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Impact of biotic and abiotic factors on the expression of fungal effector-encoding genes in axenic growth conditions

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ABSTRACT

In phytopathogenic fungi, the expression of hundreds of small secreted protein (SSP)-encoding genes is induced upon primary infection of plants while no or a low level of expression is observed during vegetative growth. In some species such as *Leptosphaeria maculans*, this coordinated *in-planta* upregulation of SSP-encoding genes expression relies on an epigenetic control but the signals triggering gene expression *in-planta* are unknown. In the present study, biotic and abiotic factors that may relieve suppression of SSP-encoding gene expression during axenic growth of *L. maculans* were investigated. Some abiotic factors (temperature, pH) could have a limited effect on SSP gene expression. In contrast, two types of cellular stresses induced by antibiotics (cycloheximide, phleomycin) activated strongly the transcription of SSP genes. A transcriptomic analysis to cycloheximide exposure revealed that biological processes such as ribosome biosynthesis and rRNA processing were induced whereas important metabolic pathways such as glycogen and nitrogen metabolism, glycolysis and tricarboxylic acid cycle activity were down-regulated. A quantitatively different expression of SSP-encoding genes compared to plant infection was also detected. Interestingly, the same physico-chemical parameters as those identified here for *L. maculans* effectors were identified to regulate positively or negatively the expression of bacterial effectors. This suggests that apoplastic phytopathogens may react to similar physiological parameters for regulation of their effector genes.

1. Introduction

Phytopathogenic fungi are responsible for huge crop losses worldwide. To infect successfully their host plant, fungi have developed diverse strategies to escape plant innate immunity and subvert host plant metabolism in order to enable the efficient uptake and utilization of host-derived nutrients. These strategies are based on the use of a cocktail of molecules named effectors that, in a broad sense, are defined as proteins and small molecules that can alter the structure and function of host cell (Hogenhout et al., 2009). Genome analyses of fungal phytopathogens have revealed that these pathogens have complex and diversified secretomes composed of hundreds of secreted proteins. Among them, several protein classes play important roles as effectors in pathogenicity [e.g. enzymes for secondary metabolite production, phytotoxins, carbohydrate-active enzymes, peptidases, lipases, and small secreted proteins (SSPs)]. Large variations in the gene number of these different protein classes exist between the phytopathogenic fungi and could be correlated to the various pathogenic strategies adopted by fungi. For example, genomes of necrotrophs like *Botrytis cinerea* are enriched in genes encoding degrading enzymes such as protein and carbohydrate hydrolases along with non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) which encode enzymes involved in biosynthesis of secondary metabolites including non-specific metabolic toxins. In contrast, very few SSPs are produced, the main exception being cell-killing proteinaceous toxins (Amselem et al., 2011; Guyon et al., 2014). In biotrophs such as rust fungi, species differentiation towards pathogenicity has been accompanied by loss of numerous genes involved in nutrient acquisition, but expansion and diversification of effector genes encoding SSPs (Duplessis et al., 2011). For hemibiotrophs that display various modes of colonization of tissues including an early biotrophic stage

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at the beginning of infection, followed by a later necrotrophic stage, families of genes encoding peptidases, secondary metabolites enzymes, pectin-degrading enzymes and SSPs are expanded (Ohm et al., 2012). Genome-wide expression profiling has revealed that the expression of these pathogenicity genes is tightly regulated during infection. Different gene classes are coordinately transcribed in successive waves that are linked to pathogenic transitions of hemibiotrophs (O'Connell et al., 2012; Gervais et al., 2016).

Leptosphaeria maculans is a hemibiotroph with an unusually complex parasitic cycle tightly associated to that of its host plant *Brassica napus* (oilseed rape) (Rouxel and Balesdent, 2005). The fungus survives off season as a saprobe on stem residues of harvested plant. Sexual mating occurs at this stage resulting in the production of ascospores that are released and widely dispersed by wind. Ascopores germinate on leaf and host infection is initiated when growing hyphae penetrate inside the leaf via wounds and natural apertures such as stomata. At this stage, *L. maculans* colonizes the tissues as a biotroph, and grows exclusively within the apoplast. As it is the case for many other biotrophs or hemibiotrophs (Hacquard et al., 2012; Cantu et al., 2013; Dong et al., 2015; Mirzadi Gohari et al., 2015; Dobon et al., 2016), this initial step of tissue colonization is accompanied by a wave of concerted expression of effector genes encoding SSPs taking place before the fungus switches to necrotrophy (Rouxel et al., 2011).

The *L. maculans* genome has an unusual bipartite structure with guanine and cytosine (GC)-equilibrated regions alternating with adenine and thymine (AT)-rich isochores which contain mosaics of transposable elements (TE) mutated by repeat-induced point mutation process (Rouxel et al., 2011). Only 5% of the genes are encountered in AT-rich isochores which cover 36% of the genome. Bioinformatic analysis of protein sequences has enabled to predict 651 SSPs in the secretome of this fungus. Twenty percent of the genes located in AT-isochores encode putative SSPs whereas 4.2% of the genes in the GC-isochores encode SSPs. In addition to SSPs, AT-isochores are also enriched in genes involved in response to chemical and biotic stimuli as well as PKSs and NRPSs. Similar features were found for other filamentous fungi with complex genomes in which specific compartments, usually TE-rich, are also enriched in genes involved in niche adaptation such as effectors (Spanu et al., 2010; Ma et al., 2010). This genome location has a major consequence on regulation of expression of effector genes. AT-isochores are heterochromatic and the gene location results in repression of the expression of effector genes located in AT-isochores during vegetative growth of the fungus (Soyer et al., 2014). In contrast, during the first days of plant tissue colonization, microarray analyses indicated that 72.7% of the SSP-encoding genes located within AT-isochores (compared with 19.1–22.2% in GC-isochores) are over-expressed compared to *in vitro* mycelium growth. The repression of expression of effector genes in axenic conditions seems to be a general trait in filamentous phytopathogens (Lo Presti et al., 2015), and there is currently no information on the environmental or plant-derived signals produced by leaves that could induce effector gene expression. During the systematic analysis of T-DNA tags in our collection of 5000 *L. maculans* agrotransformants (Bourras et al., 2012), one T-DNA insertion was found in the promoter of a gene (*hyp1*) predicted to encode a hypothetical protein harboring a reverse transcriptase-like domain. Preliminary *in vitro* and *in vivo* analyses indicated that this integration event led not only to an overexpression of this gene but also to a deregulation of expression of genes encoding effectors like *AvrLm4-7* and *AvrLm6* (S. Bourras and M. Meyer, unpublished data). Recently, an orthologue of *hyp1* present in *Neurospora crassa* was found to be strongly induced when protein synthesis was inhibited by the addition of antibiotics (cycloheximide, blasticidin) to the exponentially growing mycelium (Gladyshev and Arkhipova, 2011).This prompted us to investigate whether these antibiotics could also have an effect on the expression of *L. maculans* effectors. In addition, to propose a first evaluation of environmental signals that may be involved in derepression of expression of effector genes located within AT-isochores, we investigate here whether selected biotic and abiotic factors (including antibiotics) can influence the expression of *L. maculans* effector genes in axenic growth condition.

2. Materials and methods

2.1. Reagents

Cycloheximide, anisomycin, and puromycin were purchased from Sigma-Aldrich, Saint-Quentin Fallavier, France; hygromycin B was from Invitrogen, Cergy Pontoise, France; nourseothricin was from Werner BioAgents, Jena, Germany; blasticidin, phleomycin, and zeomycin were from Cayla, Toulouse, France.

2.2. Fungal cultures and growth assays

The general conditions used during our experiments were the following: 10^8 conidia of the v23.1.3 isolate of *L. maculans* (Balesdent et al., 2002) were added to 50 ml of minimal medium II (20 g of glucose, 2 g of $NaNO_3$, 0.1 g KH_2PO_4, 0.05 g of $MgSO_4$, 0.05 g KCl, and 0.0001 g of $FeSO_4$, $7H_2O$ per liter) or Fries medium (30 g of sucrose, 5 g of $C_4H_{12}N_2O_6$, 5 g of yeast extract, 1 g of NH_4NO_3, 1 g KH_2PO_4, 0.13 g of $CaCl_2$, 0.1 g NaCl, and 0.05 g $MgSO_4$ per liter) in flasks incubated at 28 °C and shaked at 100 rpm. After 3 to 4 days of growth, the mycelium was harvested by filtration through sterile miracloth, dried, and immediately immersed in liquid nitrogen. When MMII medium was supplemented with different antibiotics at final concentrations of 0.1, 1 and 10 μg/ml, the culture was incubated for a further 3 h. A time-course experiment was also performed by collecting fungal mycelium grown in MMII medium at 5, 10, 20, 30, 60, 120, 180 min after the addition of cycloheximide at 1 μg/ml. Conidia were also grown in modified MMII medium (i) whose pH was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 by the addition of 10 N NaOH; (ii) containing NH_4^+ at 1, 5, 20, 50 and 100 mM as unique nitrogen source; (iii) containing glucose, fructose, sodium acetate, sodium succinate, sodium propionate and sodium citrate at 10 mM as unique carbon source. For this later condition, the dry weight of mycelium collected was also measured. Finally, conidia were incubated in MMII medium at five different temperatures (13 °C, 16 °C, 19 °C, 22 °C, 25 °C) but due to slower growth, mycelium was collected after 6 and 5 days of incubation at 13 °C and 16 °C, respectively.

On agar media, growth assays were performed by depositing 10 μl of 10^7/ml pynidiospores suspension at the center of 55 mm-Petri dishes containing agar MMII medium. Antibiotics were added to solid MMII medium at final concentrations of 0.1, 1 and 10 μg/ml. Radial growth of *L. maculans* was measured after incubation at 25 °C for two weeks.

2.3. cDNA synthesis, PCR amplification, and real-time RT-PCR analysis

Total RNA was extracted from freeze-dried mycelium by using TRIzol reagent (Invitrogen, Cergy Pontoise, France). Extracts were treated with DNAseI (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Three micrograms of total RNA were reverse transcribed using an oligo-$dT_{(23)}$ anchor (Sigma-Aldrich, St. Louis) and 100 units of PowerScript Reverse Transcriptase (Ozyme, St-Germain-en-Laye, France) in a final volume of 20 μl. One microliter of RT reaction was used for PCR amplification in the final volume of 10 μl using specific primer pairs (Supplemental Table S1). PCR conditions for *AvrLm1*, *AvrLm4-7*, *AvrLm6*, *Lema086540* and *Lemau086550* were as follows: 1 cycle