition-induced metabolite production during co-inoculation experiments with *P. variabile* and *F. oxysporum*. We showed that two oxylipins, 13-oxo-9,11-octadecadienoic acid (13-oxo-ODE) and 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) were overproduced in the interaction zone whereas beauvericin, a mycotoxin from *F. oxysporum,* was present in lower amounts as compared to single culture (Combès *et al.,* 2012). It was not clear-cut, at that moment, if these two oxylipins were produced by the endophyte *P. variabile* or the

phytopathogen *F. oxysporum*. Where they come from during the interaction and how they regulate beauvericin produced by the phytopathogen were the questions we addressed in this study.

Oxylipins constitute a large family of oxidized fatty acids and metabolites derived therefrom which show diverse signaling properties in mammals, microbes and plants (Tsitsigiannis & Keller, 2007, Christensen & Kolomiets, 2011; Fischer & Keller, 2016, Wasternack & Feussner, 2018). Compelling evidence has been found, that plants, animals and fungi use oxylipins as a common language (Schulz & Appel, 2004; Tsitsigiannis & Keller, 2007; Fischer & Keller, 2016). In mammals, they mediate important immunological responses (Funk, 2001) and in plants, jasmonic acid in particular has been extensively reviewed, with regard to its impact on growth processes, responses to abiotic stress and pathogen attacks (Blée, 2002). Indeed, they play a crucial role in plant-fungal chemical crosstalk (Christensen & Kolomiets, 2011; Fischer & Keller 2016). Fungi as well produce a wide diversity of oxylipins (Brodhun & Feussner, 2011) and have been mostly studied in *Aspergillus nidulans* where they serve as intra-species extracellular signals to regulate spore development and secondary metabolite production, such as mycotoxins (Tsitsigiannis et *al.,* 2005).

Oxylipins are biosynthesised by a variety of oxygenases incorporating oxygen into polyunsaturated fatty acid (PUFA) substrates (Brodhun & Feussner, 2011). In fungi as in plants and mammals, the formation of 13-HPODE is catalyzed by non-heme LOXs, from linoleic acid (Ivanov *et al.,* 2016). In plants it has also been shown that the same lipoxygenase can convert linoleic acid into 13-HPODE and further into 13-oxo-ODE (Kühn *et al.,* 1991). The 13-HPODE and 13-oxo-ODE found in the *P. variabile – F. oxysporum* interaction zone, are thus most likely produced by a LOX. So far, the role of LOX formed hydroperoxy- and keto oxylipins in fungal interspecies crosstalk has not been investigated.

We show in this work that the endophyte *P. variabile* possesses two LOX genes, whereas one of them (*pvlox2)* is specifically upregulated in the presence of *F. oxysporum*. The fact that no *F. oxysporum lox* gene is upregulated during the interaction and that *P. variabile* produces higher amounts of 13-HPODE, in co-culture indicates the endophytic origin of the oxylipin. Unexpectedly, 13-HPODE as well as the endophyte itself induced beauvericin production in *F. oxysporum,* and beauvericin shows a moderate toxic activity on the endophyte's growth. This latter, however, counteracts in degrading the mycotoxin during the interaction. These results show a specific role for the 13-HPODE *P. variabile* oxylipin in *F. oxysporum* mycotoxin regulation and contribute to the decoding of microbial inter-species crosstalk.

Results

P. variabile possesses txo lipoxygenase genes

In order to verify if *P. variabile* is capable to produce 13-HPODE and 13-oxo-ODE oxylipins, we first had to verify the presence of potential 13-LOX genes in the endophyte's genome. Sequencing of the *P. variabile* genome had been initialized before in our laboratory. BLAST searches in the genome of the endophyte using *Gaeumannomyces graminis* manganese LOX (GenBank: AAK81882.2) as template for a 13-LOX was undertaken. The purified 13*R*-manganese LOX from the cereal phytopathogen *G. graminis* had been shown before to mainly produce 13*R*-HPODE (Su & Oliw, 1998; Hörnsten *et al.,* 2002; Cristea *et al.,* 2005). BLAST results revealed two *P. variabile* sequences exhibiting significant similarity to the *G. graminis* 13-LOX. The two open reading frames identified showed 36% and 39% identity to the *G. graminis* MnLOX and were named respectively *pvlox1* and *pvlox2*.

We then aligned the C-terminal region (catalytic domain) of the two hypothetical lipoxygenases PVLOX1 and PVLOX2 with seven other fungal LOXs (Figure 1A) which have been characterized biochemically. All these LOXs form both 13-HPODE and 9-HPODE, and sometimes 11-HPODE. They differ in their preference to oxygenate a certain carbon and are named accordingly, for example 13-LOX mainly forms 13-hydroperoxydes etc. We found that both PVLOXs contain the highly conserved motif W-X_2-AK found in all LOXs (Heshof *et al.*, 2014), as well as the five catalytic amino acids responsible for metal binding, in other words the three histidines, the asparagine and the C-terminal valine or isoleucine amino acids (Figure 1A). At the position of the "Coffa-Brash" determinant, an important amino acid involved in the stereochemistry of LOXs (Coffa & Brash, 2004), a glycine or an alanine has been reported in evrey known mammalian, plant or fungal Loxs and was found in PVLOX1 and PVLOX2 as well. We also found for both enzymes an N-terminal signal sequence, and using Expasy software we can predict molecular sizes of 68 and 64 kD for PVLOX1 and PVLOX2 respectively, and pI values of 5,78 both enzymes (NCBI).

We then aligned PVLOX1 and PVLOX2 with 101 putative fungal amino acid LOX sequences retrieved from the protein database NCBI to generate a phylogenetic tree (Figure 1B). PVLOX1 and PVLOX2 clustered together with other hypothetical and assessed manganese fungal lipoxygenases. Indeed, LOXs are non-heme enzymes, but use iron or manganese as co-factors binding this metal ions in their catalytic center (Su & Oliw, 1998). All fungal FeLOXs included in the tree have Isoleucine as their C-terminal amino acid, whereas this amino acid in MnLOXs is mostly Valine, or Isoleucine, with the exception of the F.oxysporum-LOX1 showing another

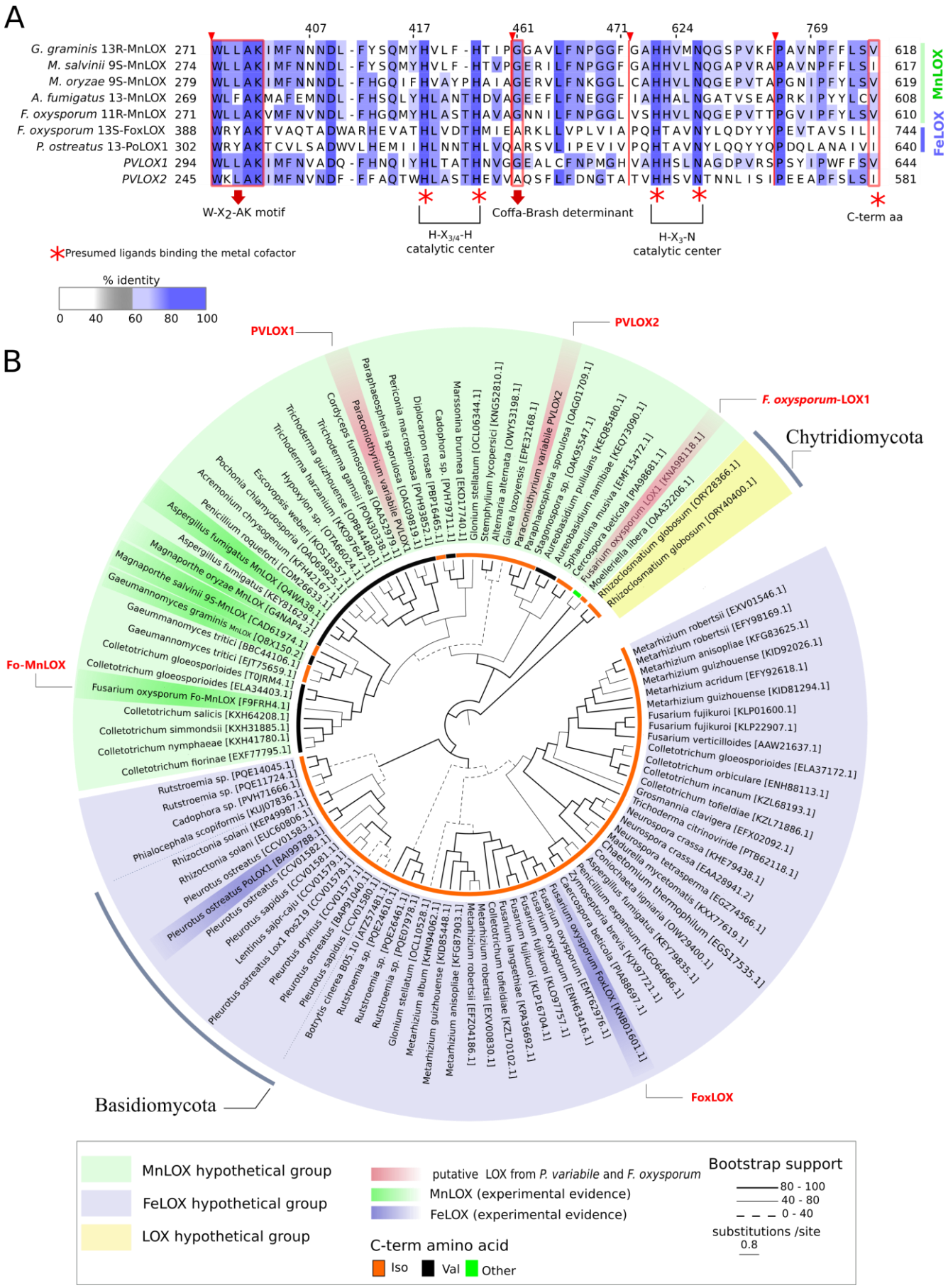
amino acid at the C-terminal end. PVLOX1 differs from PVLOX2 in that it has Valine as opposed to Isoleucine at its C-terminal end.

Altogether, we identified two *lox* genes in *P. variabile* (*pvlox1* and *pvlox2)* coding for putative LOXs (PVLOX1 and PVLOX2) with primary structures indicating that these proteins are secreted Mn-lipoxygenases capable of forming 13-HPODE. These results allow us to speculate that competition induced 13-HPODE and 13-oxo-ODE might be produced by the endophyte *P. variabile* during its interaction with F. *oxysporum*.

Pvlox2 **is specifically induced during the interaction with** ***F. oxysporum***

We have shown above that *P. variabile* has two *lox* genes potentially at the origin of the competition induced 13-HPODE and 13-oxo-ODE. *F. oxysporum*, however, also contains lipoxygenase genes that could be at the origin of the upregulated oxylipins 13-HPODE and 13-oxo-ODE during the interaction (Figure. 1B). To elucidate the origin of these oxylipins we first investigated any specific expression of *P. variabile* and/or *F. oxysporum lox* genes during the interaction. In *F. oxysporum f. sp. lycopersici*, an iron 13*S*-lipoxygenase termed FoxLOX, producing 13*S*-HPODE (GenBank: KNB01601.1) has been enzymatically characterized (Brodhun *et al.,* 2013). The same authors also mention an additional lipoxygenase, named *F. oxysporum*-LOX1 (GenBank: KNA98118.1), exhibiting significant similarity to 13R-MnLOX from *G. graminis*, but this LOX was not characterized. Later on, Wennman *et al.,* (2015) identified a manganese 11R-lipoxygenase referred to as Fo-MnLOX (GenBank: F9FRH4.1) producing mainly 11R-HPODE and 13S-HPODE, in *F. oxysporum* Fo5176.

Figure 1: *P. variabile* possesses two putative lipoxygenases. A) Partial amino acid alignment of PVLOX1 and PVLOX2 with seven fungal LOXs biochemically characterised. Asteriks indicate the position of the five presumed ligands binding the letal cofactor (Mn or Fe). The Coffa-Brash determinant, involved in stereospecificity of LOXS, and the conserved W-X_2-AK motif are higlighted. Red arrows indicate the position at which sequences are shortened for graphical reasons. GenBank accession number from top to bottom: *Gaeumannomyces graminis Q8X150.2, Magnaporthe salvinii CAD61974.4, Magnaporthe oryzae G4NAP4.2, Aspergillus fumigatus Q4WA38.1, Fusarium oxysporum F9FRH4.1, Fusarium oxysporum KNB0160.1, Pleurotus ostreatus BAI99788.1.* B) BIONJ phylogenetic tree of 101 fungal LOX amino acid sequences including LOX from *P. variabile* (PVLOX1 and PVLOX2) and *F. oxysporum*, supported by 1000 bootstraps. Basidiomycota and Chytridiomycota phylum are highlighted, all the other sequences belong to the Ascomycota phylum. The internal circle indicates the type of amino acid present at the C-terminal position.


A
407
417
461
471
624
769
G. graminis 13R-MnLOX 271 618
M. salvinii 9S-MnLOX 274 617
M. oryzae 9S-MnLOX 279 619
A. fumigatus 13-MnLOX 269 608
F. oxysporum 11R-MnLOX 271 610
F. oxysporum 13S-FoxLOX 388 744
P. ostreatus 13-PoLOX1 302 640
PVLOX1 294 644
PVLOX2 245 581
MnLOX
FeLOX
W-X2-AK motif
H-X3/4-H catalytic center
Coffa-Brash determinant
H-X3-N catalytic center
C-term aa
Presumed ligands binding the metal cofactor
% identity
0 40 60 80 100
B
PVLOX1
PVLOX2
F. oxysporum-LOX1
Chytridiomycota
Fo-MnLOX
Basidiomycota
FoxLOX
MnLOX hypothetical group
FeLOX hypothetical group
LOX hypothetical group
putative LOX from P. variabile and F. oxysporum
MnLOX (experimental evidence)
FeLOX (experimental evidence)
C-term amino acid
Iso
Val
Other
Bootstrap support
80 - 100
40 - 80
0 - 40
substitutions /site
0.8

In our study, we used the *F. oxysporum* f.sp. *medicaginis* strain for which a genome sequence is available on NCBI (GCA_001652425.1) and a BLAST analysis revealed the presence of three sequences exhibiting high identities (97-99%) to *fo-mnlox*, *foxlox* and *foxlox1*. We then tested the presence of those three *lox* genes using specific primers. We were able to amplify the *foxlox* and *foxlox1* gene but not the *fo-Mnlox* gene (data not shown).

To test whether the interaction influences the expression of the *P. variabile* and *F. oxysporum lox* genes, the two fungi were grown together on PDA medium on a cellophane sheet. Mycelium samples were collected at the confrontation zone just before the two fungi were touching and used for RT-qPCR analysis (Figure 2A/2B). Our results showed that none of the *F. oxysporum lox* genes were significantly induced during the interaction, with *foxlox1* not expressed at all. In contrast, we found significant induction of *pvlox*2 expression during co-culture whereas *pvlox1* expression pattern was identical in co-culture or in control experiments (Figure 2C).

All together these experiments show that during the antagonistic interaction between *P. variabile* and *F. oxysyporum,* only *pvlox2* is specifically upregulated. We can therefore assume that the 13-HPODE oxylipin upregulated during the interaction is produced by the *P. variabile* PVLOX2.

P. variabile **produces 13-HPODE in co-culture with** ***F. oxysyporum***

We show specific *Pvlox2* gene induction in *P. variabile* during the interaction with *F. oxysporum* and anlaysed if at the same time oxylipin production was raised in the endophyte mycelium as well as the culture medium indicating secreted metabolites. In line with the previous results, we found higher amounts of 13-HPODE in co-culture versus single culture in the mycelium of *P. variabile* (Figure 2D). This difference in oxylipin production is not visible for *F. oxysporum,* which corresponds to the lack of *foxlox over*-expression. 13-HPODE measurements in the culture medium underneath the mycelium (see Figure 2B) were not exploitable as oxylipins were below the quantification level in our experimental approach. The oxylipin 13-HPODE can be further oxidized by lipoxygenase activity (Künh *et al.,* 1991) or non-enzymatically, to the keto form 13-oxo-ODE. However, we found only traces of 13-oxo-ODE that were close to the detection threshold. These results indicate that *P. variabile* produces more 13-HPODE oxylipin during the interaction.

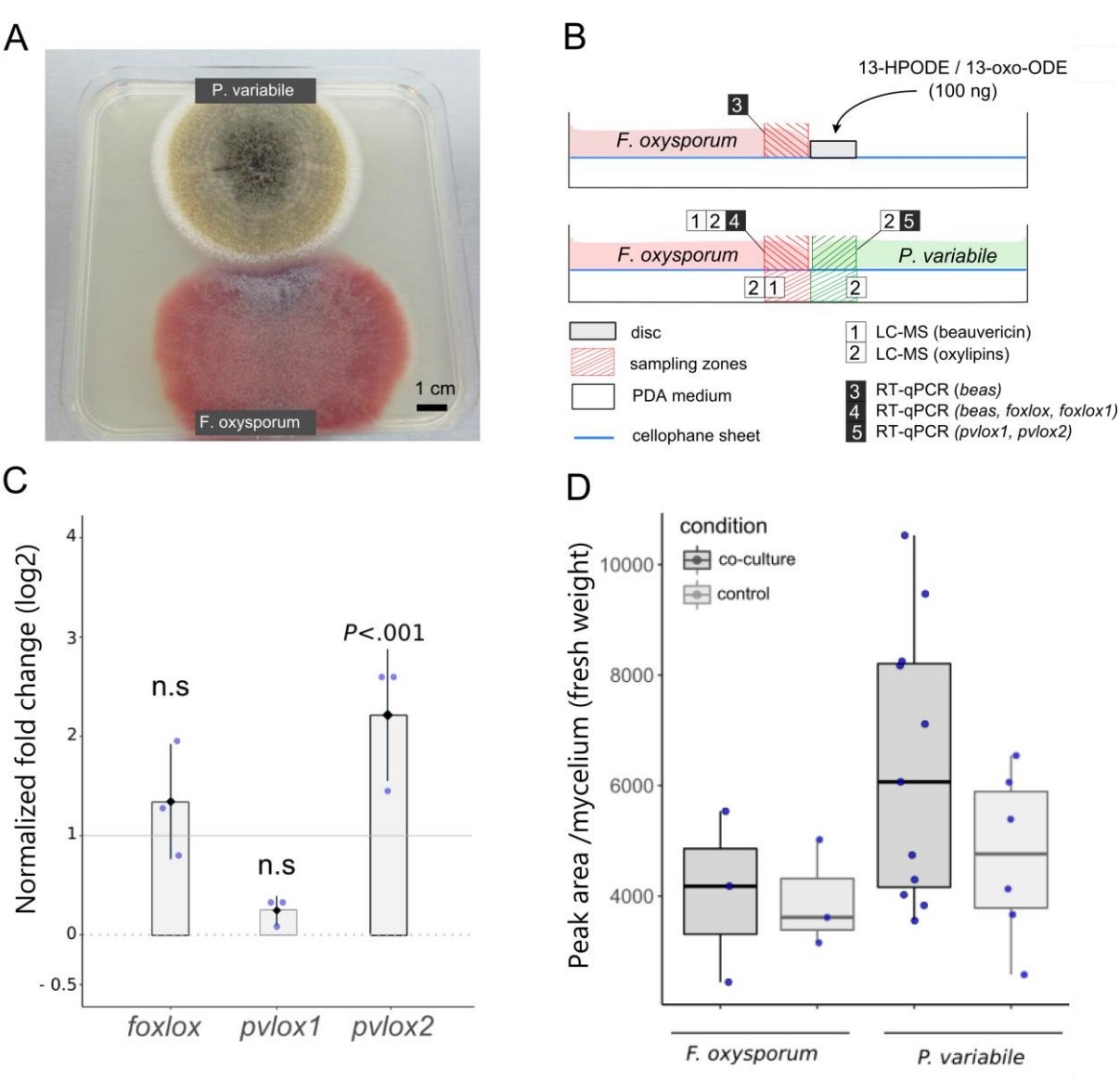


Figure 2: Oxylipin synthesis in co-culture. A) A picture of *P. variabile* and *F. oxysporum* co-culture just before sampling is shown. For co-culture experiments, control condition (not shown) corresponds to each fungus grown alone. B) The diagram shows the experimental design depicting a cross-section of *in vitro* cultures on PDA indicating sampling zones and the type of analysis for which they are intended. To measure the effect of oxylipins on beauvericin synthesis, a disc soaked with 13-HPODE, 13-KODE or ethanol (control) was added in front of the mycelium. The mycelium was collected to assess *beas* gene expression by RT-qPCR. For co-culture experiments, the mycelium of both fungi were collected to assess *beas* and *lox* gene expressions by RT-qPCR. Mycelium and medium samples were collected to quantify beauvericin and oxylipins by LC-MS. C) Data represent the derived log2 fold changes (mean value ± SD) relative to gene expression in control conditions determined by RT-qPCR. Values are means of three biological repetitions (pictured as blue circles), each consisting of four technical replicates. A bootstrap analysis, conducted on the derived log2 mean, assigned significance to the fold change values with comparison to the theoretical value of 1. D) Comparison of 13-HPODE content in mycelium samples

between control condition and co-culture condition. Values are expressed as the peak area for the ion at *m*/z=312.23 (13-HPODE), normalized by fresh mycelium mass. 13-HPODE was also detected in culture medium samples but the values were inferior to LOQ (limit of quantification). Traces of 13-oxo-ODE were detected in all samples (mycelium and culture medium) but inferior to LOQ.

13-HPODE induces beauvericin synthesis in *F. oxysporum*

To investigate whether oxylipins produced by the endophyte during the interaction can regulate beauvericin production on a transcriptional and/or post-transcriptional level, we analysed *beas* gene expression in *F. oxsyporum* in the presence of exogenous 13-oxo-ODE or 13-HPODE. *Beas* gene encodes the beauvericin nonribosomal peptide synthetase (Herrmann *et al.,* 1996; Xu *et al.,* 2008) described in *F. oxysporum* (Lopez-Berges *et al.,* 2013; Niehaus *et al.,* 2016). We grew *F. oxysporum* in the presence of *P. variabile* or in the presence of the two oxylipins as shown in figure 2B, and analysed induction of *beas* gene expression using RT-qPCR. Figure 3 shows that the presence of the endophyte, when the two mycelia start to touch, significantly up-regulates *beas* expression. 13-oxo-ODE had no effect on *beas* gene expression, whereas 13-HPODE, after 48h of exposure to *F. oxysporum* mycelium, lead to an increase of *beas* gene expression.

Using the same experimental design, we measured beauvericin production in the *F. oxysporum* mycelium, as well as in the culture medium below the fungus performing LC-MS analysis in the presence of *P. variabile* (Table 1). The two fungi were grown in co-culture on PDA medium on a cellophane sheet in order to separate the mycelia from the culture medium below the mycelium (Figure 2B). This allowed us to measure metabolites distinctly in the mycelia and culture medium. Mycelia were collected at the confrontation zone just before the two fungi were touching and used for LC-MS analysis. The three independent biological repetitions showed 9.10 to 24.20 fold change increase in beauvericin content in the *F. oxysporum* co-culture mycelium (Table 1) and a 2.50 to 14.30 fold change in the culture medium below the *F. oxysporum* mycelium, indicating that more beauvericin was secreted.

These results show that, *P. variabile* induces beauvericin synthase gene expression in *F. oxysporum*, followed by increased mycotoxin production and secretion. Interestingly, 13-HPODE but not the 13-oxo-ODE oxylipin also up-regulates *beas* gene expression.

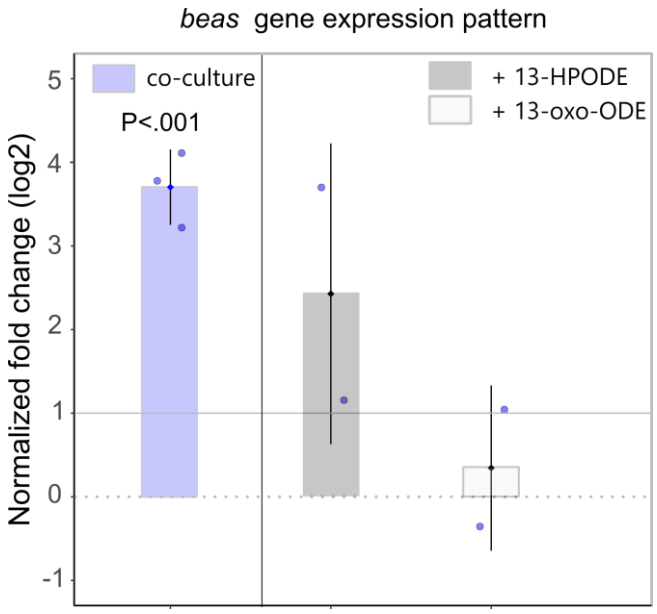


Figure 3: Beauvericin synthetase gene expression. Data represent the derived log2 fold changes (mean value ± SD) relative to beas gene expression in control condition determined by RT-qPCR. For co-culture experiments, values are means of 12 technical replicates from three independent biological repetitions. A bootstrap analysis conducted on the derived log2 mean assigned significance to the fold change values with comparison to the theoretical value of 1. For oxylipin measurements, values are means of 8 technical replicates from two independent biological repetitions.

	Beauvericin detection in *F. oxysporum*					
	mycelium samples			culture medium samples		
experimental repetition	control condition	co-culture	fold change	control condition	co-culture	fold change
1	$1.28x10^5$	$3.1x10^6$	24.2	nd	nd	-
2	$1.39x10^4$	$8.18x10^4$	5.9	$2.42x10^3$	$3.45x10^4$	14.3
3	$6,37x10^3$	$5.76x10^4$	9.1	$1.16x10^3$	$2.85x10^3$	2.5

Table 1: Beauvericin synthesis. Comparison of beauvericin content in mycelium and culture medium samples between control condition and co-culture condition. Values are expressed as the peak area for the ion at *m*/z=783.41 (beauvericin), normalized by fresh mycelium mass for mycelium samples. Measurements are from three different biological repetitions. Fold change is calculated as the ratio between values for co-culture and values for control condition.

P. variabile overcomes growth inhibition by beauvericin

We then wanted to determine if beauvericin had an effect on the growth of *P. variabile* and if the latter could degrade beauvericin. In order to quantify growth we used the nephelometer display, which allows monitoring of growth parameters in filamentous fungi (Joubert *et al.,* 2010), such as measuring maximal growth rate, time to reach maximal growth rate and total growth. Control growth curves of *P. variabile* in liquid PDB cultures in a 96-well plate exhibited an initial lag phase, an exponential growth phase and a saturation phase, in the same way, as it was shown by Joubert *et al.* (2010) for *Alternaria brassicicola* (Figure 4A). This confirms that nephelometry is a reproducible and efficient method for the monitoring of filamentous growth of *P. variabile.*

The effect of beauvericin, at 100 μg ml^{-1}, on *P. variabile* growth was measured at 10^5 ml^{-1} and 10^3 ml^{-1} initial conidia concentrations (Figure 4A). Results show that time to reach the maximal growth rate was significantly delayed by 13 hours with 10^5 $spores.ml^{-1}$ in the presence of beauvericin, and total growth was significantly reduced by 10 %. With a lower inoculum concentration, this delay was even more important with 10^3 $spores.ml^{-1}$ as the maximal growth rate was reached 19.3 hours later with beauvericin and the total growth was reduced by 23 %. The maximal growth rate, however, was not significantly reduced. Beauvericin, therefore, slows down initial growth of *P. variabile*, which then recuperates its normal growth rate, but never reaches the same total growth as without beauvericin. The same results were found in three independent biological repetitions, at both conidia concentrations (see Table S1 in Supporting information). When we measured beauvericin content by LC-MS during the nephelometric growth assay (with 10^5 $spores.ml^{-1}$), we found that beauvericin amounts declined drastically after 25 hours of incubation, to reach 20 % of its initial concentration after 96 hours post inoculation (Figure 4B).

Taken together, those results demonstrate that beauvericin has a moderate inhibitory effect on *P. variabile* growth and that the endophyte can partially overcome this effect by degrading the mycotoxin. Nevertheless, despite recuperating a normal growth rate, total growth is always reduced in the presence of beauvericin, which might be explained by a certain amount of remaining mycotoxin in the culture medium.

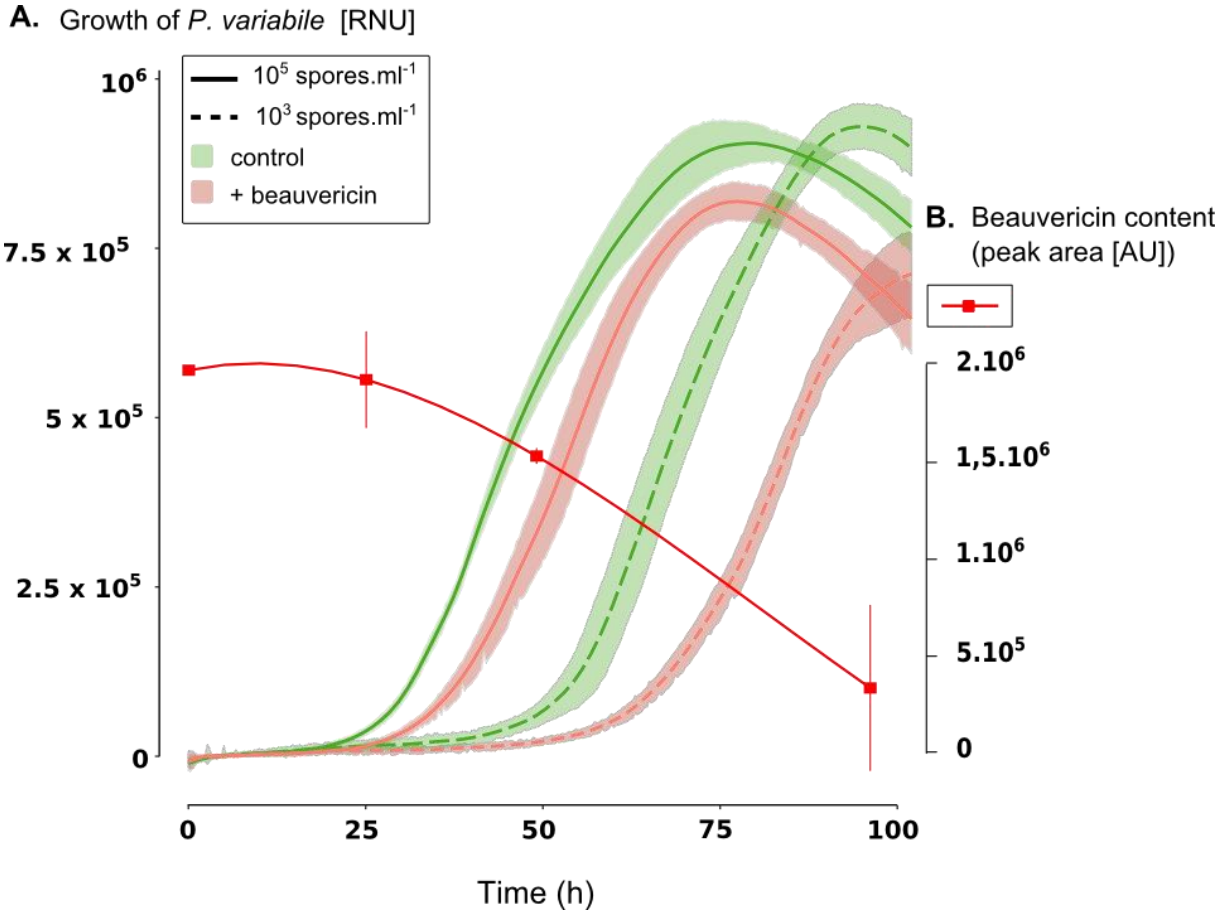


Figure 4: Beauvericin susceptibility in *P. variabile* is balanced by its degradation. A) Nephelometric growth curves of *P. variabile* at 10^5 spores.ml^{-1} or 10^3 spores.ml^{-1} in 96-well plate. Values were recorded every 20 minutes. Ribbons represent the SD values calculated from 6 replicates. Control: *P. variabile* in PDB culture medium, + beauvericin: *P. variabile* in the presence of beauvericin at 100 µg.ml^{-1}, RNU: *Relative Nephelometric Units*. B) Beauvericin content was measured in two replicates of the 96-well plate at different time points in the presence of *P. variabile* at 10^5 spores.ml^{-1}. Values shown are the peak area for the ion at *m/z*=783,41 (beauvericin). AU: arbitrary Units.

Discussion

The plant biome comprises the plant and multiple fungal and bacterial players, including both pathogens and mutualists, and is characterized by a dense network of multitrophic interactions, which are still poorly understood. Signaling and recognition processes herein are highly important and are extensively studied in plant-pathogen interactions (see Dodds & Rathjen, 2010; Peyraud *et al.,* 2016 for reviews) and only scarcely in plant-endophyte interactions (Gao *et al.,* 2010; Friesen *et al.,* 2011; Hardoim *et al.,* 2015). Oxylipin signaling in fungal pathogen/host interactions has been reported in model interactions such as *Arabidopsis* /*F. oxysporum* and *M. oryzae* on rice (Thatcher *et al.,* 2009; Riemann *et al.,* 2013, Patkar *et al.,* 2015) but nothing is known about oxylipins as mediators in plant-endophyte interactions and microbial "chatter" within the plant host. First attempts to understand sophisticated chemical communication between the endophyte *P. variabile* and the phytopathogen *F. oxysporum* was attempted in our earlier work identifying oxylipins as competition induced metabolites leading to decreased amounts of mycotoxin (beauvericin) production in the phytopathogen (Combès *et al.,* 2012). To identify the origin of the oxylipins in the competition zone, we first had to verify if the endophyte *P. variabile* produces the identified 13-HPODE and 13-oxo-ODE oxylipins via lipoxygenase activity.

P. variabile produces two potential Mn-lipoxygenases

Two *lox* genes, *pvlox1* and *pvlox2,* were found in the endophyte *P. variabile,* and their corresponding enzymes clustered with already described fungal Mn-LOXs based on primary sequences. Experimental evidence from a few purified Mn-LOXs (*Gaeumannomyces graminis* Q8X150.2, *Magnaporthe salvinii* CAD61974.4, *Magnaporthe oryzae* G4NAP4.2, *Aspergillus fumigatus* Q4WA38.1, *Fusarium oxysporum* F9FRH4.1) indicate that they all form 13-HPODE, the oxylipin found in the competition zone between *P. variabile* and *F. oxysporum.* We can thus assume, that both PVLOXs can produce this oxylipin. However, the two enzymes differ in several points. Heshof *et al.,* (2014) split fungal LOXs in two different groups: the Ile- and Val-group. The Ile-group has a unique conserved WRYAK sequence in contrary to other known LOXs and all these sequences have a C-terminal Ile. The Val-group has the conserved WL-L/F-AK sequence and end with a Val, with some some exceptions. Heshof's comparisons of the two fungal groups also indicated a preference for the Ile-group LOXs for Ala at the Coffa-Brash site and a preference for the Val-group LOXs for Gly at this site. The Coffa-Brash site is important with respect to the stereochemistry of the formed oxylipins (Coffa & Brash, 2004; Cristea & Oliw 2006). He also hypothesized, based on experimental evidence, that the Val-group uses manganese as a cofactor. Considering the two *P.*

variabile LOXs, PVLOX1 fits nicely in the Val-group, but PVLOX2 with the Ile-terminal amino acid and Ala at the Coffa-Brash site seems to fit into the Ile-group. However, it does not show the WRYAK motif but a yet undescribed WKLAK motif, and cluster together with other known manganese LOXs. So both enzymes might use manganese as a co-factor and can potentially form 13-HPODE. However, they differ at their Coffa-Brash site and therefore, might form stereochemically different oxylipins. Further purification and biochemical characterization is needed and will shed new light on these structure-function questions.

Oxylipin synthesis during the *P. variabile – F. oxysyporum* interaction

Our results indicate that *foxlox*, *pvlox1* and *pvlox2* genes were expressed during the interaction. On the contrary, the *foxlox1* gene from *F. oxysporum* was neither expressed in single culture nor in co-culture experiments. We can make the assumption that this gene is indeed not expressed in our culture conditions, or we can question whether this gene possesses a functional promoter.

Although we identified an ortholog of *fo-mnlox*, encoding a Mn-LOX in *F. oxysporum* FO5176 (Wennman *et al.,* 2015) in the sequenced strain *F. oxysporum f. sp. medicaginis*, by BLAST, we never amplified this gene in our laboratory strain. It is not unexpected that different isolates of *F. oxysporum f. sp. medicaginis* possess different haplotypes. Indeed, a study of O'Donnell *et al.* (2009), reports the presence of three different haplotypes for the forma specialis *medicaginis*. This suggests that our isolate might possess a different haplotype than the one available in NCBI and different *lox* genes (*foxlox* and *foxlox1*).

Of the two *pvlox* genes, only one (*pvlox2*) was specifically induced by the presence of *F. oxysporum*. To verify if this overexpression could be at the origin of the competition-induced 13-HPODE found by Combès *et al.,* (2012), we measured in this study the oxylipin production individually in each species and not only in the competition zone as done beforehand. Hereby, we observed that 13-HPODE was synthesized in control single cultures of *P. variabile* and *F. oxysporum* and upon interaction in co-cultures, in both fungi, which matches with the expression of *lox* genes. However, 13-HPODE content raised only in the mycelium of *P. variabile* when in co-culture. These results let us conclude that PVLOX2 is at the origin of increased 13-HPODE during dual culture. In order to have more evidence for this, it is now important to further characterize biochemically PVLOX2 to proof that it can synthesize 13-HPODE and to determine if it produces *R* or *S* stereospecific forms. So far, we have not identified if the 13-HPODE in the interaction zone is of

the *R* or *S* form. In addition, distinct fungal LOX can produce different types of hydroperoxides. Very few fungal LOXs have been characterized but all were found to catalyse the oxygenation of linoleic acid into 13-HPODE, 9-HPODE and sometimes 11-HPODE, in different proportions. For example, the Fo-MnLOX from *F. oxysporum* synthesizes mainly 11R-HPODE and few amounts of 13-HPODE and 9-HPODE (Wennman *et al.,* 2015), whereas the 13S-FoxLOX produces mainly 13-HPODE and few amounts of 9-HPODE (Brodhun *et al.,* 2013). In addition, a 9-MnLOX from *Magnaporthe salvinii* has been reported to convert 13-HPODE into 9-HPODE (Wennman & Oliw, 2012). To know the exact catalytic activity of PVLOX1 and PVLOX2 is, therefore, not only of biological interest, but will also give new insight into fundamental understanding of how oxylipin communication molecules are formed.

Modulation of beauvericin during the *P. variabile – F. oxysyporum* interaction

We found that 13-HPODE oxylipins, as well as the presence of *P. variabile,* up-regulated *beas* gene transcription in *F. oxsyporum*. Oxilipins, acting as elicitors of biological responses that lead to secondary metabolite regulation have been shown in several species (Brodhagen & Keller, 2006). "Psi factor" oxylipins in particular, produced by Ppo enzymes, such as 8-HODE (8-hydroxy-octadecadienoic acid) and 5,8-DiHODE (5,8-di-hydroxyoctadecadienoic acid) were subjected to intensive research. In the model system *Aspergillus nidulans*, for instance, they regulate sporulation and trigger sterigmatocystin production (Tsitsigiannis *et al.,* 2006). Oxylipins can up or downregulate mycotoxin production in other fungi as well. The deletion of a Ppo enzyme led to decreased levels in T2-toxin in *Fusarium sporotrichoides* (McDonald *et al.,* 2004) and 13S-HPODE repressed the synthesis of aflatoxin and sterigmatocystin, a precursor of aflatoxin, in *Aspergilii* (Burow *et al.,* 1997; Tsitsigiannis *et al.,* 2005; Scarpari *et al.,* 2014). However, investigation of the effect of 13-HPODE on mycotoxin synthesis has been restricted to aflatoxin on an intra-species level, and to our knowledge, a role in beauvericin synthesis has never been reported, and certainly not on an inter-species communication level. The transcriptional up-regulation triggered by the presence of *P. variabile* of the beauvericin synthase gene *beas* found in this study, can now, at least partly, be attributed to the PVLOX2 13-HPODE oxylipin. That we found higher beauvericin contents in *F. oxysporum* mycelium and in the culture medium is therefore not astonishing, but was somewhat in contradiction with our results (Combès *et al.,* 2012) where we found lower beauvericin content in the interaction zone. In this study, nonetheless, we demonstrate that *P. variabile* can degrade beauvericin, and during the interaction of the two antagonists beauvericin overproduced by *F. oxysporum* can be deactivated by the endophyte.

In plant-pathogen interactions, plant and fungi can mimic each other's oxylipins (Christensen & Kolomiets, 2011) leading, for example, to higher mycotoxin production and increased virulence, and plant colonization by the fungus. Another example for the role of oxylipins as plant-fungus signaling molecules comes from Gao *et al.,* (2007; 2009) who demonstrated that maize oxylipins synthesized by a 9-LOX (*ZmLOX3*) are perceived by the pathogen *F. verticilloides* and lead to increased levels of fumonisin, contributing to the pathogen's virulence. We can therefore assume that similar oxylipin-mediated crosstalk also occurs between microorganisms, although it has not been investigated to date. However, our results are consistent with the generally observed fact that microbial communication can lead to the activation of "cryptic" gene clusters and set off the synthesis of secondary metabolites (Netzker *et al.,* 2015). Consequently, we may wonder if other secondary metabolites relevant to *F. oxysporum* virulence, such as siderophores, are upregulated in *F. oxysporum,* upon interaction with the endophyte. Indeed, siderophores constitute a class of secondary metabolites involved in iron uptake and fungal virulence (Oide *et al.,* 2006), and have been shown to be regulated by the velvet complex, together with beauvericin biosynthesis in *F. oxysporum* (Lopez-Berges *et al.,* 2013).

The regulatory mechanisms of secondary metabolite biosynthetic gene clusters are poorly understood. In *F. oxysporum* it was shown that the velvet complex regulates chromatin structure and transcription of the beauvericin biosynthesis gene cluster, as well as beauvericin production in a light-dependent manner (Lopez-Berges *et al.,* 2013). As a consequence, in the dark, when the velvet complex is active, more beauvericin is formed. A role of MAP kinases in regulating the transcriptional expression of the beauvericin biosynthetic genes was also shown in *F. oxysporum*, by Ding *et al.* (2015). Recently, Niehaus *et al.,* (2016) demonstrated the role of the histone deacetylase HDA1 and the methyltransferase Kmt6, which both modifiy chromatin structure, in *beauvericin* gene cluster repression, in *Fusarium fujikuroi.* As observed in plants and mammals, fungal oxilipins are perceived by G-protein coupled receptors (Affeldt *et al.,* 2012) and there is compelling evidence that the MAP kinase pathway and velvet could be connected pathways involved in regulation of mycotoxin synthesis in fungi (Bayram & Braus, 2011). It would be interesting therefore, to determine velvet regulation in *F. oxysporum*, upon interaction with *P. variabile.*

Beauvericin cytotoxicity

Even though insecticidal, antitumor and antibacterial activity has been shown (Wang & Xu, 2012) little work on the beauvericin activity against fungal endophytes and phytopathogens are available. Hamil *et al.* (1969), who described the structure of beauvericin, attributed moderate antifungal

activity to the mycotoxin, without specifying the fungal species tested. Antifungal activity of beauvericin has also been tested against fungal infections in mice, and no significant therapeutic effect on the survival rate of infected mice has been found (Zhang *et al,*. 2007), but the authors report a strong synergistic effect of beauvericin in combination with other therapeutic fungicides. In this work, we showed that the mycotoxin is harmful upon *P. variabile* growth, in a mycelium mass dependent manner. In the presence of low initial spore concentrations and a low start of mycelium growth, the mycotoxin delayed fungal growth and decreased total growth of *P. variabile*. Upregulation of beauvericin is thus not to the advantage of the endophyte, and might indicate an adaptive defense mechanism induced in the phytopathogen in the presence of other microorganisms. Mycotoxins are defined by how they affect animals feeding on contaminating crops, but few studies question their role for the fungal fitness, both in a plant environment and in the competitive soil environment (Drott *et al.,* 2017). Our results indicate that beauvericin could give a competitive advantage to *F. oxysporum* over non-mycotoxigenic isolates.

Beauvericin degradation by *P. variabile*

Several microorganisms capable to degrade beauvericin have been described, in particular the yeast *Saccharomyces cerevisiae* (Meca *et al.,* 2013) as well as a patented bacteria (Duvick & Rood, 1998). Here we show that incubation of beauvericin with *P. variabile* leads to the degradation of the mycotoxin. This is in accordance with previous results where we demonstrated the ability of *P. variabile* to hydrolyse surfactins from *Bacillus subtilis* (Vallet *et al.,* 2017). Surfactins are cyclic lipopeptide molecules with a similar structure to beauvericin. In a screen of 85 soil samples searching for laccase producing fungi, *P. variabile* has been isolated from maple leaf compost as the most effective laccase producer (Forootanfar *et al.,* 2011). In contrast to most substrate-specific oxidases, the substrates of laccases may vary from diphenols and polyphenols to diamines, aromatic amines, benzenethiols, and substituted (Kiiskinen *et al.,* 2002). It was also shown that they have the ability to degrade toxic fungal metabolites, such as aflatoxin B1 (Alberts *et al*., 2009). Overall, the endophyte *P. variabile* seems to be well equipped to confront the *F. oxysporum* and *Fusarium* sp. antifungal compounds including beauvericin but also less frequently reported toxins such as fumonisin and moniliformin (Logrieco *et al.,* 1998; Moretti *et al.,* 2002; O'Donnell *et al.,* 2009).

A model to depicture *P. variabile* – *F. oxysporum* interaction

It is nowadays widely accepted that the outcome of a plant host-phytopathogen interaction is strongly influenced by the present microbiota. Interest in endophytes is steadily increasing and many works show the complexity and diversity of microorganisms in plants (Vanderkoornhuyse et al., 2015; Agler *et al.,* 2016). Their importance for the plant health is now accepted as well as the fact that they are part of the plant's phenotype (Rosenberg *et al.,* 2009). Very few studies, so far, have investigated functional interactions within the microbiota, and even less between endophytes and phytopathogens (Kaul *et al.,* 2016). Nevertheless, functional studies of these interactions *in vitro* are essential steps to address functions of plant microbiomes, before moving on to more holistic models including the host plant and other microorganisms (Lebeis, 2014).

Our different findings of the molecular interactions between the endophyte *P. variabile* and the phytopathogen *F. oxysporum* are depicted in Figure 5 and include hypothetic influences on the host plant and microbiota. In our model, *F. oxysporum* reacts to the presence of *P. variabile* and produces more mycotoxin beauvericin, which has an inhibitory effect on P. *variabile* growth. Yet, *P. variabile* produces, in a constitutive way, the necessary enzymes to degrade the mycotoxin and continues its growth normally. During the interaction, a yet unknown *F. oxysporum* signal induces specifically the lipoxygenase gene *pvlox2* in *P. variabile*, which leads to a higher 13-HPODE concentration and secretion. The oxylipin is recognized by *F. oxysporum* and induces the *beas* gene expression in the phytopathogen. Finally, we also found 13-HPODE in the culture medium samples, indicating that oxylipins can be secreted by both fungi during the interaction.

Beauvericin has been shown to be a virulence factor for *F. oxysporum* and the *beas* gene was induced during colonization of the plant (Lopez-Berges *et al.,* 2012). Since plants also can produce 13-HPODE (Wasternack & Feussner, 2018), *F. oxysporum*, again, might hijack the plant produced 13-HPODE to induce his virulence factor beauvericin. The mycotoxin beauvericin induces cell death in tomato and melon protoplasts (Paciolla *et al.,* 2004, Sagakuchi *et al.,* 2000) and has a fatal effect on *in vitro* grown *Arabidopsis thaliana* seedlings (data not shown). The presence of *P. variabile* and deterioration of beauvericin is thus in favour to the host plant. Moreover, antibacterial activity has been attributed to beauvericin and again, *P. variabile* might protect some of the other endophytes from the toxic activity of beauvericin. In the prospect to investigate potential amelioration of plant health in the presence of *P. variabile*, we have initiated a project to establish a tripartite interaction system involving the model plant *Arabidopsis thaliana,* its potential pathogen *F. oxsyporum* and the endophyte *P. variabile.*

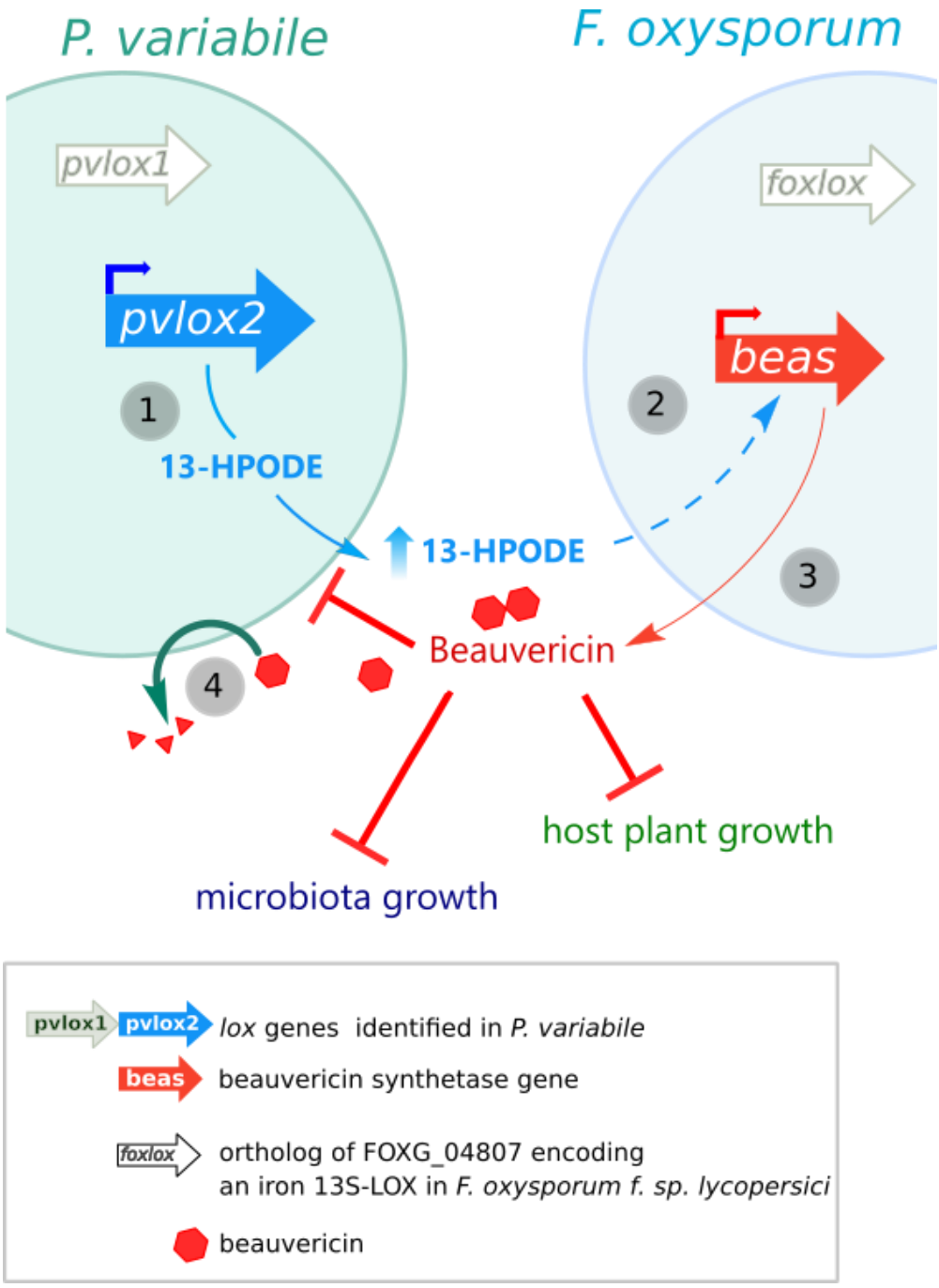


Figure 5: Molecular crosstalk between the endophyte *P. variabile* and the phytopathogen *F. oxysporum*. 1/lipoxygenase gene *pvlox2* in *P. variabile* is specifically induced during the interaction, which leads to higher amount of 13-HPODE in its mycelium. 2/13-HPODE induces beas expression in *F. oxysporum*. 3/More beauvericin is produced and secreted during the interaction. 4/ Beauvericin inhibits growth of *P. variabile* and might also have a negative effect on the host plant and microbiota growth. 5/ *P. variabile* in turn degrades the mycotoxin beauvericin.

Experimental procedure

Fungal strains and culture conditions

In this study we used a monospore strain MS1 derived from the endophyte *Paraconiothyrium variabile*, isolated from the conifer tree *Cephalotaxus harringtonia* (Combès *et al.,* 2012). This strain is conserved in the fungal culture collection of the National Museum of Natural History in Paris, under the identifier LCP5644. The strain *Fusarium oxysporum f. sp. medicaginis* is conserved in the same collection under the identifier LCP531. Both microorganisms were maintened on PDA solid medium (Potato Dextrose Agar Difco, CONDA: potato starch 4 $g.L^{-1}$, dextrose 20 $g.L^{-1}$, agar 15 $g.L^{-1}$, pH 5.6). Nephelometry assays were performed using PDB medium (Potato Dextrose Broth, CONDA: infusion from potatoes 26.5 $g.L^{-1}$, pH 5.6). For RT-qPCR and metabolite extractions, strains were cultivated on PDA medium on Cellophane sheets (Hutchinson). Spores from *P. variabile* were obtained from a 20 to 30-day old culture and 5 µl of this suspension was inoculated at 2.5 cm from the Petri dish edge. *Fusarium oxysporum* inoculum was prepared from a 7-day old culture and inoculated seven days later.

Chemicals

A stock standard solution of beauvericin (BEA) was purchased from Cayman and kept at -20°C at a concentration of 1 $mg.ml^{-1}$ in DMSO or methanol. Commercial solutions of 13-HPODE and 13-KODE were purchased from Cayman and stored at 100 µg/ml in ethanol at -80°C.

Nephelometry assays

Spores of *P. variabile* were collected by grinding a 1 cm^2 piece of mycelium form a 20-day-old culture in 1 ml of glycerol 25%. Spores were filtered on bolting clothes (25 µm and 10 µm) and the suspension was adjusted by counting spores in a hemocytometer cell counting chamber. A spore suspension at 105 $spores.ml^{-1}$ into 300 µl of PDB was poured in each wells of a 96-well plate (Falcon). Beauvericin was added at a final concentration of 100 $\mu g.ml^{-1}$ with 1% DMSO. We performed two types of control: *P. variabile* in PDB medium and in the presence of 1% DMSO. Growth was automatically recorded for 72h at 25°C using a nephelometric reader (NEPHELOstar Galaxy, BMG Labtech, Offenburg, Germany), equipped with a 635-nm laser source. The 96-well plates were subjected to double orbital shaking at 400 rpm for the whole incubation period. Measurements were done every 20 minutes with a laser intensity of 40%. Each well was measured

for 0.5 s with a laser beam focus of 2.5 mm. The experiment was repeated with three independent biological repetitions, with 6 technical replicates for each condition. Data were exported from Nephelostar Mars data software in ASCII format and further processed in R 3.5.0. Maximal growth rate (defined as the highest slope) and time to reach maximal growth rate variables were calculated from the growth curves using the first derivative. In order to compare growth curves, mean of the initial ten measurements was subtracted from each curve value. Means and standard errors where calculated from the replicates and a statistical analysis was performed to compare growth parameters in the presence or in the absence of beauvericin.

Extraction of metabolites in co-culture

For co-culture experiments, mycelium from *F. oxysporum* and *P. variabile* in co-culture or grown alone was collected as illustrated on figure 2A from two Petri dishes and pooled to reach around 100 mg of wet mycelium mass. We waited for microorganisms to come into contact before collecting the samples. We also collected three agar plugs from the same two Petri dishes with a 0.3 cm diameter die-cutting and pooled them. Samples were frozen and kept at -80°C before extraction.

Beauvericin

Mycelium samples from *F. oxysporum* were crushed in a mortar with liquid nitrogen and the powder was transferred in glass tubes with 2 ml of distillated ethyl acetate. After a 20-minute sonication, samples were centrifugated 10 minutes at 4°C and 4000 rpm. The extraction was performed twice and the collected supernatants were evaporated using a SpeedVac at 40°C. Crude extracts were kept dried at -20°C before LC-MS analyses. Samples were solubilized in 100 µl of methanol and we injected 2 µl in the HPLC-MS to detect beauvericin.

Oxylipins

Mycelium samples from *P. variabile* and *F. oxysporum* were crushed in a mortar with liquid nitrogen and the powder was transferred in glass tubes with 1.5 ml of hexane: isopropanol (3:2) additioned with 0.5 ml of MilliQ water. After a 25-minute sonication in cold water, samples were centrifugated 10 minutes at 4°C and 4000 rpm and we collected the hexane layer. The extraction was performed twice and the extracts were evaporated using a SpeedVac at room temperature. Crude extracts were kept dried at -20°C before LC-MS analyses. Samples were solubilized in 30 µl of methanol and we injected 4 µl in the HPLC-MS to detect 13-HPODE and 13-oxo-ODE.

LC/MS quantification

Samples were analyzed by liquid chromatography (Ultimate 3000, ThermoScientific) coupled to a high resolution Quadrupole-Time of Flight (Q-TOF) hybrid mass spectrometer (maxis II ETD, Brucker), equipped with an electrospray ionization source (ESI). The chromatographic separation was carried out by reverse phase liquid chromatography using an Acclaim RSLC Polar Advantage type III column (2.1 x 100 mm, 120 Å) (Dionex). Optimum separation was achieved with a gradient elution using MilliQ water with 0.1% (v/v) formic acid (A) and acetonitrile containing 0.07% (v/v) formic acid (B). The separation was achieved with a flow rate of 300 µl.min^{-1} and the following gradient: 5% B during 9 minutes, 50% B during 6 mn, 90% B during 4 minutes, 5% B during 2 minutes. Samples of *F. oxysporum* for beauvericin detection were acquired in positive mode. The optimal ionization source working parameters were: nebulizer 2.4 bar, temperature 200°C, capillary voltage 3500 V. Full scan mass spectra were performed from 50 to 1500 m/z at 5 spectra/sec. All oxylipin samples were acquired in negative mode with slight parameters modifications: capillary voltage was set to 2500 V, m/z range was 50-1200 with focus active. Commercial beauvericin and oxylipins (13-HPODE and 13-oxo-ODE) were injected to establish the retention times of the compounds and clusters of sodium-formate were used for calibration. Data were analyzed with the software Bruker Compass DataAnalysis 4.3.

Biodegradation of beauvericin

A 96-well plate was prepared as for the beauvericin cytotoxicity assays. Controls were carried out by adding beauvericin at a final concentration of 100 µg.ml^{-1} in 300 µl of PDB. The whole content of a well (mycelium and PDB medium) containing either mycelium of *P. variabile* and beauvericin or beauvericin alone was collected at different time points of incubation (0h, 25h, 49h and 95h). Extractions were performed in duplicate. Samples were extracted with 500 µl of distillated methanol, sonicated for 30 minutes and kept at 4°C before LC-MS analysis. Samples were diluted 1:100 in methanol and 1 µl was injected in the HPLC-MS for beauvericin quantification.

Reverse-transcriptase quantitative PCR

Experimental design and samples

Mycelium samples of *P. variabile* and *F. oxysporum* from three biological repetitions were collected as illustrated on figure 2A. For each condition (fungus alone or in co-culture), we pooled mycelium samples from two distinct Petri dishes, thus recovering 30 mg to 100 mg of fresh mycelium.

Samples were kept frozen at -80°C for no more than a month before being processed. Samples were grinded in liquid nitrogen and extracted with the Rneasy Plant Mini kit (Qiagen). Extracted RNA (~ 5 µg) was treated with the DNAse Max kit from MOBIO (#15 200-500) for 1h at 37°C, according to the furnisher's instructions. RNA quality was assessed by migration on agarose gel and determination of the A280/260 ratio using a spectrophotometer (Nanovue Plus CE Healthcore). The same apparatus was used to quantify the extracted RNA. Resulting RNAs were reverse transcripted on the same day.

Reverse transcription

Total RNA (400 ng for *F. oxysporum* and 600 ng for *P. variabile*) was reverse transcripted using 1 µl of RevertAid reverse Transcriptase (Thermo Fischer scientifc, #EP0441), 4 µl of random hexamers at 50 µM (Invitrogen), 8 µl of reaction buffer 5X, 1 µl of Ribolock RNAse inhibitor (Thermo Fischer Scientific #E00381) and 4 µl of dNTPs at 10 mM (Thermo Fischer Scientific #R0191) in a final volume of 20 µl. The thermocycler program included a preliminary step of 10 minutes at 25°C, followed by 60 minutes at 42°C and a final step of 10 minutes at 70°C to inactivate the reverse transcriptase. Finally, the resulting cDNA were diluted 1:10 with MilliQ water and stored no longer than two weeks at -20°C. In order to assess for genomic DNA contamination absence, a control sample was prepared during RT step without the enzyme. The samples were then subjected to qPCR and checked on agarose gel. Moreover, to check the absence of inhibition of the RT reaction, successive dilutions of RNA were used. For *F. oxysporum*, we tested 84, 168, 343, and 682.5 ng. For *P. variabile*, we tested 100, 200, 400, 600 and 800 ng. It enabled us to ensure the linearity of the relation between the amount of RNA used in the RT reaction and the corresponding amount of cDNA detected by qPCR.

qPCR

For the relative quantification of gene expression of selected genes, specific primer pairs were designed using Primer 3 version 4.0.0 software (Supporting information Table S1). Oligonucleotides were synthesized by Eurofins. qPCR reactions were performed in a final volume of 10 µl including 4 µl of cDNA diluted 1:10, 2 µl of SyberGreen I (LightCycler FastStart DNA SyberGreen I, Roche) and a 2 µl mix of reverse and forward primers at 0.25 µM each. No-template controls were included in each run for each primer pair. We used capillaries from Roche and a LightCycler 2.0 instrument from Roche. The thermocycler program is given in Supporting information, Table S2. Each run was performed twice, with two technical replicates for each sample. To estimate amplification efficiency of primers, different dilutions ranges were prepared in sterile deionized water depending on gene expression levels and standard curves were performed

in duplicates. Details are given in table S3. Primers specificity was assessed by checking the melting curves and making qPCR products migrate on agarose gel. Data were analyzed with R 3.5.0 according to the method described by Hellemans *et al.,* (2007). Data are presented as the log2 derived fold change normalized by the corresponding fold change for reference genes. Log2 fold changes were considered significant when above 1 or below -1.

Effect of 13-HPODE and 13-KODE on beas gene expression

Discs for antibiotic susceptiblity testing were soaked with 10 µl of 13-HPODE or 13-KODE at 10 $ng.\mu l^{-1}$ in ethanol and added in front of 6-day old cultures of *F. oxysporum* on PDA medium and cellophane. After 48 h of exposure, mycelium was collected from two distinct petri dishes and pooled, before RT-qPCR analyses. Experiment was performed in two distinct biological repetitions.

LOXs sequences retrieval and phylogenetic tree construction

LOXs amino acid sequences were retrieved from the protein database NCBI using the R package « rentrez 1.2.1 » (Winter, 2017). We searched for proteins having the tag « lipoxygenase » and choosing « fungi » as organisms. We added a filter to delete sequences having the following tags: partial, hypothetical, putative, predicted, uncharacterized, related to, unnamed, XP. We then selected proteins on their sequences content with a custom R script inspired from Heshof *et al.,* 2014 (see Supporting information S5). They should start with methionine, finish with either a valine or an isoleucine. They should also contain the « W-X_2-AK » motif conserved in all described LOXs and the specific amino acids involved in the catalytic center « H-$X_{3/4}$-H » and « H-X_3-N/H/S ». The remaining 115 sequences were then aligned with PVLOX1 and PVLOX2 by MUSCLE in SeaView software (Gouy *et al.,* 2009). We added manually sequences from LOXs that benefit experimental evidence but that were not selected with the script, probably because they are registered as hypothetical proteins in NCBI. It concerns the following sequences : *Fusarium oxysporum* FoxLOX (KNB01601.1), *Magnaporthe salvinii* 9S-MnLOX (CAD61974.1). Despite its terminal amino acid beeing and alanine, we also aligned the putative LOX1 from *Fusarium oxysporum* (KNA98118.1) described by Brodhun *et al.*, 2013. We trimed sequences manually to remove duplicates and poorly aligned sequences. Conserved blocks were selected using the Gblocks method (Castresana, 2000) implemented in SeaView with the least stringent parameters. 101 protein sequences from fungal origins were finally used to construct a phylogenetic tree in SeaView

using the BIONJ algorithm (Poisson model, 1000 bootstrap replicates). The tree was edited in Figtree 1.4.3.

LOXs sequences alignement

We aligned 9 protein sequences from known LOXs using MUSCLE. The alignment was edited with Jalview 2.10. (Genbank accession number of sequences from top to bottom: Q8X150.2, CAD61974.4, G4NAP4.2, Q4WA38.1, F9FRH4.1, KNB0160.1, BAI99788.1)

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Supporting information

Table S1: Growth parameters recorded by nephelometry, describing the effect of beauvericin (at 100 µg.ml^{-1}) on the growth of *P. variabile*. For each biological repetition, all displayed values are estimated over 6 technical replicates and standard errors are indicated. Percentage of inhibition and time delays between the control samples and the samples with beauvericin are indicated in brackets. Asterisks indicate statiscally significant differences with the control (**: *P*<.01; Kruskal-Wallis) RNU: Relative Nephelometric Unit. Repetition 2 is shown on Figure 4A.

biological repetition	spores.ml^{-1}	condition	maximal growth rate (RNU/h)	maximal growth (RNU)	time to reach maximal growth rate (h)
1	10^5	control	30309 ± 1389	863452 ± 18194	37 ± 1,6
		+ BEA	27058 ± 6346 **(-11 %)**	705228 ± 87227 **(-18 %)** ***	46,4 ± 3,9 **(+ 9,4 h)** **
	10^3	control	32144 ± 3310	876463 ± 30996	61 ± 4,8
		+ BEA	26329 ± 2740 **(-18 %)**	638828 ± 52409 **(-27 %)** **	83 ± 3,4 **(+22 h)** **
2	10^5	control	29129 ± 3184	909920 ± 29480	41,3 ± 1,2
		+ BEA	28851 ± 2393 **(-1 %)**	818116 ± 28575 **(-10 %)** **	54,3 ± 2,5 **(+ 13 h)** **
	10^3	control	31278 ± 2290	931197 ± 32534	64,5 ± 4
		+ BEA	26989 ± 3419 **(-14 %)** **	713411 ± 62018 **(-23 %)** **	83,8 ± 2,1 **(+ 19,3 h)** **
3	10^5	control	28824 ± 1358	912016 ± 26668	38 ± 1,9
		+ BEA	25203 ± 1231 **(-13 %)** **	827999 ± 22266 **(-9 %)** **	41,8 ± 5,6 **(+ 3,8 h)**
	10^3	control	31418 ± 1688	925976 ± 34851	56,9 ± 2,2
		+ BEA	25291 ± 1632 **(-20 %)** **	770376 ± 40186 **(-17 %)** **	69,6 ± 3,8 **(+ 12,7 h)** **

Table S2 : Primer sequences of target and reference genes specific to *F. oxysporum f. sp. medicaginis* and *P. variabile* used in RT-qPCR. Efficiencies of primers (E) are given with their standard error and the corresponding R^2. na: no amplification

gene name	fwd sequence (5'-3')	rev sequence (5'-3')	Size amplicon	E	R^2
F. oxysporum					
foxlox	CCGACTGGTGCAAGGAGATT	GGAGGCTTGGATGGAACGAA	175 bp	1.89±0.321	0.994
fusarium-lox1	CTCATCTACATGTCAGCTACCC	GATTGCTTGGTCGCCATTC	171 bp	na	na
beas	TTCCCTATCCCAACGCAAGG	CTCTGTCACTACGCCGAACA	155 bp	1.891±0.17	0.992
actin	CCATTGAGCACGGTGTTGTC	TTCTCACGGTTGGACTTGGG	145 bp	1.94±0.068	1
tubulin	CAATGCTACCCTCTCCGTCC	CGAGGTAGTTGAGGTCACCG	137 bp	1.92±0.172	0.998
ef1α	CGGTACTGGTGAGTTCGAGG	TTGATGGCGACGATGAGGTT	105 bp	1.916±0.167	0.998
P. variabile					
pvlox1	ATTACACCCTTGACTCCTTCTTCGG	GCAATCTTCTTCGCCTCCTCATCA	121 bp	1.751±0.227	0.996
pvlox2	CTGCGGCGTGTGATGCCTACTTT	GCGTCCTCGTCTACTGGTGTGTA	115 bp	1.749±0.214	0.997
actin	TAGAAGCACTTGCGGTGAAC	TATCTCCGACCGTATGCAGAA	204 bp	1.906±0.024	1
tubulin	GGGCGAAGGGTCACTCACACT	CCAGTTCCACCACCAAGGGAGT	130 bp	1.833±0.26	0.994
ef1α	CGTTCCCTTCGTGCCCATCT	GGTCTCCTTCTCCAGCCCTTGTA	94 bp	1.889±0.297	0.992

Table S3: qPCR program parameters.

°C	Hold (hh:mm:ss)	Slope (°C/s)	Cycle	Acquisition mode
95	00:10:00	20	1	None
95	00:00:10	20	45	None
60	00:00:05	20		None
72	00:00:10	20		Single
95	00:00:00	20	1	None
65	00:00:15	20	1	None
95	00:00:00	0,1	1	Continuous
40	00:00:30	20	1	none

Table S4: Details of standard curves preparation for efficiency estimation of primers. In both microorganisms, expression of genes of interest was too low to perform dilution ranges on cDNA. To circumvent this problem, we used either plasmids carrying the cDNA of the corresponding gene, or directly genomic DNA. To ensure that this matrix was as close as possible to the cDNA matrix, we performed dilutions in a mix of cDNA combining all samples.

gene	DNA origin	dilution range
F. oxysporum		
actin, tubulin, ef1α	Pool of cDNA from all analysed samples	$1:10^0$,$1:10^1$,$1:10^2$,$1:10^3$,$1:10^4$,$1:10^5$
foxlox	gDNA of *F. oxysporum* diluted in a matrix of cDNA	$1:10^0$,$1:10^1$,$1:10^2$,$1:10^3$,$1:10^4$
beas	Pool of cDNA from all analysed samples	$1:10^0$,$1:10^1$,1:50,$1:10^2$,1:500,$1:10^3$
P. variabile		
actin, tubulin, ef1α	Pool of cDNA from all analysed samples	1:5,1:10,1:40,1:160,1:320,1:1280,1:5120
pvlox1, pvlox2	Plasmids carrying cDNA sequences diluted in a matrix of cDNA	$1:10^0$,$1:10^1$,$1:10^2$,$1:10^3$,$1:10^4$,$1:10^5$, $1:10^6$

File S5: Script used in isolating fungal lipoxygenases from NCBI

```
#packages
library("rentrez")
library("seqinr")
library("Biostrings")
library("xlsx")

#searching proteins matching criteria in NCBI
fungal_lox<-entrez_search("protein",term=("(((((((((lipoxygenase AND fungi [ORGN]) NOT partial[ALL]) NOT hypothetical[ALL]) NOT putative [ALL]) NOT predicted[ALL]) NOT uncharacterized[ALL]) NOT related to [ALL]) NOT unnamed [ALL]) NOT similar to [TITL])"), retmax=300)

#to know how many sequences are found
fungal_lox$count
#[1] 179
```

```
#compile sequences in a unique fasta file
lox_fasta<-entrez_fetch("protein",id=fungal_lox$ids,rettype = "fasta",retmax=300)
write(lox_fasta, "LOX_strategy1.fasta")

#trim data in a dataframe
fastaFile <- readAAStringSet("LOX_strategy1.fasta")
seq_name = names(fastaFile)
sequence = paste(fastaFile)
df <- data.frame(seq_name, sequence)

#extract protein ID and add ID column to the dataframe
ids<-fungal_lox$id
df$id<-ids
#add a column indicating last sequence updatedate
lox_sum<-entrez_summary(db="protein",id=fungal_lox$ids,retmax=fungal_lox$count)
updatdate<-extract_from_esummary(lox_sum, "updatedate")
df$update<-updatdate

#add a column with ACCN number
accn<-extract_from_esummary(lox_sum,"extra")
df$identifiants<-accn

#filter sequences
df$WXXAK<-grepl("W..AK",df$sequence)
df$Mstart<-grepl("^M",df$sequence)
df$IVend<-grepl("(I|V)$",df$sequence)
df$H1<-grepl("(H(...|....)H).+(H...(N|S|H))",df$sequence)
df$XP<-grepl("^[^X]",df$seq_name)

#get ID of sequences matching all criteria
filter<-df[!rowSums(df=="FALSE"),]
filter$id
```

```
# 115 are matching, they are compiled in a new fasta file
lox_fasta_cured<-entrez_fetch("protein",id=filter$id,rettype = "fasta",retmax=200)
write(lox_fasta_cured, "LOXstrategy1_cured.fasta")

#convert in dataframe
fastaFile <- readAAStringSet("LOXstrategy1_cured.fasta")
seq_name = names(fastaFile)
sequence = paste(fastaFile)
df2 <- data.frame(seq_name, sequence)

#add column with protein ID
df2$id<-filter$id

#add column with update
lox_sum2<-entrez_summary(db="protein",id=filter$id,retmax=200)
updatdate2<-extract_from_esummary(lox_sum2, "updatedate")
df2$update<-updatdate2

#add column with ACCN
accn2<-extract_from_esummary(lox_sum2,"extra")
df2$identifiants<-accn2

#export to excel
write.xlsx(df2, "115_lox_ncbi_strategy1.xlsx")
```

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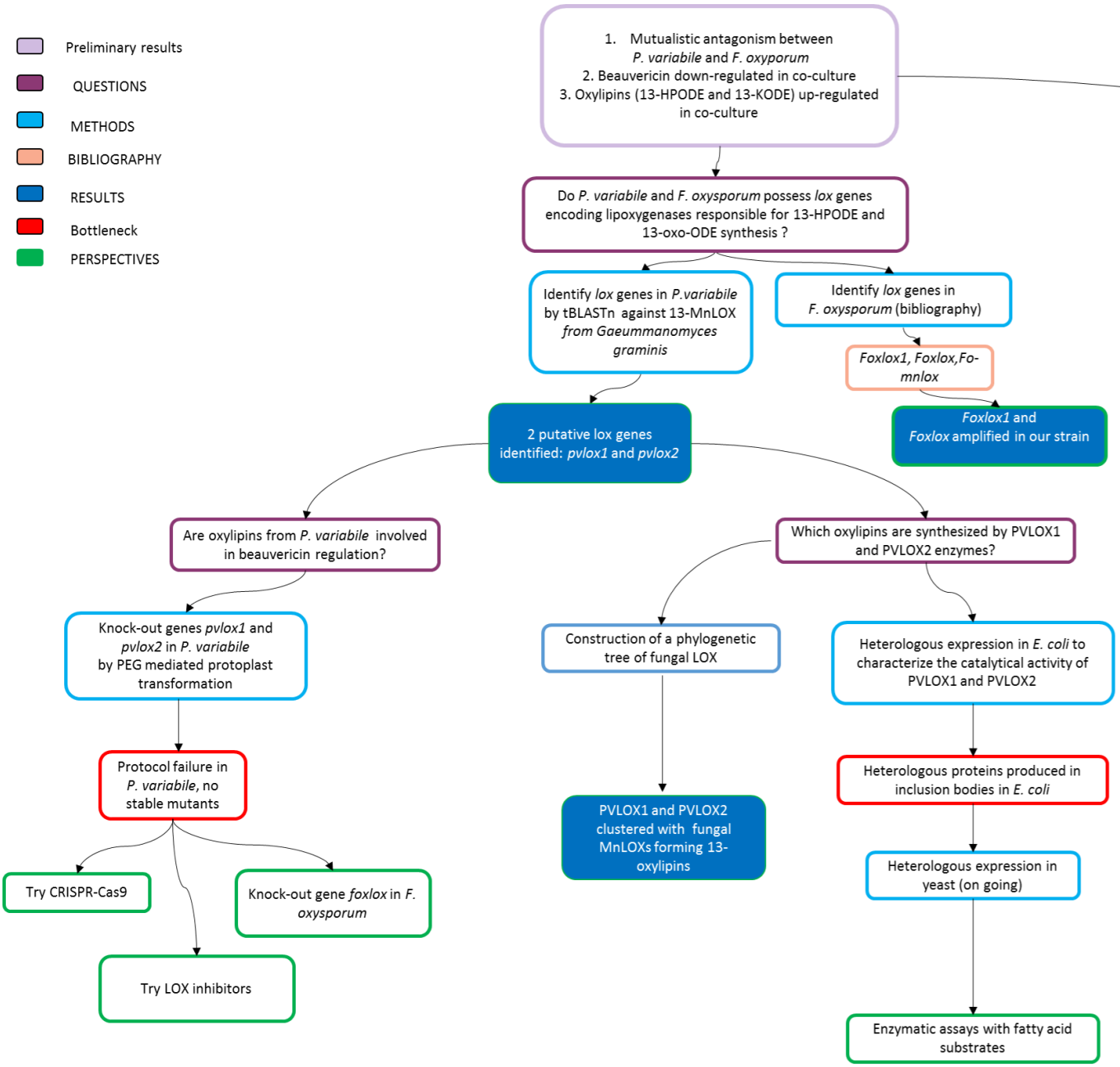
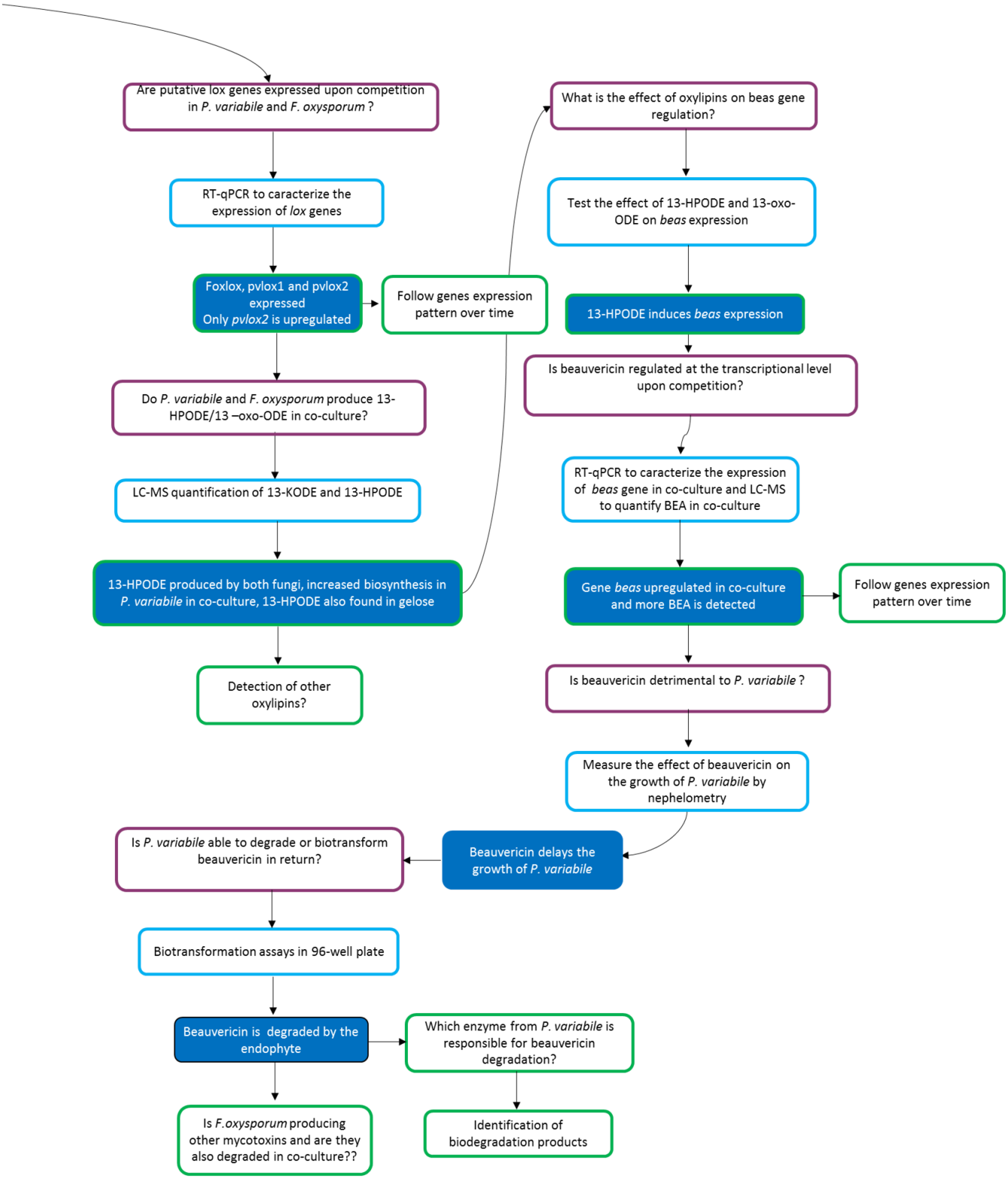
Conclusion & perspectives



Chapter I

Chapter II


Are putative lox genes expressed upon competition in P. variabile and F. oxysporum ?
RT-qPCR to caracterize the expression of lox genes
Foxlox, pvlox1 and pvlox2 expressed
Only pvlox2 is upregulated
Follow genes expression pattern over time
Do P. variabile and F. oxysporum produce 13-HPODE/13 –oxo-ODE in co-culture?
LC-MS quantification of 13-KODE and 13-HPODE
13-HPODE produced by both fungi, increased biosynthesis in P. variabile in co-culture, 13-HPODE also found in gelose
Detection of other oxylipins?
What is the effect of oxylipins on beas gene regulation?
Test the effect of 13-HPODE and 13-oxo-ODE on beas expression
13-HPODE induces beas expression
Is beauvericin regulated at the transcriptional level upon competition?
RT-qPCR to caracterize the expression of beas gene in co-culture and LC-MS to quantify BEA in co-culture
Gene beas upregulated in co-culture and more BEA is detected
Follow genes expression pattern over time
Is beauvericin detrimental to P. variabile ?
Measure the effect of beauvericin on the growth of P. variabile by nephelometry
Beauvericin delays the growth of P. variabile
Is P. variabile able to degrade or biotransform beauvericin in return?
Biotransformation assays in 96-well plate
Beauvericin is degraded by the endophyte
Which enzyme from P. variabile is responsible for beauvericin degradation?
Identification of biodegradation products
Is F.oxysporum producing other mycotoxins and are they also degraded in co-culture??

Chapter III

Figure CP-1 (previous pages): Thesis summary outline. This diagrams highlights the main problematics, the methods used, the main results and possible perspectives.

In this thesis work, we have explored the hypothesis of a transcriptional modulation of beauvericin synthesis mediated by oxylipins, in the course of the interaction between a foliar endophyte, *Paraconiothyrium variabile*, and an ubiquitous soil-born phytopathogen, *Fusarium oxysporum*.

Chapter I

The initial design was to obtain *lox* mutants in the endophyte strain by deletion of genes *pvlox1* and *pvlox2*, to determine beauvericin regulation upon interaction with the mutants. So far, no stable integration of exogenous DNA was achieved in *P. variabile* using a PEG-mediated protoplast transformation, and CRISPR-Cas9 is being considered as an alternative.

With no *P. variabile* mutant at hand, another approach to address the question about the role of 13-HPODE in beauvericin regulation during the interaction could be to obtain a *foxlox* mutant in *F. oxsyporum*. In this species an efficient genetic transformation system exists (Kistler & Benny, 1988) but no *foxlox* mutant has been obtained so far. First we would need to test the lack of production of 13-HPODE in the *foxlox* mutant and could then test it in co-culture experiments with *P. variabile*. Consecutive analysis of *beas* gene induction would then give valuable information about the importance of PVLOX2 in the production of 13-HPODE involved in beauvericin synthesis regulation and confirm the role of 13-HPODE synthesized by the endophyte, in the interaction. However, it is possible that multi-copies of the *foxlox* gene exist, which has been reported for the arachidonate 15-LOX of *Aspergillus fumigatus* (Reverberi *et al.,* 2010).

A third alternative would be to use LOX inhibitors. Such inhibitors have been developed since a long time as potential drugs to target lipoxygenase pathways in inflammatory diseases (Eleftheriadis *et al.,* 2015). Cafeic acid for example, and norhydroguaiaretic acid have been used to highlight the role of LOXs in the insect immune response to *Beauveria bassiana*, as well as in human infection by the fungal pathogen *Candida (*Lord *et al., 2002;* Noverr *et al., 2002)*. Although the use of protein inhibitors for *in vivo* experiments can lead to undesired side effects, it may be worthwhile to perform such tests in co-culture experiments with *P. variabile* and *F. oxysporum*.

Chapter II

The second objective was to express and purify lipoxygenases PVLOX1 and PVLOX2 to characterize their catalytic activity. A strategy of expression in *E. coli* was adopted and led to the expression of misfolded recombinant proteins exclusively found in inclusion bodies. We have turned to a second strategy using the eukaryotic host *Saccharomyces cerevisiae* for heterologous expression, which was developed by two master students. The proteins were readily expressed in the soluble fraction and should be soon purified in larger quantities for enzymatic assays. Our aim is to identify LOX-derived products as well as stereochemistry, using chiral chromatography.

Chapter III

The objective of this third part was to study the transcriptional regulation of lipoxygenase and beauvericin synthetase encoding genes during the interaction, and assess their corresponding metabolites. Our results demonstrate that, in contrast with our starting assumption, beauvericin is not down-regulated at the transcriptional level, and the lower levels of beauvericin detected are due to its degradation by the endophyte. On top of that, we found that 13-HPODE was produced by both microorganisms during the interaction, with a specific upregulation of *pvlox2,* encoding a putative 13-LOX. 13-HPODE was also found to upregulate beauvericin synthase gene transcription, indicating a probable role of the endophyte oxylipins in beauvericin modulation. One possible additional experiment, but maybe difficult to interprete, would be to test the effect of an oxylipin extract from *P. variabile* grown in the presence of *F. oxysporum* and compare it with an extract of oxylipins produced by the endophyte grown alone. This would show if specific oxylipins produced by *P. variabile* upon competition can trigger the expression of the *beas* cluster.

We could also consider additional mechanisms involved in beauvericin regulation. For instance, we observed a strong violet pigmentation in the mycelium of *F. oxysporum*, appearing when the two fungi were almost touching. In *F. fujikuroi,* pigmentation has been attributed to several secondary metabolites such as fusarubins (Studt *et al.,* 2012). Interestingly, in the same work, fusarubins where upregulated upon nitrogen starvation. We might therefore investigate the role of nitrogen availability in co-culture. However, beauvericin has been shown to be upregulated by nitrogen (Lopez *et al.,* 2014). We do not know if *F. oxysporum* in our experimental approach experiences nitrogen limitation, or on the contrary, benefits from enzymatic liberation of nitrogen, or other nutrients, by *P. variabile*. In addition to nitrogen, pH has also appeared to be an essential stimulus for the activation of biosynthesis of secondary metabolites and mycotoxins (Brackhage, 2013). It might be possible, upon close interaction with *P. variabile*, that changes in pH (we typically observed

an alkalinization of the medium in the competition zone) could lead to secondary metabolite modulation.

The signal that triggers *pvlox2* expression remains unknown so far. Ppo enzymes have been shown to be regulated by feedback loops (Tsitsigiannis *et al.,* 2004), and we wondered if 13-HPODE could have such a role in the interaction. In this perspective, we tested the effect of 13-HPODE and 13-oxo-ODE on *foxlox* but found no difference with the control (data not shown). However, we did not test the effect of 13-HPODE on *pvlox1* and *pvlox2*.

Conclusion

This study paves the way for further molecular investigations in a tripartite model, *in planta*, and a project was initiated in our laboratory by C. Kunz to examine the effect of co-inoculation with *P. variabile* and *F. oxysporum* on the survival of *Arabidopsis thaliana* seedlings. Preliminary results indicate a clear protective effect of *P. variabile,* as well as an herbicidal effect of beauvericin*,* and it would be particularly interesting to address beauvericin and oxylipin modulation in the plant.

To conclude, in a plant-centric view, endophytes are usually seen as "nice" microorganisms, nice collaborators, that are here to promote plant growth and ensure its ecological success by providing it with new enzymatic functions, new defense mecanisms to face pathogens and new molecular tools to increase their trophic niche width. It is therefore tempting to use them as biocontrol agents to reduce impacts of pathogens on crops, as an alternative to chemicals for a more sustainable agriculture.

On the other hand, it is also true that:

(i) endophytes have profound impacts on plant communities and can affect food web by deterring feeding by herbivores and insects
(ii) they can travel long distances through wind and pollination possibly establishing in non-desired areas
(iii) they can turn into pathogens on the basis of a single mutation
(iv) they can produce/ trigger the synthesis of a wide array of "cryptic" secondary metabolites of unknown functions and toxicity in the presence of other microbes
(v) equilibrium between pathogenicity and mutualism is fragile, and endophytes may turn out to be latent pathogens
(vi) their beneficial effects can be offset depending on environmental conditions and the plant's health status

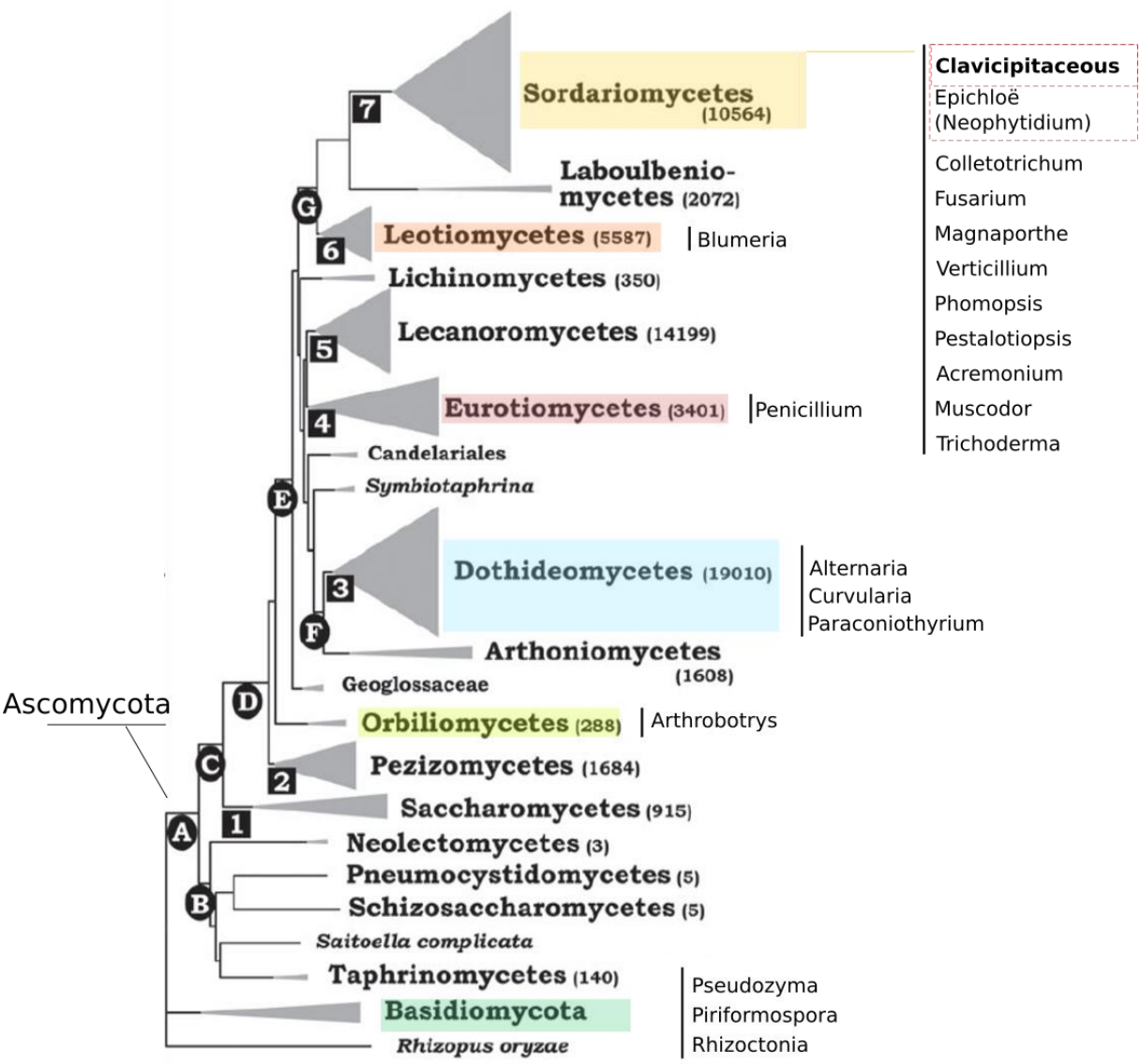
(vii) they are in constant interaction with a complex network of microorganisms within the plant biome and could have deleterious impacts depending on interactions with other microbes

Finally, to fully apprehend the role of endophytes, it is necessary to think plant-endophyte interactions in the context of ecological complexity. Plant also interact with other beneficial fungi such as myccorhyzae, bacteria and protist from the rhizosphere and from the phyllosphere, and all those partners create a complex network of interactions. New roles of endophytes already emerged from studies looking at the effect of endophytes on microbiome composition in plants. For example, the lettuce microbiome experienced an important shift in its composition when infected by the pathogen *Rhizoctonia solani*, especially concerning the "hub" microbes (Erlacher *et al.,* 2014). Very interestingly, the co-inoculation with the endophyte *Bacillus amyloliquefaciens* compensate these effects. It demonstrates how interaction of endophytes with their plant host may also affect its relationship with other microbes. I personally find exploration of the effects of endophyte symbiosis on the plant microbiota and rhizosphere a captivating topic, especially concerning the influence of cropping practices on microbial communities (Hartman *et al.,* 2018).

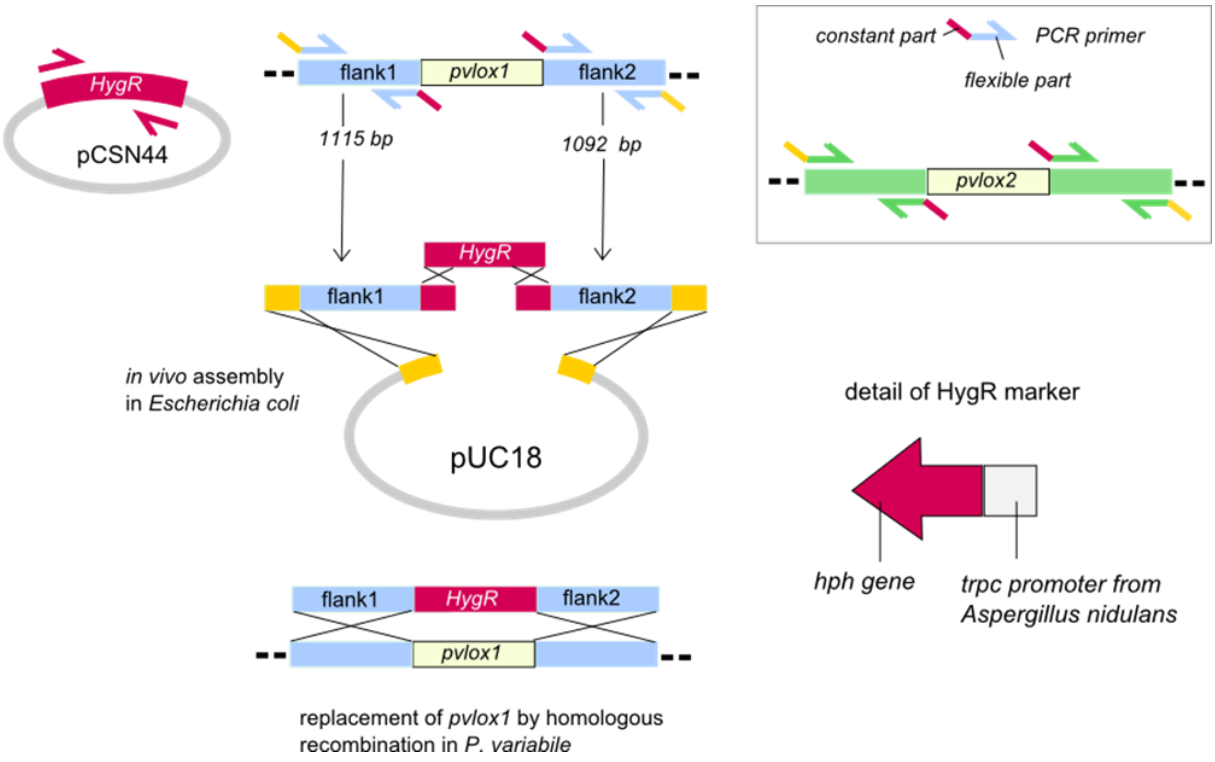
Appendices

Appendices

Appendix 1 - Simplified phylogeny of Ascomycota and Basidiomycota (from Schoch *et al.,* 2009) indicating the fungal species mentioned in the introduction



Appendix 2 - Strategy used for construction of plasmids pMB1 and pMB2



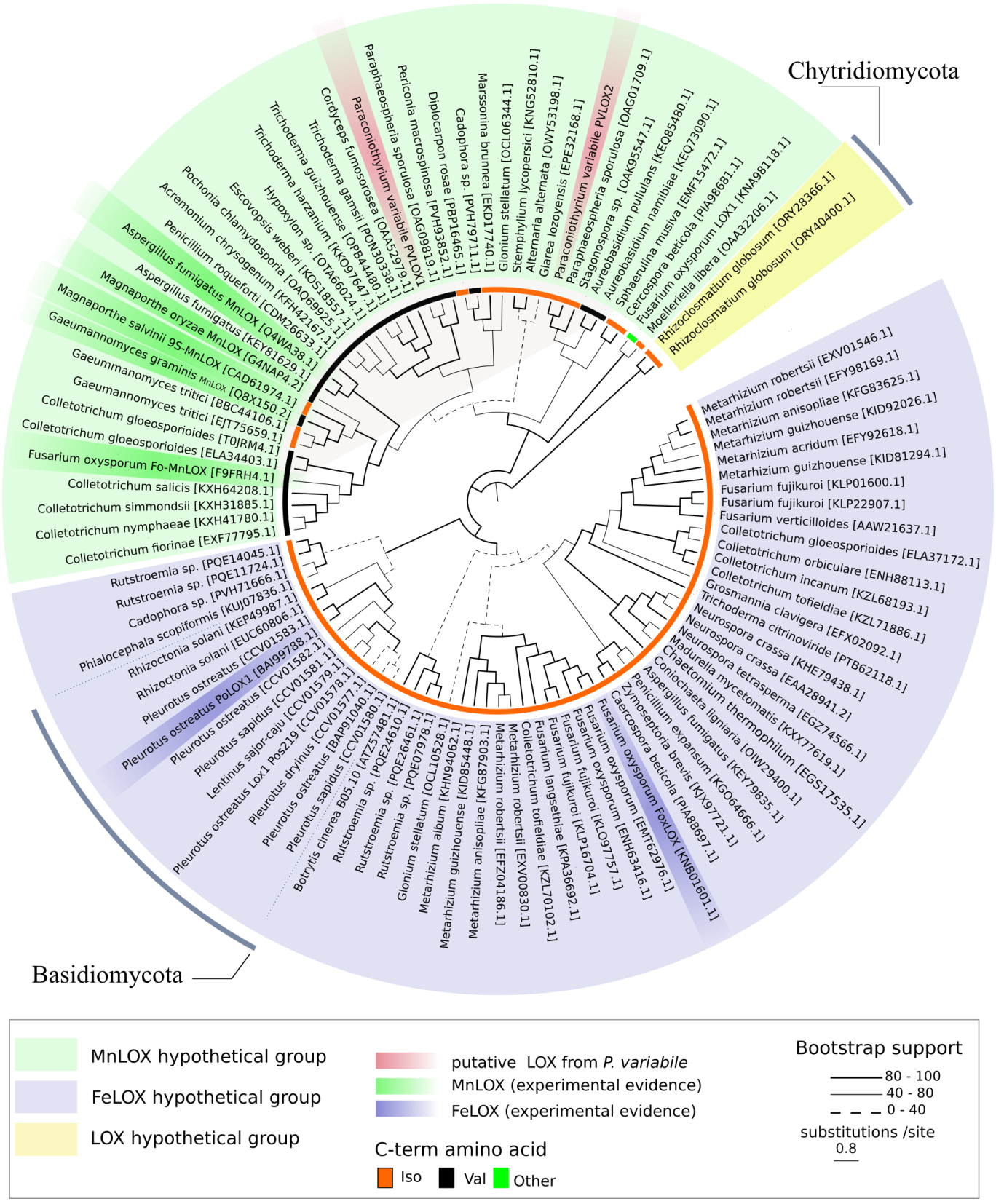
Construction of *pvlox1* and *pvlox2* cassette by multiple-cloning in *E. coli*. Upstream and downstream fragments of 1 kb were amplified from *P. variabile's* genome. Those fragments are flanking the *pvlox1* gene. They were cloned by homologous recombination inside a pUC18 vector together with the *hph* gene. Note that *hph* gene is transcribed in the antisense direction relative to the target gene. *pvlox1* gene will be replaced by this concstruction by homologous recombination in the endophyte nucleus. The inset shows the PCR strategy to amplify flanking regions of *pvlox2* gene. The modus operandi was identical to *pvlox1*. Primers were designed to add 20-30 bp homologies with pUC18 vector (yellow) or *hph* gene (red).

To construct the two deletion cassettes for genes *pvlox1* and *pvlox2*, we adopted a multi-fragment cloning protocol. This is an efficient and elegant way to construct vectors without using restriction enzymes (Zhang *et al.,* 2012; Kostylev *et al.,* 2015). In summary, two fragments of around 1 kb were amplified from flanking regions of genes *pvlox1* and *pvlox2*. They were assembled together with the hygromycin marker (HygR) by homologous recombination in the shuttle vector pUC18 from *E. coli*. Homologous recombination was made possible by the addition of short end homologies (20-30 pb) to flanking regions. Those short homologies were added by PCR using primers containing a flexible part as illustrated in the inset, and a constant part, either homologous to *hph* gene (in red) or to pUC18 vector (in yellow). With this technic, a one-step cloning with the three different fragments is sufficient to build the deletion cassette. In addition, the "constant part" from primers could be used for both *pvlox1* and *pvlox2* deletion cassettes. It has been reported for *A. niger* that increasing the length of homologous flanks from 100 bp to 1000 bp improved HR efficiency (Meyer *et al,* 2008). Therefore, we decided to use 1 kb long DNA fragment for *P. variabile*.

Appendix 3 - Alignment of proteins PVLOX1 and PVLOX2 highlighting the main LOX features

The alignment was performed with MUSCLE algorithm. The peptide signal is shown in red. Asterisks indicate the five amino acids binding the metal co-factor. Amino acids important for region and stereospecificity are highlighted in purple. The conserved W-X_2-AK motif is shown in green.

Appendix 4 - BIONJ phylogenetic tree of 101 fungal LOX amino acid sequences including the new hypothetical lipoxygenases from *P. variabile* PVLOX1 and PVLOX2.



Appendix 5 – Bioinformatic script used in isolating 101 fungal LOXs from NCBI database (R 3.5.0)

```
#packages
library("rentrez")
library("seqinr")
library("Biostrings")
library("xlsx")

#searching proteins matching criteria in NCBI (retmax must be fixed, otherwise only the first 20 sequences are retrieved)
fungal_lox<-entrez_search("protein",term=("(((((((((lipoxygenase AND fungi [ORGN]) NOT partial[ALL]) NOT hypothetical[ALL]) NOT putative [ALL]) NOT predicted[ALL]) NOT uncharacterized[ALL]) NOT related to [ALL]) NOT unnamed [ALL]) NOT similar to [TITL])"), retmax=300)

#to know how many sequences are found
fungal_lox$count
#> fungal_lox$count
#[1] 179

#compile sequences in a unique fasta file
lox_fasta<-entrez_fetch("protein",id=fungal_lox$ids,rettype = "fasta",retmax=300)
write(lox_fasta, "LOX_strategy1.fasta")

#trim data in a dataframe
fastaFile <- readAAStringSet("LOX_strategy1.fasta")
seq_name = names(fastaFile)
sequence = paste(fastaFile)
df <- data.frame(seq_name, sequence)

#extract protein ID and add ID column to the dataframe
ids<-fungal_lox$id
df$id<-ids
```

```
#add a column indicating last sequence updatedate
lox_sum<-entrez_summary(db="protein",id=fungal_lox$ids,retmax=fungal_lox$count)
updatdate<-extract_from_esummary(lox_sum, "updatedate")
df$update<-updatdate

#add a column with ACCN number
accn<-extract_from_esummary(lox_sum,"extra")
df$identifiants<-accn

#filter sequences
df$WXXAK<-grepl("W..AK",df$sequence)
df$Mstart<-grepl("^M",df$sequence)
df$IVend<-grepl("(I|V)$",df$sequence)
df$H1<-grepl("(H(...|....)H).+(H...(N|S|H))",df$sequence)
df$XP<-grepl("^[^X]",df$seq_name)

#export dataframe to excel
write.xlsx(df, "170lox_strategy1.xlsx")

#get ID of sequences matching all criteria
filter<-df[!rowSums(df=="FALSE"),]
filter$id

# 115 are matching, they are compiled in a new fasta file
lox_fasta_cured<-entrez_fetch("protein",id=filter$id,rettype = "fasta",retmax=200)
write(lox_fasta_cured, "LOXstrategy1_cured.fasta")

#convert in dataframe
fastaFile <- readAAStringSet("LOXstrategy1_cured.fasta")
seq_name = names(fastaFile)
sequence = paste(fastaFile)
df2 <- data.frame(seq_name, sequence)
```

```
#add column with protein ID
df2$id<-filter$id

#add column with update
lox_sum2<-entrez_summary(db="protein",id=filter$id,retmax=200)
updatdate2<-extract_from_esummary(lox_sum2, "updatedate")
df2$update<-updatdate2

#add column with ACCN
accn2<-extract_from_esummary(lox_sum2,"extra")
df2$identifiants<-accn2

#export to excel
write.xlsx(df2, "115_lox_ncbi_strategy1.xlsx")
```

Appendix 6 – Nephelometry principle and examples of application

Nephelometry has been used for a long time in clinical chemistry to determine serum immunoglobulin for example, or in organic chemistry to quantify macromolecules, like monitoring polymerisation reactions (Angersbach *et al.,* 2005). Nephelometry has also been routinely used for counting yeast suspensions and follow the growth of *Saccharomyces cerevisiae* (Peskett, 1927). The first report of the use of nephelometry for the monitoring of filamentous fungi dates back to 2010 (Joubert *et al.,* 2010). This paper describes a tailored procedure to follow the growth of *Alternaria brassicicola*, *Neurospora crassa* and *Aspergillus* sp., and demonstrates the correlation between the RNU (Relative Nephelometric Unit) values registered and the mycelial mass (dry weight). By contrast with spectrophotometry (or turbidimetry), which measures the transmission of light, nephelometry uses light scattering, as illustrated on figure 1. Nephelometry is based on the principle that a suspension of particles will scatter light rather than absorbing it. Particle density is then a function of the light scattered into the detector, placed at 90°. The strongest scattered light signal is obtained when the particles are relatively large. As a consequence, a nephelometric signal usually increases to a maximum and decreases again as a consequence of slowly changing particle size, due to fungal senescence for instance. Nephelometry allows the achievement of reproducible measurements, in contrast with spectrophotometry, which is not adapted to follow the growth of non-homogeneous organisms such as filamentous fungi. It has been proposed for large-scale phenotypic profiling of mutants and high-throughput screening of new natural compounds.

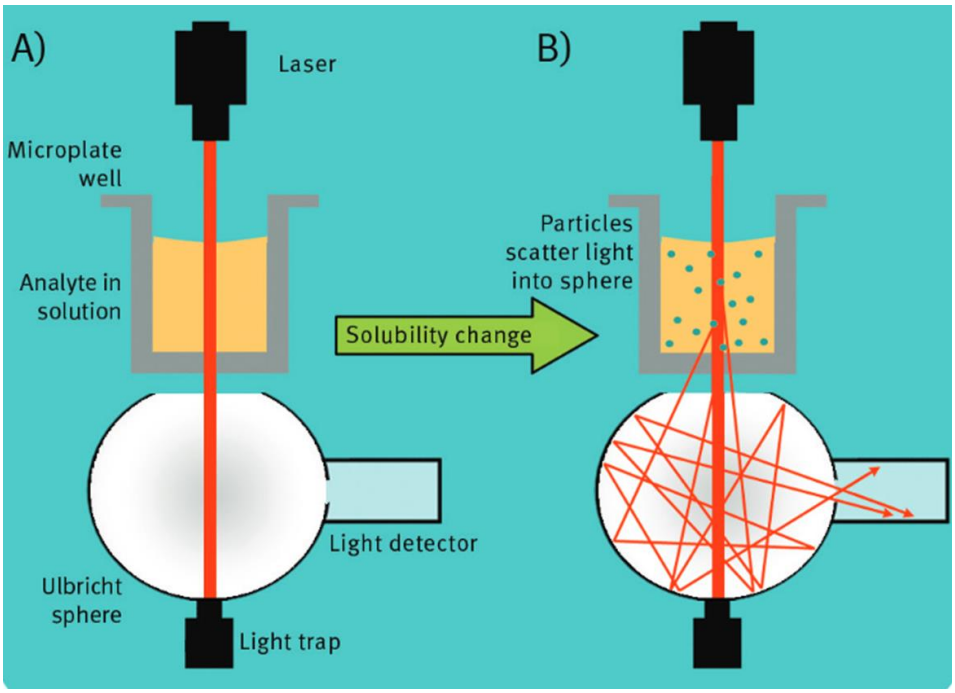


Figure 1: Principle of nephelometry (From BMG LABTECH). **A)** A clear solution with minimal scattering, results in low signal. **B)** A solution with particles scatters light and results in higher signals

In our laboratory, a NepheloStar was recently acquired. I developed a method to monitor the growth of three filamentous fungi used routinely in the laboratory: *Paraconiothyrium variabile*, *Acremonium zeae* and *Fusarium oxysporum*. To determine the reproducibility of method, I used different inoculum and followed the growth of the different fungi in each well using a stereoscopic microscope. This technic proved its worth for the three different species, indicating its high reproducibility. Typical growth curves obtained for *F. oxysporum* are shown, with pictures of the corresponding wells at the end of the experiment (figure 2).

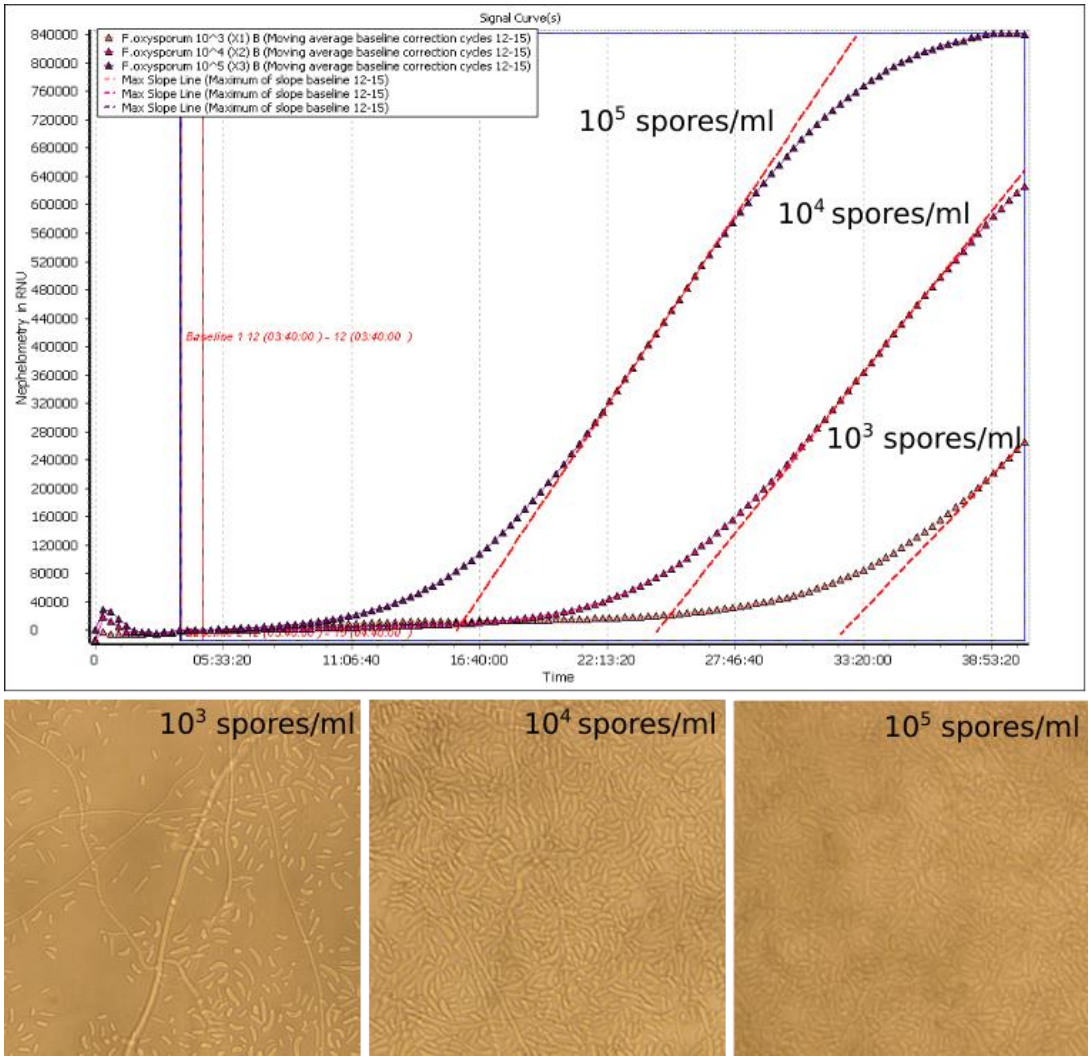


Figure 2: Nephelometric growth curves of *F. oxysporum* with different inoculum concentration. Pictures are taken with a stereoscopic microscope after 40 hours of growth.

Recently, I used nephelometry to assess the cytotoxicity of nine new acetylenic compounds isolated by A. El Demerdash from the fungus *Phaeospheria nodorum* on the growth of *F. oxysporum* and *Botrytis cinerea*. This work will be part of a publication.

Highly Oxygenated Isoprenylated Cyclohexanoids from the Fungus *Stagonospora nodorum* SN15

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ABSTRACT: The chemical investigation of the wheat plant pathogen *Phaeosphaeria nodorum* SN15 afforded Phaeosphaerins A-I (**1-9**), nine previously unreported highly oxygenated acetylenic cyclohexanoids. Their structures and absolute configurations were determined on the basis of spectroscopic analyses, electronic circular dichroism (ECD) as well as GIAO NMR shift calculation followed by DP4 analysis. Some compounds displayed significant herbicidal activities.

Appendix 7 – Participation to scientific congresses

1. Bärenstrauch, M., Nay, B., Prado, S., Mann, S. and Kunz, C. (April 2016). *Oxylipin-mediated regulation of mycotoxins in Fusarium phytopathogen species during the interaction with the endophyte Paraconiothyrium variabile*.
   Poster presentation at the 13th European Congress on fungal genetics (ECFG), Paris, France

2. Bärenstrauch, M., Nay, B., Prado, S., Mann, S. and Kunz, C. (October 2016, Paris) *Role of oxylipins produced during the interaction between the endophytic fungus Paraconiothyrium variabile and the phytopathogen Fusarium oxysporum.* Poster presentation at the GERLI congress "Microbe and host lipids", Toulouse, France

3. Bärenstrauch, M., Nay, B., Prado, S., Mann, S. and Kunz, C. (April 19-20-21, 2017). *Oxylipins at the core of a phytopathogen-endophyte interaction*. Poster presentation at the International Conference on Holobionts, National Museum of Natural History, Paris, France

4. Bärenstrauch, M., Nay, B., Prado, S., Mann, S. and Kunz, C. (May 2017, Paris) *Role of oxylipins produced during the interaction between the endophytic fungus Paraconiothyrium variabile and the phytopathogen Fusarium oxysporum.* Poster presentation at the "Colloque du réseau des laboratoires de microbiologie de Sorbonne Universités-1ère édition- La microbiologie dans tous ses états", Université Pierre et Marie Curie, Paris, France

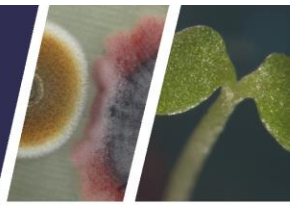
5. Bärenstrauch, M., Nay, B., Prado, S., Mann, S. and Kunz, C. (January 2018). *Mycotoxin beauvericin regulation in a phytopathogen-endophyte interaction.* Oral presentation* at the "12ème rencontres de phytopathologie, Journées Jean Chevaugeon", Aussois, France
   *Awarded best oral presentation

6. Bärenstrauch, M., Nay, B., Prado, S., Mann, S. and Kunz, C. (July 2018). *Down-regulation of the mycotoxin beauvericin in a phytopathogen-endophyte interaction.* Poster presentation at the International Congress of Plant Pathology (ICPP): "Plant health in a global economy", Boston, USA

Other

5th YNHM (Young Natural History Scientists' Meeting), 6-10 March 2018, National Museum of Natural History, Paris*- international congress entirely free dedicated to PhD students with 250 attendees
*member of the organizing committee (management of fundings, communication, logistic)
https://ynhm2018.sciencesconf.org/

Fusarium oxysporum - Paraconiothyrium variabile interaction: a story about beauvericin, oxylipins and plant protection



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Endophytes as beneficial organisms

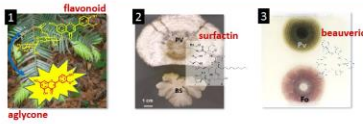
Endophytic fungi are **non-pathogenic microorganisms** involved in **mutualistic associations** with plants.

They have been shown to provide **fitness benefits** to their host plant, ranging from influx of nutrients to protection against phytopathogens. Indeed, they produce many metabolites displaying **fungicidal or antibacterial activity**. Therefore, the endophytic diversity is a trove for new medecines and **biocontrol agents**.

Fig. 1 : Isolation of fungal endophytes from *Cephalotaxus harringtonia*

Studying the cultivable fungal diversity of the conifer *Cephalotaxus harringtonia* (Langenfeld *et al.* 2013), our team identified among 104 MOTUs the Ascomycete, Dothideomycete, ***Paraconiothyrium variabile***.

P. variabile, a resourceful endophyte

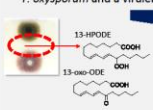


1 **Modifies the plant metabolome to its own benefit**, through the synthesis of aglycone: displays antifungal activities and promotes the growth of the endophyte *(Tian et al, 2014)*

2 **Inactivates surfactins** (membrane disrupters) from *Bacillus subtilis*, by hydrolysis *(Vallet et al, 2017)*

3 **Responsible for beauvericin (BEA) decrease**, a potent mycotoxin from *F. oxysporum* and a virulence factor on plant *(Combès et al, 2012)*

Hypothesis

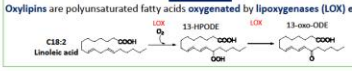


- ✓ In co-culture, the biosynthesis of **two oxylipins (13-HPODE/13-oxo-ODE)** is induced
- ✓ Oxylipins are known to **regulate mycotoxin biosynthesis** in filamentous fungi

Involved here?

Oxylipins?

Oxylipins are polyunsaturated fatty acids **oxygenated** by **lipoxygenases (LOX) enzymes**



OBJECTIVES

1. Characterize the cytotoxicity **of beauvericin (BEA)** for *P. variabile*
   Investigate the **role of oxylipins** in BEA regulation
   → *cytotoxicity assays*
   → *gene regulation*
   → *quantification of beauvericin*
2. Identify lipoxygenases (LOX) in *P. variabile* and study their role in the interaction with *F. oxysporum*
   → *gene regulation*
   → *quantification of oxylipins (13-HPODE)*
3. Explore the protective effect of the endophyte *P. variabile* against *F. oxysporum* on *Arabidopsis thaliana*

Experimental design for part 1 and 2

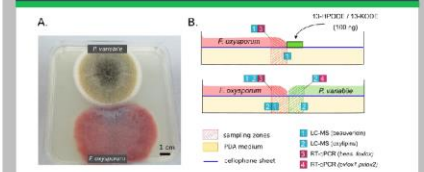


Fig.3: Experimental design illustrating sampling zones in co-culture experiments and in experiments with oxylipin addition
A: Picture of microorganisms just before sampling.
B: Cross-section of Petri dish showing sampling zones and indicating the experiments for which they are intended.

1 Beauvericin cytotoxicity balanced by its degradation

a) BEA cytotoxicity for *P. variabile*

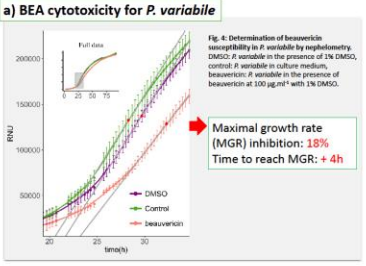


Fig. 4: Determination of beauvericin susceptibility in *P. variabile* by nephelometry.
DMSO: *P. variabile* in the presence of 1% DMSO, control: *P. variabile* in culture medium, beauvericin: *P. variabile* in the presence of beauvericin at 100 µg.mL^{-1} with 1% DMSO.

Maximal growth rate (MGR) inhibition: 18%
Time to reach MGR: + 4h

RESULTS

a) BEA delays growth of *P. variabile* and displays a moderate cytotoxicity

b) *P. variabile* is able to degrade BEA, neutralizing its cytotoxicity

c) BEA synthesis is upregulated in the presence of *P. variabile* and in the presence of oxylipin 13-HPODE

b) BEA degradation by *P. variabile*

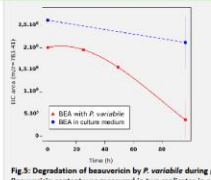


Fig.5: Degradation of beauvericin by *P. variabile* during growth.
Beauvericin content was measured in two replicates in a 96-well plate at different time points in the presence or absence of *P. variabile*.
Values shown are the EIC (Extracted Ion Chromatogram) area for the ion at m/z=783,41, corresponding to beauvericin.

c) BEA transcriptional regulation and synthesis

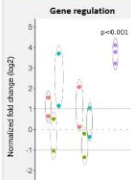


Figure 6: Expression analysis of *bea5* gene from *F. oxysporum* in co-culture or in the presence of 13-HPODE (100 ng) or 13-KODE (100 ng).
Data represent the derived log2 fold changes (mean value ± SD) relative to gene expression in control condition determined by RT-qPCR.

2 Oxylipin signaling in the interaction

a) LOX in *P. variabile*?

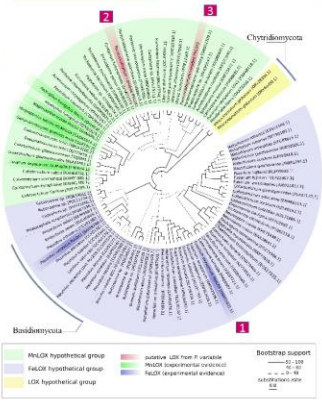


Fig.7: Position of PVLOX1 and PVLOX2 within the lipoxygenases family. BIONJ phylogenetic tree of 101 fungal LOX amino acid sequences supported by 1000 bootstraps. Sequences were aligned with MUSCLE. **The tree includes the new hypothetical lipoxygenases from *P. variabile* PVLOX1 (2) and PVLOX2 (3) , previously identified by BLAST, and the already described FoxLox from *F. oxysporum* (1).**
Basidiomycota and Chitrydiomycota phylum are highlighted, all the other sequences belong to the Asmocycota phylum.

b) LOX transcriptional regulation

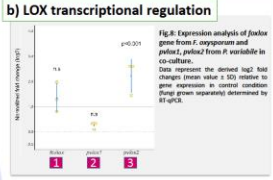


Fig.8: Expression analysis of *foxlox* gene from *F. oxysporum* and *pvlox1, pvlox2* from *P. variabile* in co-culture.
Data represent the derived log2 fold changes (mean value ± SD) relative to gene expression in control condition (fungi grown separately) determined by RT-qPCR.

c) Oxylipins quantification (13-HPODE)

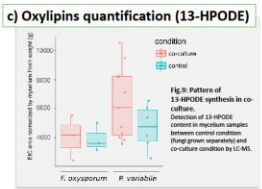


Fig.9: Pattern of 13-HPODE synthesis in co-culture.
Detection of 13-HPODE content in mycelium samples between control condition (fungi grown separately) and co-culture condition by LC-MS.

RESULTS

a) Two putative LOX identified from *P. variabile's* genome

b) Gene *pvlox2* specifically upregulated in the presence of *F. oxysporum*

c) Oxylipin 13-HPODE detected in both microbes, upward trend in the endophyte's mycelium during the interaction

3 *P. variabile* protects *A. thaliana* against phytopathogens

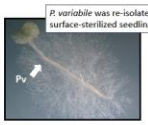
Protective effect of *P. variabile* on seedlings survival after infection by phytopathogens

Fig. 10: A) Phenotype of *Arabidopsis thaliana* seedlings infected by *F. oxysporum (Fo)*, by *P. variabile (Pv)* or by both microorganisms.
B) % of survival among seedlings infected by *P. variabile (Pv)*, *P. variabile and F. oxysporum (Pv-Fo)*, *P. variabile* and *Botrytis cinerea (Pv-Bc)*."Control" refers to un-inoculated plant. % were estimated on day M, when pathogen has killed all seedlings (see "Experimental design").

Experimental design

- Fungi were inoculated either on non-dormant seeds (Day 0) or 4 days after (Day 4)
- Day M is when pathogen has killed 100% of seedlings

P. variabile was re-isolated from surface-sterilized seedlings



RESULTS

P. variabile develops asymptomatically in the plant and protects seedlings from infection by *F. oxysporum* and *B. cinerea*

Conclusion & perspectives

- ✓ Mycotoxin **beauvericin** (BEA) is **upregulated** in the presence of the endophyte *P. variabile (a defense mechanism ?)* as well as in the presence of oxylipins (13-HPODE)
- ✓ Only the **endophyte lipoxygenase PVLOX2 is upregulated** in the interaction, potentially producing the 13-HPODE found in the dual zone
- ✓ *P. variabile* is able to **degrade BEA**, bypassing its cytotoxicity
- ✓ In a tripartite model, *P. variabile* **protects** *A. thaliana* against *F. oxysporum (through BEA degradation?)*

1. Clarify the role of oxylipins by genetic mutation of *lox* genes in both *P. variabile* and *F. oxysporum*
2. Characterize the enzymatic activity of PVLOX1 and PVLOX2 new lipoxygenases (their heterologous expression is under progress)
3. Determine the microscopic localization of *P. variabile* in *A. thaliana* seedlings (FISH)
4. Explore the regulation of BEA in the tripartite interaction involving the endophyte, the phytopathogen and the plant

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RESUME

Les champignons endophytes sont des microorganismes non-pathogènes impliqués dans des associations mutualistes avec les plantes. Les endophytes foliaires, en particulier, représentent un groupe très divers, mais leurs interactions avec la plante hôte et ses micro-organismes associés sont peu connues. Des travaux préliminaires initiés par notre équipe, explorant la diversité microbienne foliaire du conifère *Cephalotaxus harringtonia*, ont permis d'isoler la souche fongique *Paraconiothyrium variabile* (Ascomycota), un antagoniste du phytopathogène *Fusarium oxysporum*. Au cours de leur interaction, on détecte des quantités moindres de beauvéricine, une mycotoxine synthétisée par *F. oxysporum*. En parallèle, on observe une augmentation de la synthèse de deux oxylipines, l'acide 13-hydroperoxyoctadécadiénoïque (13-HPODE) et l'acide 13-oxo-octadécadiénoïque (13-oxo-ODE), dans la zone de confrontation. L'objectif de ce travail était de comprendre les mécanismes conduisant à la diminution de la beauvéricine au cours de l'interaction et d'explorer le rôle des oxylipines dans la régulation de cette dernière. Dans mon travail de thèse, je montre la présence de deux gènes *lox* chez *P. variabile* (*pvlox1* et *pvlox2*) codant tous deux pour des manganèse lipoxygénases, potentiellement à l'origine des acides 13-HPODE et 13-oxo-ODE. *Pvlox2* est spécifiquement induit pendant l'interaction, et ces résultats sont corroborés par une synthèse accrue de 13-HPODE chez *P. variabile*. Par ailleurs, l'interaction avec l'endophyte, ainsi que l'ajout de l'oxylipine 13-HPODE, régulent positivement la voie de biosynthèse de la beauvéricine, comme l'indiquent les teneurs plus élevées en mycotoxines observées chez *F. oxysporum*. Enfin, nous avons montré que la beauvéricine inhibait la croissance de l'endophyte, mais que ce dernier était capable de dégrader la mycotoxine, expliquant ainsi les faibles quantités de beauvericine observées initialement dans la zone de compétition. Ce travail contribue à la compréhension du rôle des oxylipines dans la communication inter-microbienne.

ABSTRACT

Endophytic fungi are non-pathogenic microorganisms involved in mutualistic associations with their host. Foliar endophytes, in particular, represent a very diverse group but little is known about their interactions with the host and its associated micro-organisms. In preliminary work, exploring the leaf microbial diversity of the conifer *Cephalotaxus harringtonia,* our team isolated the fungal strain *Paraconiothyrium variabile* (Ascomycota), an antagonist of the phytopathogen *Fusarium oxysporum*. During their interaction, decreased amounts of the *F. oxysporum* mycotoxin beauvericin, and higher amounts of the two oxylipins, 13-hydroperoxyoctadecadienoic acid (13-HPODE) and 13-oxo-octadecadienoic acid (13-oxo-ODE), were observed in the confrontation zone. The objective of the present work was to understand the mechanisms leading to beauvericin decrease during the interaction and to explore the role of oxylipins in beauvericin regulation. In my thesis work I show the presence of two *lox* genes in *P. variabile* (*pvlox1* and *pvlox2*) coding both for manganese lipoxygenases, potentially at the origin of 13-HPODE and 13-oxo-ODE. *Pvlox2* is specifically induced during the interaction, which lead to an increased synthesis of 13-HPODE in *P. variabile*. The endophyte itself, as well as the oxylipin 13-HPODE, up-regulated the beauvericin biosynthesis gene *beas,* which was paralleled by higher mycotoxin content in the mycelium of *F. oxysporum*. Finally, we showed that beauvericin inhibited the endophyte's growth, but the latter was capable to degrade the mycotoxin, which explains the lower amounts of beauvericin found in the competition zone. This work presents pioneer undertaking to elucidate the role of oxylipins in inter-microbial crosstalk.