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# Epigenetic regulation of fungal genes involved in plant colonization

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## ABSTRACT

Plant pathogenic fungi have a major impact on agriculture and human health and remarkable abilities to adapt to new conditions. Their adaptability relies, at least in part, on the fine-tune expression of genes involved in plant colonization. The massive sequencing of fungal genomes associated with transcriptomic data during plant infection highlighted that genes involved in plant colonization: i) exhibited sophisticated waves of expression and ii) were often enriched in particular regions of the fungal genomes (including repeat-rich regions, accessory chromosomes or sub-telomeric regions, heterochromatin). Their specific location suggests that an epigenetic control might be involved in the regulation of their expression. In this chapter, we provide a historical perspective on the tremendous work accumulated on chromatin organization and remodeling, and on recent data on genomes and epigenomes of plant-interacting fungi, which have refined our understanding of the impact of chromatin structure on the regulation of genes involved in host colonization. Then, we highlight how functional analyses have supported hypothesis of a chromatin-based control, associated to the action of specific transcription factor(s), of plant-associated genes. We conclude by outlining the next challenges to be addressed concerning the epigenetic regulation of fungal genes involved in plant colonization.

**Keywords:** plant-interacting fungi, effectors, chromatin, epigenetic, fungal epigenome, regulation, transcription factors

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## 1. Introduction

Plant pathogenic fungi have a major impact on agriculture and human health and show a remarkable ability to adapt rapidly to new environmental conditions (Fisher et al. 2018). They occupy most ecological niches, present a great diversity of lifestyle, nutritional strategies or interaction with their host. Their success in infecting a host plant, adapting to new environmental conditions or to new hosts depends, at least in part, on their ability to fine-tune the expression of pathogenicity genes and to dynamically regulate their transcriptional profiles. During plant colonization, plant-associated fungi secrete molecules, commonly called effectors, that can modulate plant immunity and promote colonization (Sánchez-Vallet et al. 2018; Rocafort et al. 2020). In plants carrying major resistance (R) genes, some of these effectors can be specifically recognized and are then called avirulence (AVR) proteins. Effectors initially referred to (small) secreted proteins ((S)SPs) but some secondary metabolites and small RNAs (siRNA) were also found to play a role of effectors (Weiberg et al. 2013; Wang et al. 2015; Sánchez-Vallet et al. 2018; Collemare et al. 2019). The massive sequencing of fungal genomes with a growing assembly quality associated with transcriptomic data during plant infection highlighted that effector genes: i) exhibited complex waves of expression during host colonization and ii) were enriched in particular regions of the fungal genomes (repeat-rich regions, accessory chromosomes, sub-telomeric regions). The location of effector genes in particular genomic regions suggested that an epigenetic control, through chromatin remodeling, might be involved in the regulation of their expression. Use of the term 'epigenetic' - literally meaning 'above genetic' - is still controversial and represents a "semantic morass" (Lederberg 2001). This term was initially proposed by Waddington (1942, 1957) to describe interactions between the environment and the genes leading to the development of a new phenotype, with no involvement of the underlying DNA sequence.

In this chapter, we provide a historical overview of the deciphering of chromatin organization, and the accumulation of data regarding genomes and epigenomes of plant-associated fungi. Pioneer analyses performed in the model fungus *Neurospora crassa* helped scientists working on plant-related fungi building hypotheses on the epigenetic control of plant colonization. Here, our ambition is to reconcile data gathered on *N. crassa* with the latest knowledge obtained from -omic analyses on plant-associated fungi (genomic, transcriptomic, epigenomic) to set the ground on how plant-related genes are regulated. Then, we report on the functional analyses that support hypothesis of an epigenetic control of plant-associated genes and highlight the next challenges to be tackled concerning our understanding of this regulation.

## 2. Current knowledge on fungal chromatin organization and key actors involved in chromatin remodeling

### 2.1 Historical overview of the chromosome organization

Within eukaryotic nucleus, chromatin folding from the DNA fiber to the chromosome territories represents intertwined levels of compactations of which each level is regulated and each scale is an important regulator of physiological processes. The two centuries between Brown's discovery of the nucleus in 1831 (cited in Jost et al. 2012) and the decoding of the human genome in 2001 (International Human Genome Consortium 2001) have led to an accumulation of knowledge about the composition of the nucleus and its role. Nucleic acids constituting the substrate of the genetic code were first isolated by Friedrich Miescher in 1869 (cited in Jost et al. 2012). With advances in staining and cytological techniques, Walther Flemming was able to visualize a highly organized fibrous structure within eukaryotic nuclei in 1879, which he referred to as chromatin. The DNA molecule itself is static whereas the chromatin fiber is a highly dynamic structure. Beyond its role in maintaining the physical compaction of the DNA molecule, chromatin forms a platform where the main cellular functions are regulated: replication, transcription, chromosome segregation or DNA repair. Chromatin consists of a complex made up by DNA and proteins. The nucleosome is the fundamental unit of chromatin,

composed of histones, described by Kossel in 1884 (cited by Jost et al. 2012) and represents the brick supporting the information carried by DNA. It was Kornberg, in 1974, who first postulated that chromatin was composed of a portion of DNA of about 200 bp wrapped around the "core" nucleosome, composed of an octamer of four histones (two H3-H4 and two H2A-H2B) giving it the appearance of a "pearl necklace". In the nucleus, the chromatin fiber undergoes several levels of compaction and folding until it forms the chromosome (according to the term proposed by Waldeyer in 1888).

On the basis of all this accumulated knowledge, it is undeniable that the regulation of gene expression is a multifactorial process and relies on intertwined factors. Gene transcription relies on the binding of one (or more) transcription factor(s) (TF) to a cis-regulatory element in a given chromatin context. The first layer governing gene expression relies on the accessibility of TFs to promoter sequences. This accessibility is influenced by the post-translational modifications targeting histone proteins of the chromatin. These modifications define at least two distinct condensation states of the chromatin, firstly identified based on cytological studies: euchromatin, a relaxed structure, permissive for gene expression, and heterochromatin, highly condensed and repressive for gene expression (Heitz 1928; Jenuwein and Allis 2001; Huisinga et al. 2006; Grewal and Jia 2007). Heterochromatin constitutes the highly condensed fraction of the genome and, in contrast to euchromatin, is gene-poor, has a low rate of recombination and is usually repeat-rich (Richards and Elgin 2002; Huisinga et al. 2006; Grewal and Jia 2007). Two forms of heterochromatin can be distinguished: constitutive and facultative heterochromatin (Craig 2005). The former is a permanent structure, mainly found in centromeres and telomeres, to ensure chromosome segregation and genome stability by preventing transposition of repetitive sequences, whereas facultative heterochromatin is a dynamic and regulated structure, involved in regulation of gene expression. These two types of chromatin states are also distinguished by molecular "signatures", conserved from *Schizosaccharomyces pombe* to humans, which are the presence of proteins and post-translational modifications targeting the DNA or histone tails that protrude from nucleosomes. These modifications alter the chromatin folding and thus accessibility of the genetic information stored therein (Luger and Richmond 1998). Since the work of Allfrey (1964) who first described histone modifications and their association with the regulation of gene expression, many different types of histone modifications have been described. These modifications are referred to as the "histone code" because the state of chromatin condensation is not conditioned by an isolated type of modification but rather by a density and local combination of modifications (Jenuwein and Allis 2001). According to Jenuwein and Allis, the histone code "extends the information potential of the genetic code" and determines the two opposite states of chromatin.

At least eight post-translational modifications can affect histone tails, i.e. acetylation, phosphorylation, methylation, de-amination, ADP ribosylation, ubiquitylation, sumoylation, isomerization (see for review (Kouzarides 2007; Bannister and Kouzarides 2011)) and thus influence gene expression. These modifications were initially highlighted by the use of specific antibodies, by mass spectrometry approach and more recently, the development of the Chromatin ImmunoPrecipitation (ChIP) technique that has made it possible to map and characterize more precisely these modifications, their specificity and associated function (Solomon et al. 1988; Ren et al. 2000; Johnson et al. 2007; Park 2009).

The location of nucleosomes along the chromatin also influence gene expression by altering the accessibility of promoters or regulatory sequences to TFs or histone modifying enzymes (HMEs; Radman-Livaja and Rando 2010; Struhl and Segal 2013). Nucleosome positioning and occupancy are determined by a combination of DNA sequence features, TFs, chromatin remodelers and histones modifiers (Singh and Mueller-Planitz 2021). Genome-wide maps of nucleosome occupancy and positioning have only been developed in a few Hemiascomycota yeast species, including *Saccharomyces cerevisiae* (Yuan et al. 2005; Struhl and Segal 2013), in the ascomycete *Aspergillus fumigatus* (Nishida et al. 2009), the basidiomycete *Mixia osmundae* (Nishida et al. 2012) and recently in a few plant-pathogenic fungi (*Leptosphaeria maculans* 'brassicae', *Leptosphaeria maculans* 'lepidii', *Botrytis cinerea*, *Fusarium graminearum* and *Verticillium verticillioides*; Cook et al., 2020; Clairet et al.,

2021a). These results showed that nucleosomes are generally depleted in promoters, enhancers and terminators of transcriptionally active genes indicating nucleosomal control of gene expression and that the nucleosomal DNA length distribution was similar in *M. osmundae*, *A. fumigatus*, *B. cinerea* and *F. graminearum* but differed from that of hemiascomycetous yeasts. *L. maculans* 'brassicae' and *L. maculans* 'lepidii' distinguished themselves with shorter nucleosomal DNA length distribution, suggesting a narrower chromatin fiber structure.

Within the nucleus, chromosomes are not randomly organized but display specific spatial localization in interphase as first observed in animal cells by Rabl in 1885. This territorial organization was referred to as "chromosome territories" (CT) by Theodor Boveri (1909). While histone modifications have long been known to influence chromatin compaction and thereby gene expression, our understanding of the role of the 3D organization of the chromosomes has dramatically changed over the last ten years. Long distance contacts occur in chromatin loops bringing together the cis-regulators and the target genes. A central role of chromatin loops and long-distance chromosomal interactions has been recognized as important for several physiological processes (replication, DNA repair, transcription...; see for review (Bonev and Cavalli 2016)). In terms of gene regulation, CTs are also dynamic and genes expressed together can be closely located within these territories although located on different chromosomes (Cremer and Cremer 2010). Hence CT represent an important template for gene regulation (Duan et al. 2010).

## **2.2 Key actors ensuring the equilibrium between heterochromatin and euchromatin**

Although many interconnected levels are involved in chromatin organization and regulation of cellular processes, so far, in plant-pathogenic fungi, mainly analysis of DNA methylation and post-translational modifications (PTMs) of histones have attracted attention. Indeed, chromatin state has a direct, and quantifiable impact on gene expression, and can be monitored in different cell types or growth conditions. HMEs catalyze deposition or removal of any covalent histone modifications (Kouzarides 2007). Different actors are involved in remodeling the chromatin structure. This encompasses "writer" proteins, such as the best-described histone acetyltransferases (HATs), methyltransferases (HMT), deacetylases (HDACs) or demethylases, which consist in enzymes directly responsible for the deposition or removal of histone or DNA modifications. They act together with "reader" proteins (such as the heterochromatin protein 1, HP1, or the Polycomb proteins) which recognize specific modifications after their deposition and subsequently recruit other enzymes to define the local chromatin state (see for review (Musselman et al. 2012; Lalonde et al. 2014)). Among HMTs, the SET-domain (Su(var)3-9, Enhancer of zeste, Trithorax) family is specific for methylation of lysine residues (histone methyltransferases, KMTs). Apart from DNA methylation, associated with heterochromatin, different histone modifications serve as markers of the chromatin state. Heterochromatin is characterized by the presence of histone hypo-acetylation, methylation at lysine 9 of histone H3 (H3K9me) or lysine 27 of histone H3 (H3K27me) and the presence of HP1. In filamentous fungi, heterochromatin assembly and maintenance are well deciphered in *N. crassa* (Tamaru and Selker 2001; Kouzminova and Selker 2001; Tamaru et al. 2003; Freitag et al. 2004a, b; Honda and Selker 2008; Lewis et al. 2009; Honda et al. 2010; Jamieson et al. 2013; Jamieson et al. 2016). In this fungus, KMT1 (also called DIM5 or ClrD) is responsible for H3K9me3 deposition (Tamaru and Selker 2001; Tamaru et al. 2003) at domains of constitutive heterochromatin; this modification is then specifically recognized by the chromodomain of HP1 which in turn recruits the DNA-methyltransferase DIM-2 via its chromo-shadow domain (Honda and Selker 2008). Recruitment of DIM-2 catalyzes DNA methylation, hereby promoting and stabilizing heterochromatin. Although involved in maintenance of constitutive heterochromatin, HP1 is also important for H3K27me3 maintenance in domains of facultative heterochromatin (Basenko et al. 2015; Jamieson et al. 2016). The histone lysine methyltransferase KMT6 (also called EzhB) is part of the Polycomb Repressive Complex 2 (PRC2) and catalyzes H3K27 methylation (Jamieson et al. 2013) at facultative, gene-rich heterochromatin domains. In *N. crassa*, DNA methylation and DIM-2 are not essential for heterochromatin assembly contrary to KMT1 and

HP1 (Foss et al. 1993; Kouzminova and Selker 2001; Tamaru and Selker 2001; Honda et al. 2012). This might explain why DNA methylation has not been found conserved widely in filamentous fungi (Bewick et al. 2019). In *N. crassa*, action of HDACs is also paramount to this process as inactivation of had-1 induces a loss of DNA methylation but is also associated with an increase of H3K9ac which jeopardizes methylation of this lysine (Selker 1998). Hence, de-acetylation of H3K9 seems to be a pre-requisite to DNA methylation, H3K9 methylation and heterochromatin assembly (Smith et al. 2010). Contrary to histone acetylation, histone methylations have been originally considered as irreversible for the following reasons: i) the C-N bond is more stable than the amine bond established during acetylation, ii) the half-life of the methyl mark is equal to that of the histones (Byvoet et al. 1972). However, recent biochemical analyses in model organisms have allowed identifying histone demethylases, describing their specificity and showed that they are key actors of transcription regulation (see for review (Kooistra and Helin 2012)).

### **3. Contribution of omics data to our understanding of the genomic, epigenomic and transcriptional context of fungal genes involved in plant colonization**

#### **3.1 Fungal genomes are as puzzling as the fungal kingdom**

Fungal species are important for human activities (as benefits they bring for scientific research or industry), explaining that fungi represent the kingdom for which the largest number of genomes are sequenced, aiming at solving their inner puzzling nature. The genome of the yeast *S. cerevisiae* was the first genome of a eukaryotic organism to be sequenced (Goffeau et al. 1996), followed six years later by that of another yeast, *S. pombe* (Wood et al. 2002). This data paved the way to the first functional analyses of genes from eukaryotes. This was followed by the publication of the first genomes of Ascomycete fungi (*N. crassa* and *Aspergillus sp.*; Galagan et al. 2003; 2005; Machida et al. 2005) and of *Ustilago maydis*, a plant pathogenic basidiomycete (Kämper et al. 2006). The sequencing of fungal genomes has been accelerating with the advent of high-throughput sequencing technologies, leading to an exponential accumulation of genomic data from plant-associated fungi, of any lifestyle. To date, more than 10 thousand fungal genomes are available on the NCBI, including sequencing of several strains within the same species and re-sequencing data using different technologies. While the first genome analyses focused on one genome, the increasing data available opened the possibility to lead comparative genomic studies within closely related species (e.g. for the *Leptosphaeria* species complex, *Zymoseptoria sp.*, *Epichloe sp.* or *Ustilago sp.*; Stukenbrock et al. 2011; Laurie et al. 2012; Grandaubert et al. 2014; Treindl et al. 2021) to outstanding comparative analyses of fungal genomes from multiple lineages and host-interaction modes (e.g. Miyauchi et al. 2020). The size of fungal genomes ranges from less than 10 Mb (for the Basidiomycete *Wallemia sebi*, 9.82 Mb; Padamsee et al. 2012) to several hundreds of Mb (e.g. for the mycorrhizal fungus *Gigaspora rosea*, 567 Mb or for *Phakopsora pachyrhizi* 1057,43Mb; <https://mycocosm.igj.doe.gov/Phakopsora/Phakopsora.info.html>; Miyauchi et al. 2020). Finally, complete genome assemblies were recently obtained with the advent of third-generation sequencing strategies, e.g. for *F. graminearum*, *Verticillium dahliae*, *Colletotrichum higginsianum*, *B. cinerea*, *Epichloe sp.* (Faino et al. 2015; King et al. 2015; Dallery et al. 2017; Van Kan et al. 2017; Treindl et al. 2021). These re-sequencing allowed complete genome assemblies including telomeric / sub-telomeric sequences, shedding light on previously unidentified Transposable Element (TE) sequences - which could have been under-estimated by previous sequencing strategies (e.g. in the *C. higginsianum* genome, 7% of TE in the new assembly vs. 1.2% in the first assembly; or for *L. biglobosa* 'brassicae', with 17.8% of TE in the new assembly vs. 2% in the first assembly; Dallery et al. 2017; Dutreux et al. 2018)-, or TE-rich accessory chromosomes. Besides representing a major driver of rapid evolution of effector genes making it difficult to identify long-lasting and efficient disease control methods (see for review (Rouxel and Balesdent 2017; Möller and Stukenbrock 2017)), TEs have also been shown to play a crucial role for genome architecture and expansion of genome size. TEs are often

found to be organized in clusters, compartmentalizing the genome into gene-rich regions and TE-rich regions (e.g. in *L. maculans*, *Mycosphaerella fijiensis*, *Epichloe festucae*; Rouxel et al. 2011; Ohm et al. 2012; Grandaubert et al. 2014; Winter et al. 2018). On the opposite, some large genomes, although characterized by an expansion of TEs (e.g. *Melampsora larici-populina*, having 45% of TEs, or *Blumeria graminis*, having 75% of TEs) do not present a specific location of these TEs, which are homogeneously scattered throughout the genome (e.g. in Spanu et al. 2010; Duplessis et al. 2011a). An extreme feature of fungi, although not limited to this kingdom, is the prevalence of accessory chromosomes (also called B chromosomes or dispensable chromosomes) in their genomes (see for review (Galazka and Freitag 2014; Soyer et al. 2018)). Some species harbor TE-rich accessory chromosome(s) (e.g. *L. maculans*, *Zymoseptoria tritici*, *Fusarium oxysporum*, *Fusarium poae*, *Magnaporthe oryzae*; Ma et al., 2010; Rouxel et al. 2011; Balesdent et al. 2013; Dhillon et al. 2014; Grandaubert et al. 2015; Vanheule et al. 2016; Peng et al. 2019). However, there is a cost associated with the maintenance of TE-rich regions and Eukaryotes set up different strategies to limit the spread of TEs within genomes, including their packaging in heterochromatin regions (Hollister and Gaut 2009).

### 3.2 Organization of the epigenomic landscape in plant-interacting fungi

So far, most genome-wide descriptions of the epigenomic landscapes in plant-interacting fungi have been performed using Chromatin Immunoprecipitation followed by high-throughput sequencing (ChIP-seq). This technique allows precise location, throughout the genome, of histone modifications associated with euchromatin or heterochromatin (Johnson et al. 2007; Park et al. 2009; Soyer et al. 2015a). The set-up of this technique has proven challenging, even during axenic growth, explaining that genome-wide studies of the chromatin structure in fungi are still sparse. ChIP-seq analyses were so far focused mostly on location of the euchromatin modification H3K4me2/3 and of the heterochromatin modifications H3K9me3 and H3K27me3, as for example in the two phytopathogenic fungi *Z. tritici* and *L. maculans*, under axenic conditions (Schotanus et al. 2015; Soyer et al. 2021). These analyses, together with numerous others, have allowed to draw generic characteristics of the organization of the epigenome in fungi, and corresponding to the knowledge gathered previously in the model ascomycete *N. crassa*. However, they have also highlighted the fact that each fungus can display specificities in its epigenomic organization. The most consistent feature regarding the distribution of these hallmarks is that domains enriched in H3K4me2/3 and domains enriched in H3K9me3 are mutually exclusive in the genomes. This was shown for instance in *N. crassa*, *Fusarium fujikuroi*, *Z. tritici* and *L. maculans* (Smith et al. 2008; Jamieson et al. 2013; Wiemann et al. 2013; Schotanus et al. 2015; Soyer et al. 2021). So far, and as in *N. crassa*, TE-rich regions have always been found associated with heterochromatin (e.g. in *Z. tritici*, *F. fujikuroi*, *L. maculans*; Wiemann et al. 2013; Schotanus et al. 2015; Soyer et al. 2021). Centromeres are enriched with H3K9me2/3 for *N. crassa*, *F. graminearum*, *V. dahliae*, *F. fujikuroi*, *Z. tritici* (Smith et al. 2011; Wiemann et al. 2013; Schotanus et al. 2015; Seidl et al. 2020) and are usually devoid of coding sequences, except for *Z. tritici* (Schotanus et al. 2015). Sub-telomeric regions are often found associated with both H3K9me3 and H3K27me3, overlapping over repetitive sequences, which is a classic feature observed also in *N. crassa*, *Z. tritici* or *L. maculans* (Smith et al. 2008; Jamieson et al. 2013; Schotanus et al. 2015; Soyer et al. 2021). Only H3K27me3 has been shown to locate in sub-telomeric areas in *F. graminearum* and *F. fujikuroi* (Connolly et al. 2013; Niehaus et al., 2016a; Studt et al. 2016).

Along with the increased amount of ChIP-seq data, specific features of plant-associated fungal epigenomes were brought to light. Although TE-rich regions are typically associated with heterochromatin, in *Z. tritici*, TE-rich regions are enriched in both H3K9me3 and H3K27me3 modifications (Schotanus et al. 2015). TEs of *V. dahliae* are enriched in DNA methylation, H3K9me3 or H3K27me3, although association depends on the genomic context and on the TE families considered (Cook et al. 2020). Accessory chromosomes of *F. oxysporum*, *F. fujikuroi*, *F. graminearum* or *Fusarium asiaticum* are enriched in H3K27me3 (Connolly et al. 2013; Galazka and Freitag 2014; Niehaus et al., 2016a; Studt et al. 2016; Fokkens et al. 2018). Adaptive genomic regions of *V. dahliae* are also enriched



in H3K27me3 (Cook et al. 2020). In *Z. tritici*, accessory chromosomes are associated with both H3K9me3 and H3K27me3, while it is associated with H3K9me3 only in *L. maculans* (Schotanus et al. 2015; Soyer et al. 2021). An epigenomic analysis was set up in *B. cinerea*, allowing detection of H3K9me3 and H3K27me3 in this species (Schumacher et al. 2019) but a precise location of these two histone modifications is lacking so far. As for H3K9me3 or H3K27me3 showing variations among the different fungi in which it has been analyzed, DNA methylation is not always conserved either. It has been found for instance so far in *N. crassa*, *M. fijiensis*, *Ascobolus immersus*, *Coprinus cinereus*, *M. oryzae* and *V. dahliae* (Selker and Stevens 1985; Zolan and Pukkila 1986; Goyon et al. 1996; Dhillon et al. 2010; Jeon et al. 2015; Cook et al. 2020) although absent in some others (e.g. *S. pombe*, *L. maculans*; Antequera et al. 1984; Bewick et al. 2019). In *Z. tritici*, DNA methylation was initially not detected (Dhillon et al. 2010) but a recent analysis has pointed out that DNA methylation was observed on TEs in isolates having a DIM-2 functional protein, and that presence / absence of DNA methylation has an impact on genome evolution of this species (Möller et al. 2021).

As for genomic analyses, the next step forward for epigenomic analyses is the set-up of comparative epigenomic analyses. This has been done in the closely related species *L. maculans* 'brassicae' and *L. maculans* 'lepidii' (Soyer et al. 2021) through ChIP-seq to map H3K4me2, H3K9me3 and H3K27me3. The comparative epigenomic analysis corroborated previous comparative genomic analysis and previous epigenetic studies led in this species complex (Grandaubert et al. 2014; Soyer et al. 2014). Difference in terms of genome organization between *L. maculans* 'brassicae' and *L. maculans* 'lepidii' (i.e. alternation of large stretches of TEs and large gene-rich compartments in the former vs. little TE content, no bipartite organization of the genome in the latter) is consistent with the underlying organization of the epigenomic landscape in both species. Location of H3K4me3 was analyzed and compared among different subspecies of the *Zymoseptoria* genus (i.e. *Z. tritici*, *Zymoseptoria ardabiliae*, *Zymoseptoria pseudotritici*) showing that accessory chromosomes are consistently depleted in H3K4me2 (Feurtey et al. 2020). Recent groundbreaking comparative epigenomic analyses were performed in several species of the *Fusarium* genus showing that genes conserved among the genus are not associated with H3K27me3 and that two thirds of the genes associated with this modification are consistently found associated in the other species analyzed (Moser Tralamazza et al. 2022), as it was shown in *Z. tritici* (Soyer et al. 2019). On this basis, comparative epigenomics appear essential to understand the impact of the epigenomic landscape on adaptation toward rapid environmental changes, modulation of interactions with the holobiont, host adaptation and specialization. This type of analyses provides an opportunity to understand the evolutionary significance of epigenetic modifications and will undoubtedly be extended to other models in the near future.

In filamentous fungi, 3D organization of the chromosomes remains poorly investigated although development of new technics, such as Hi-C, allow to decipher chromosome organization in the nucleus and its impact on genes expression. Hi-C allows the mapping of the chromosome interactions within the nucleus, at the genome-wide scale (Lieberman-Aiden et al. 2009). As often, this new approach was first applied in filamentous fungi on the model *N. crassa* (Galazka et al. 2016; Klocko et al. 2016) showing that H3K9me3-rich regions physically interact. Functional analyses of the role of KMT1 and KMT6 have shown that KMT1 is not involved in the 3D organization of the nucleus. In contrast, H3K27me3-domains bind to the nuclear membrane and are essential for the integrity of the 3D genome organization (Klocko et al. 2016). In *N. crassa*, inactivation of *KMT1* and loss of H3K9me3 marks results in relocation of H3K27me3 marks, which, once relocated to the location of H3K9me3 marks, do not participate in the 3D genome organization. These results indicate a robustness of the 3D genome structure that is not undermined by inactivation of key factors involved in establishing chromatin structure (Basenko et al. 2015; Galazka et al. 2016; Klocko et al. 2016). The 3D genome organization of *E. festucae*, an endophytic fungus with an isochore genomic structure, was recently described (Winter et al. 2018). Winter et al. (2018) found physical contacts between different AT-isochores and GC-isochores but very few interactions between the two types of domains. Furthermore, contacts between AT-rich regions overwhelmingly dominate (50% of all inter-chromosomal contacts),

indicating that AT-isochores play a fundamental role in topologically structuring the chromosomes (Winter et al. 2018). Epigenome analyses of plant-associated fungi have defined both common features and specificities in the content and distribution of euchromatin and heterochromatin throughout the genomes. These distribution variations have consequences for genome stability, heritability of associated regions and genes, and gene expression.

### 3.3 Complex expression patterns of genes involved in host interaction

Accumulation of transcriptomic data during different stages of plant infection highlighted waves of expression of subsets of genes, including effector genes, over the course of infection, according to the host-plant infected or even between different species or strains infecting the same host. The first transcriptomic analyses revealed that the infection cycle was underpinned by specific temporal patterns of expression of effector genes (e.g. in *M. larici-populina*, *L. maculans*, *C. higginsianum*, *M. oryzae*, *Z. tritici*; Duplessis et al. 2011b; Rouxel et al. 2011; O'Connell et al. 2012; Hacquard et al. 2012; Petre et al. 2012; Dong et al. 2015; Mirzadi Gohari et al. 2015; Rudd et al. 2015). In *C. higginsianum*, different sets of effector genes are expressed in pre-penetrating appressoria, during the early biotrophic phase and during the transition to necrotrophy (O'Connell et al. 2012), with effectors expressed during the first stages of infection favoring cell viability, while effectors expressed during necrotrophy being involved in cell death induction (Kleemann et al. 2012). Expression of specific sets of (effector) genes were also found to represent a signature of the infection structures (e.g. in *M. oryzae*, *M. larici-populina* or *C. higginsianum*; Soanes et al. 2012; Hacquard et al. 2012; Kleemann et al. 2012). Transcriptomic analyses were also performed on fungi showing a complex lifecycle on their host. The fungus *L. maculans* exhibits a very complex lifecycle on oilseed rape, lasting several months in the field during which the fungus alternates different lifestyles on different plant organs (Rouxel and Balesdent 2005). Gene expression analysis throughout the life cycle of *L. maculans* -in controlled conditions or in the field- on different oilseed rape organs revealed a very complex regulation of the genes involved in pathogenesis, far more sophisticated than initially postulated from analyses carried out during early infection of cotyledons or petioles in controlled conditions (Rouxel et al. 2011; Gervais et al. 2017; Gay et al. 2021). Eight specific clusters of genes, all enriched in effector genes, are expressed during interaction with oilseed rape and associated with a given lifestyle and / or an infected tissue. Based on previous analyses, all avirulence effector genes were believed to be expressed only during early infection stages on cotyledons (Rouxel et al. 2011; Gervais et al. 2017) but Gay et al. (2021) showed that expression of these genes was finely regulated to be activated exclusively during the asymptomatic colonization stages. Such specific expression of subsets of candidate effector genes, associated with a specific infection stage, is also observed in *Z. tritici* for instance (Mirzadi Gohari et al. 2015; Haueisen et al. 2019). Besides expressing specific subsets of effector genes at different stages of infection, plant-associated fungi also express different sets of genes, including effectors, on different hosts (e.g. Hacquard et al. 2013; Kellner et al. 2014; Plett et al., 2015; Lorrain et al. 2018; see for review (Petre et al. 2020)). When comparing infection of wheat (compatible host) and *Brachypodium distachyon* (non-compatible host) by *Z. tritici*, Kellner et al. (2014) identified genes (including putative effector genes) specifically expressed during wheat infection. Due to their exceptionally elaborated life cycle (Kolmer 2013; Leonard and Szabo 2005; Chen et al. 2014), rust fungi represent a perfect framework to decipher gene expression associated with infection of taxonomically distant hosts, associated with a specific stage of the infection. A transcriptomic analysis was performed in *M. larici-populina* to highlight genes underlying specific types of spores and or hosts (Lorrain et al. 2018). Genes differentially expressed between the three stages of infection on poplar are enriched in effector genes and comparison between poplar and larch, the alternate host on which sexual reproduction occurs, highlights that subsets of effector genes might be involved in host specialization. Species/strain-specific effector gene expression pattern were also found through a comparative transcriptomic analysis (e.g. in *L. maculans* and *L. biglobosa*, *Z. tritici*, *M. oryzae*; Lowe et al. 2014; Dong et al. 2015; Palma-Guerrero et al. 2016, 2017; Haueisen et al. 2019) or even on different host genotypes (Schurack et al. 2021). Expression of genes associated with early stages of oilseed rape infection was compared

between two closely related species, the hemibiotroph *L. maculans* 'brassicae' and the necrotroph *L. biglobosa* 'canadensis'. Although infecting the same host and same organs, *L. maculans* 'brassicae' expresses a large number of genes with no known domains, many of them encoding (putative) effectors, while *L. biglobosa* 'canadensis' expresses cell wall degrading Carbohydrate-Active enZymes (Lowe et al. 2014). Comparative transcriptomic analyses of wheat infection by several *Z. tritici* strains differing in virulence revealed strain-specific regulation of putative effectors, proteases and cell wall-degrading enzymes, suggesting that differences in gene expression could be a major determinant of virulence variation among *Z. tritici* strains (Palma-Guerrero et al. 2016, 2017; Hauelsen et al. 2019). Sophisticated spatiotemporal or host-specific expression of effector genes indicates that their fine-tuned regulation is crucial for the successful outcome of plant infection and combined analysis of epigenomic and transcriptomic data will help deciphering impact of fungal epigenome on regulation of gene expression.

### 3.4 Lessons from combined analysis of genomic, transcriptomic and epigenomic data

Heterochromatin is subdivided into two states: constitutive and facultative. Constitutive heterochromatin is a permanent structure, found in all cell types and tissues, at centromeres, telomeres and TE, and as such involved in genome integrity and stability and ensures proper chromosome segregation. Contrary to constitutive heterochromatin, facultative heterochromatin designates genomic regions in the nucleus of a eukaryotic cell that have the opportunity to adopt open or compact conformations within temporal and spatial contexts (Trojer and Reinberg 2007). However, dogmas regarding conventional definitions of facultative or constitutive heterochromatin seem not to be valid in all plant-associated fungi. While histone modifications H3K9me3 and H3K27me3 are both a signature of heterochromatin, H3K9me3 is considered to be typical for constitutive heterochromatin, as associated with repeats and involved in genome stability, and H3K27me3 is associated with facultative heterochromatin and easily reversed toward a euchromatin state under some stresses from the environment. As H3K9me3 is found associated with TE-rich regions, the proportion of H3K9me3 in a genome strictly reflects the TE content, as described in *Z. tritici* for instance (Schotanus et al. 2015). This is also the case for *L. maculans* 'brassicae' and *L. maculans* 'lepidii'. In *L. maculans* 'brassicae', having >30% TE, 33% of the genome is associated with H3K9me3 while the genome of *L. maculans* 'lepidii', having a low TE content, presents a low enrichment in H3K9me3 (4% of H3K9me3 in its genome). The unique dispensable chromosome of *L. maculans* 'brassicae' is mostly repetitive and 90% of this chromosome is associated with H3K9me3 (Rouxel et al. 2011; Balesdent et al. 2013; Soyer et al. 2021). Co-location of H3K9me3 and TEs is in favour of a “constitutive” nature of this modification, together with the fact that H3K9me3-domains encompass a small number of genes (as in *L. maculans* 'brassicae' or *L. maculans* 'lepidii', with respectively 70 and 104 genes, or in *Z. tritici* with 86 genes located within H3K9me3-domains; Schotanus et al. 2015; Soyer et al. 2019; Soyer et al. 2021). Genes associated with H3K9me3 are almost all located in the middle of repetitive elements, in sub-telomeric areas, or very close to the edge of regions enriched in repeated elements. This is in accordance with the fact that TEs are targeted by H3K9me3 which might leak to neighbor genes. Nevertheless, some of these genes are heavily transcribed upon host interaction (Chujo and Scott 2014; de Jonge et al. 2013; Schotanus et al. 2015; Soyer et al. 2014, 2019, 2021; Winter et al. 2018; Gay et al. 2021). This does not match the “constitutive” nature of H3K9me3 modification. Contrary to H3K9me3-domains, H3K27me3-domains encompass a high proportion of predicted genes, with nearly 20% of the genes in both *L. maculans* subspecies and in *Z. tritici* and 30% of the genome for *F. graminearum* or *F. fujikuroi* (Connolly et al. 2013; Wiemann et al. 2013; Schotanus et al. 2015; Niehaus et al. 2016a; Studt et al. 2016; Soyer et al. 2019; Soyer et al. 2021). This is in accordance with the “facultative” nature of this histone modification. Nevertheless, some evidence is in favor of its important role in genome organization and stability. For instance, dispensable chromosomes of *Z. tritici* are twice as rich in TEs as core chromosomes, but although no significant enrichment of these chromosomes in H3K9me3 has been identified, they are entirely covered by H3K27me3 (Schotanus et al. 2015). Besides, loss of H3K27me3 in *Z. tritici* increases stability of some accessory chromosomes (Möller et al. 2019). These

features seem to indicate that although H3K27me3 is an important regulator of gene expression involved in development or response to various stresses, it might also play a role in the stability of some parts of fungal genomes.

As a side effect, genes located in TE-rich regions or accessory chromosomes are lowly expressed during axenic growth (Rouxel et al. 2011; Dallery et al. 2017; Haueisen et al. 2019; Gay et al. 2021). Interestingly, in many cases, upon infection, genes associated with TE-rich regions can be up-regulated (e.g. Rouxel et al. 2011; de Jonge et al. 2013; Chujo and Scott 2014; Dallery et al. 2017; Kombrink et al. 2017; Winter et al. 2018; Soyer et al. 2021; Gay et al. 2021). The up-regulated TE-associated genes include pathogenicity-related genes such as effector genes or secondary metabolite gene clusters. Even outside of TE-rich regions, heterochromatin is often found - when investigated- associated with genes involved in interaction with the host, whether they are genes encoding effectors or metabolites (Connolly et al. 2013; Wiemann et al. 2013; Chujo and Scott 2014; Schotanus et al. 2015; Studt et al. 2016; Niehaus et al. 2016a; Fokkens et al. 2018; Soyer et al. 2019; Gay et al. 2021; Soyer et al. 2021; Zhang et al. 2021). In *L. maculans*, combined analysis of ChIP-seq data together with RNA-seq data generated throughout the lifecycle of *L. maculans* on its host showed that H3K9me3- and H3K27me3-domains are significantly enriched in (predicted) effector genes and genes up-regulated *in planta* (Gay et al. 2021). Importantly, all characterized AVR genes of *L. maculans* are associated with H3K9me3-domains during axenic growth (Gay et al. 2021). Likewise, in *M. oryzae* and *F. graminearum*, genes located in H3K27me3-domains are enriched in genes up-regulated upon infection (Zhang et al. 2021; Moser Tralamazza et al. 2022). Moreover, in both species, genes not associated with H3K27me3 or associated with H3K4me2 during axenic growth show the same expression pattern between the *in vitro* and *in planta* conditions assessed (Zhang et al. 2021; Moser Tralamazza et al. 2022). And as for *L. maculans*, the well characterized AVR genes of *M. oryzae* are enriched in heterochromatin, although this is the H3K27me3-heterochromatin type (Zhang et al. 2021). In *V. dahliae*, subsets of genes differentially expressed between the axenic conditions assessed were associated with H3K27me3 and authors hypothesized that specific expression of genes during host infection might be under an epigenetic control (Kramer et al. 2022). Additional genome wide histone maps together with transcriptomic studies *in vitro* and *in planta* will allow determining whether association of effector genes not only with TE but also with different histone modifications is a general feature of plant-associated fungi and could potentially be involved in their regulation. Altogether, combined analysis of transcriptomic and epigenomic data support hypothesis that location of genes involved in plant interaction in distinct genomic regions yet sharing common epigenomic characteristics represents an efficient way to concertedly regulate their expression (Soyer et al. 2015b).

#### **4. Experimental evidence of the involvement of chromatin regulation, associated or not to the action of specific transcription factors, in the control of effector expression**

The localization of effector genes in dynamic regions of the fungal genomes, associated with heterochromatin domains, as well as the fine control of their expression during infection and a global repression of expression during axenic growth suggest that the expression of these effector genes may be under chromatin control associated or not with the action of specific TFs. Thus, several pioneering studies have been carried out in recent years in some phytopathogenic fungi using complementary strategies: (i) by moving effector genes from heterochromatin to euchromatin regions, (ii) by inactivating key proteins involved in chromatin remodeling and (iii) by inactivating or overexpressing TF-encoding genes.

#### 4.1 Effect of a change in genomic context on the expression of effector genes

The effect of genomic and epigenomic context on gene expression has, historically, been particularly documented in *Drosophila melanogaster*. This mechanism, called in *D. melanogaster* Position-effect variegation (PEV), leads to the silencing of genes normally located in an euchromatin region that are found in the proximity of a heterochromatin region after rearrangement or transposition. This mechanism is notably dependent on HP1 and KMT1 (see for review (Elgin and Reuter 2013)). In fungi, the effect of a change in genomic / epigenomic context on effector gene expression has been studied in a reverse manner, i.e. by moving effector genes from a heterochromatin to a euchromatin domain, in two plant pathogenic fungi, *L. maculans* and *Z. tritici* (Soyer et al. 2014; Meile et al. 2020).

As previously mentioned, *L. maculans* exhibits a compartmentalized genome structure, with alternating GC-, gene-rich regions, and AT-, repeat-rich regions (Rouxel et al. 2011). The latter are enriched in H3K9me3 heterochromatin domains (Soyer et al. 2021). While gene-poor (only 5% of the predicted genes), AT-rich regions are significantly enriched in putative effector genes (20% of the genes in this genomic environment against 4.2% of the genes in gene-rich regions) which are mainly expressed during plant infection and repressed during axenic growth (Rouxel et al. 2011; Gay et al. 2021). These observations led to the hypothesis that the chromatin structure in AT-rich regions could influence the expression of the associated genes. Soyer et al. (2014) developed a strategy aiming at determining the influence of the AT-rich regions on effector gene expression. Four effector genes were moved from AT- to GC-rich regions by *Agrobacterium tumefaciens*-mediated transformation, and expression of these genes was analyzed by qRT-PCR. The transfer of these effector genes from AT- to GC-rich regions strongly increased their expression in axenic culture, demonstrating that AT-rich regions repressed expression of these genes. In contrast, these effector genes were up-regulated during plant infection whatever their genomic context, suggesting that at that stage, repression due to the AT-rich regions was released.

In *Z. tritici*, Meile et al. (2020) proposed an elegant strategy to study the effect of both the genomic context and the presence of a native promoter on the expression level of effector genes and on the spatio-temporal pattern of expression. They studied effector genes located in an heterochromatin context during axenic growth and moved these effector genes under the control of their native promoter or of a constitutive promoter into a euchromatin genomic environment, fusing them either with fluorochromes or with a selection marker. These experiments showed that changing the genomic environment from heterochromatin to euchromatin allowed a higher expression in axenic condition, and thus, as in the study by Soyer et al. (2014), that heterochromatin regions repressed the expression of effector genes during axenic growth, this repression being independent of the promoter (the same effect was observed in the presence of a native or a constitutive promoter). In contrast, *in planta*, the expression of effector genes was derepressed regardless of the genomic context, but the change in genomic context modified the spatio-temporal pattern of expression of these effectors. Indeed, in the case of localization of effector genes in their native context, Meile et al. (2020) observed an induction of expression only in fungal cells near the penetration sites and inside the host leaf, and not in cells located on the leaf surface. In contrast, in case of a change in the genomic environment, a global overexpression of effector genes in all the fungal hyphae was observed.

Altogether, these data show that the genomic environment in which effector genes are located repressed their expression during axenic growth, that repression being released during plant infection. Nevertheless, *in planta* expression pattern of effector genes is not dependent on the genomic context. This suggests that although the chromatin-based repression needs to be released to allow for the expression of effector genes, other actors might be involved to ensure their concerted expression *in planta*.

## 4.2. Role of proteins involved in chromatin remodeling on the control of effector gene expression

The role of chromatin remodeling on the control of gene expression in plant pathogenic fungi has been mainly investigated through the inactivation of key players involved in the establishment of heterochromatin, notably *KMT1* and *KMT6*. For example, in *F. graminearum*, secondary metabolism gene clusters were found enriched in the H3K27me3 mark and inactivation of *KMT6* led to over-expression of a large number of clusters (Connolly et al. 2013). Similarly, in *F. fujikuroi*, inactivation of *KMT6* led to a decrease of the H3K27me3 modification that was associated with the induction of several secondary metabolism gene clusters and the production of metabolites (Studt et al. 2016; Niehaus et al. 2016b). In *E. festucae*, an endophyte that grows in the apoplast of *Lolium perenne* to establish a mutually beneficial association, an epigenetic mechanism regulates expression of at least two biosynthetic SM clusters, *i.e.* those for lolitrems (*ltm*) and ergot alkaloids (*eas*). These two gene clusters are located close to AT- and repeat-rich regions, and are up-regulated during host colonization. To decipher the role of the chromatin context on their regulation, Chujo and Scott (2014) generated knock-out mutants for *clrD* (*KMT1*) and *ezhB* (*KMT6*), and subsequently analyzed the expression of the *ltm* and *eas* genes and together with presence of H3K9me3 and H3K27me3 at their loci both *in vitro* and *in planta*, via ChIP-qPCR. Deletion of *clrD* and *ezhB* resulted in the induction, at least in part, of the genes located in these two clusters. In addition, both mutants had altered symbiotic interaction phenotypes with *L. perenne*. Moreover, derepression *in planta* of *ltm* and *eas* was found to be associated with a decrease of both H3K9me3 and H3K27me3 marks in the promoters of these cluster genes.

The first demonstration of a chromatin-based control of proteinaceous effector gene expression was done in *L. maculans* by Soyer et al. (2014). Expression of two genes encoding proteins involved in heterochromatin establishment was silenced using RNAi and a global transcriptomic analysis was performed on the silenced mutants using oligoarrays. RNAi silencing *LmDIM5* (*KMT1*) and *LmHP1* allowed derepression, during axenic growth, of 3% of the genes located in GC-rich regions compared with more than 30% of the genes located in AT-rich regions, specifically effector genes. Moreover, genes derepressed in both transformants were those that were naturally up-regulated, in the wild type strain, during primary infection of oilseed rape. ChIP-qPCR analyses showed that over-expression of at least two effector genes was associated with a decrease of the repressive histone modification H3K9me3 in the genomic environment of these genes (Soyer et al. 2014). However, the level of derepression of effector genes obtained during axenic growth in the *LmDIM5* and *LmHP1* mutants did not reach the level of *in planta* expression. This suggests that other components, such as specific TF(s), could be involved in effector gene induction *in planta*. Moreover, the silenced transformants obtained by Soyer et al. (2014) still expressed ~20% of *KMT1* which did not allow to fully decipher effects of a complete lack of H3K9me3 on the chromatin structure and gene expression. The CRISPR-Cas9 technology was recently established for *L. maculans* to generate knock-out mutants (Idnurm et al. 2017). *KMT1* was inactivated using that technology (Clairet et al. 2021b). Mutants inactivated for *KMT1* displayed reduced aggressiveness on oilseed rape but normal growth and conidiation. Clairet et al. (2021b) found that inactivation of *KMT1* had a significant impact on effector gene expression, but that only one avirulence gene and three genes located in H3K9me3 domains were up-regulated during axenic growth. In contrast, inactivation of *KMT1* had a significant effect on genes located in H3K27me3 domains. Clairet et al. (2021b) hypothesized that complete inactivation of *KMT1* had led to H3K27me3 relocation at native H3K9me3 domains, as previously reported for *N. crassa* or *Z. tritici* (Basenko et al. 2015; Möller et al. 2019), explaining that complete removal of H3K9me3 did not induce a global over-expression of genes located in repeat-rich regions.

Zhang et al. (2021) addressed the role of H3K27me3 in *M. oryzae* effector regulation both *in vitro* and *in planta*. Using ChIP-qPCR during plant infection, they found the H3K27me3 marks were replaced by H3K27ac, notably in regions containing genes overexpressed *in planta*, resulting in increased transcription. H3K27me3 covered half of the annotated TEs while H3K27ac was almost

exclusively associated with coding regions. They generated a *kmt6* mutant and found that most of the genes overexpressed in the mutant (about 88%) were covered in the wild-type (WT) by H3K27me3. They also inactivated *GCN5* that encodes the acetyltransferase responsible for H3K27 acetylation and made a *kmt6/gcn5* double mutant. The double mutant was affected in growth, conidia morphology and infection of rice, unlike the two single mutants. In addition, the H3K27me3 and H3K27Ac modifications were completely absent and genes that were upregulated in the *kmt6* mutant were not upregulated in the double mutant due to the absence of H3K27Ac. Kramer et al. (2022) found that the inactivation of *KMT6* in *V. dahliae* preferentially induced expression of genes located in H3K27me3 regions during axenic growth. However, while H3K27me3 is associated with gene repression, activation of genes located in a H3K27me3 context does not always necessitates removal of this heterochromatin mark.

In a reverse and complementary approach, several analyses have studied the effect of inactivation of proteins involved in the establishment of euchromatin marks (H3K4me2/3, H3K36me2/3). Janevska et al. (2018) investigated the role of two H3K36me3 histone methyltransferases, SET2 and ASH1, in *F. fujikuroi*. SET2 is responsible for H3K36me3 deposition in euchromatin regions of the genome, whereas ASH1 is responsible for H3K36 methylation in sub-telomeric regions. Inactivation of *ASH1* led to increased presence of H3K27me3 in sub-telomeric regions and increased chromosomal instability. Furthermore, loss of the H3K36me3 marks after inactivation of *SET2* or *ASH1* led to growth and sporulation defects, induction of secondary metabolite synthesis and reduced pathogenicity. Zhou et al. (2021) investigated the regulating role of H3K4me3 euchromatin mark through the functional analysis of several members of the COMPASS-like complex in *M. oryzae*, their inactivation leading to defaults in fungal development and pathogenicity. Similarly, in *C. higginsianum*, inactivation of *CCLA*, a sub-unit of the COMPASS complex involved in H3K4 trimethylation, led to reduced mycelial growth, sporulation and pathogenicity, but also to an enriched production of secondary metabolites, including several terpenoid compounds (Dallery et al. 2019). Lukito et al. (2019) also investigated the role of *CclA* on the control of the sub-telomeric gene clusters *IDT* and *EAS* in *E. festucae*. Inactivation of *CCLA* led the activation of *IDT* and *EAS* gene clusters in axenic culture. Inactivation of *KDMB*, the demethylase responsible of H3K4me3 removal, decreased *in planta* expression of *IDT* and *EAS* gene clusters. However, both *cclA* and *kdmB* mutants were still able to establish symbiosis with their host plant. In contrast, the inactivation of *SET2* led to hyphal growth defects similar to the ones observed for *kmt1* mutant, both mutants being unable to infect *L. perenne* (Lukito et al. 2021). Transcriptomic analysis at an early stage of plant colonization showed that many effector genes, over-expressed *in planta* in the WT strain, are down-regulated both in *set2* and *kmt1* mutants.

Thus, while various key players of chromatin remodeling are involved in controlling the expression of effector genes and secondary metabolism gene clusters, much remains to be discovered regarding the way this control can be released and the actors (chromatin readers, specific TFs...) that could be involved.

#### **4.3. First evidence of a double control of effector gene expression by specific transcription factors and chromatin remodelers**

Changes in the genomic context and the manipulation of proteins involved in chromatin remodeling have revealed a repressive role of the heterochromatin regions in which some of the fungal effectors are localized on their expression during axenic growth, this repression being lifted *in planta* allowing for a specific expression pattern of effector gene throughout infection. However, these experiments also showed that the release of the chromatin-based control of expression was not sufficient to reach the level of induction of effector gene expression *in planta*, suggesting involvement of other partners, such as specific TF(s).

Only few TFs affecting effector gene expression have been identified in fungi so far (see for review (Tan and Oliver 2017; John et al. 2021)). Most is known about the role of Sge1 / Ros1 orthologues in regulation of effector gene expression. Sge1 was first described in *F. oxysporum* f. sp. *lycopersici* as a positive regulator of effector genes (Michielse et al. 2009). Since then, Sge1 was found to function as a master regulator of effector genes in *V. dahliae*, *Z. tritici*, *B. cinerea* and *Cladosporium fulvum* (Michielse et al. 2011; Santhanam and Thomma 2013; Mirzadi Gohari et al. 2013; Okmen et al. 2014). In *U. maydis*, Sge1 negatively regulates the expression of effector genes associated with biotrophic development and positively regulates effector genes associated with late infection, indicating that Sge1 is a master regulator of infection process in *U. maydis* (Tollot et al. 2016). In *F. oxysporum* f. sp. *lycopersici*, Sge1 was able to regulate expression of effector genes located on an accessory chromosome independently of chromatin-remodeling (van der Does et al. 2016). In *Alternaria brassicicola*, a Zinc cluster TF specific of Pleosporales, AbPf2, regulates expression of 33 genes encoding secreted proteins including eight putative effectors (Cho et al. 2013). In *Parastagonospora nodorum*, the AbPf2 orthologue PnPf2 positively regulates two necrotrophic effectors, *SnToxA* and *SnTox3* and the orthologue of *SnToxA*, *ToxA* is regulated by PtrPf2 in *Pyrenophora tritici-repentis* (Rybak et al. 2017). In *P. nodorum*, a recent transcriptomic analysis comparing the WT and the *PnPf2* mutant during axenic culture and infection of wheat revealed involvement of *PnPf2* in the regulation of twelve effector genes and of genes associated with plant cell wall degradation and nutrient assimilation (Jones et al. 2019). In *Z. tritici*, the Pf2 orthologue was found essential for virulence, but also regulating dimorphic switch, axenic growth, fungal cell wall composition and carbon-sensing pathways (Habig et al. 2020). Although Pf2 was demonstrated to be a positive regulator of effector gene expression, no investigation of a link between action of a histone-modifying enzyme and Pf2 was performed.

In order to test the hypothesis that effector genes would be under the double control of a chromatin-based regulation and of specific TF(s), Clairet et al. (2021b) combined functional analyses on *KMT1* and the TF *Pf2* in *L. maculans*. *LmPf2* has an expression profile similar to that of the *L. maculans* avirulence genes and effector genes expressed during the asymptomatic phases of infection, while expression of *KMT1* is inversely correlated to their expression during axenic growth, cotyledon and petiole infection. They generated CRISPR-Cas9 inactivated mutants of *KMT1* and *LmPf2*, but also generated strains over-expressing *LmPf2*, either in a WT background or in a *kmt1* mutant background. No major defect in conidia production, growth rate or morphology was associated with the inactivation of *LmPf2*. In contrast, inactivation of *LmPf2* had a major effect on pathogenicity since the mutants were unable to invade the cotyledon further than the inoculation site, did not induce any visible symptom, and were highly impaired in effector gene expression *in planta*. In contrast, over-expression of *LmPf2* in a *kmt1* mutant background significantly induced the expression of effector genes, including eight avirulence genes (the up-regulation being much higher than when *LmPf2* was overexpressed in a WT background) and of genes associated with heterochromatin. In conclusion, in *L. maculans*, there is a major effect of the chromatin-context on the ability of *LmPf2* to regulate effector gene expression. Whether that model of double control of effector gene expression involving a specific TF and a histone-modifying protein could be generalized to other pathogenic fungi or if it is specific of *L. maculans* needs to be investigated.

## 5. Future challenges concerning the chromatin-based control of plant-associated genes

The discovery of the central role of chromatin structure as a regulator of plant-associated genes has deepened our understanding of how plant colonization takes place. Identifying the molecular determinants underlying this epigenetic regulation may eventually provide us with generic means to sustainably control fungal pathogens. However, much remains to be discovered, notably regarding the environmental / plant signals allowing targeted chromatin remodeling at the onset of plant



colonization and chromatin dynamics during colonization, as well as the possible interplay between epigenetic mechanisms and action of TFs.

Location of subsets of effector genes specifically expressed during infection in TE-rich heterochromatin domains, or gene-rich H3K27me3 domains, together with accumulation of functional analyses support hypothesis that expression of effector genes is governed by an epigenetic control ensuring repression during axenic growth and enabling expression during infection. The environmental or host signals that induce the release of the epigenetic control and the expression of effector genes at the early stages of infection remain unknown. To our knowledge, the signals emitted by the host plants and inducing the pathogenicity-related mechanisms have only been studied in root-colonizing oomycetes and fungi and no study has been reported for leaf-colonizing fungi (Morris et al. 1998; Akiyama et al. 2005; Turrà et al. 2015). Morris et al. (1998) reported that the oomycete *Phytophthora sojae* grows towards daidzein and genistein, two isoflavones secreted from the soybean roots and Akiyama et al. (2005) showed that symbiotic arbuscular-mycorrhizal fungi were attracted by strigolactones, plant hormones that are secreted from *Lonicera japonica* roots. Turrà et al. (2015) identified peroxidases secreted from tomato roots as the compounds that triggered attraction in *F. oxysporum*. Identification of the host signals that mediate interaction between plant and leaf-colonizing fungi have been hampered by the lack of reliable quantitative assays measuring a pathogenesis-related trait/aspect such as directed hyphal growth for root-colonizing fungi. Meyer et al. (2017) investigated environmental stimuli triggering effector gene expression in *L. maculans*. Among different biotic and abiotic factors tested, antibiotics such as cycloheximide, an antifungal compound that inhibits protein synthesis, increased the expression of several effector genes when used at concentrations 10 times lower than the ones needed to block the ribosome machinery. Physical and chemical stimuli, such as pH, osmolarity, carbon and ammonium sources and temperature also influenced, but to a lesser extent, the expression of effector genes. In contrast, no plant leaf signal influencing effector gene expression could be identified so far. The identification of host signals allowing changes in chromatin status and the induction of gene expression is of main scientific and applied importance since it would provide us with new, non-chemical strategies to control leaf-colonizing fungi, e.g. by allowing breeding of plant genotypes that would be less favorable to the expression of effectors genes.

While importance of histone modifications in the regulation of the expression of a few effector or secondary metabolite gene clusters has been demonstrated by ChIP-qPCR *in planta* (Chujo and Scott 2014; Soyer et al. 2019; Meile et al. 2020; Zhang et al. 2021), a complete view of the *in planta* chromatin dynamics of a plant-associated fungus has not been generated yet. The first *in planta* demonstration of a decrease of histone modifications associated with heterochromatin and underlying expression of genes involved in host colonization was performed in the endophytic fungus *E. festucae* (Chujo and Scott 2014). Using ChIP-qPCR, they have assessed enrichment of H3K9me3 and H3K27me3 for a few genomic loci of secondary metabolite gene clusters and compared this level between axenic culture and host colonization. This has shown that local decrease of H3K9me3 and / or H3K27me3 was associated with expression of genes during host infection (Chujo and Scott 2014). The same strategy was developed for three putative effector genes in *Z. tritici* during infection of wheat leaves, showing that their high expression at 13 dpi (corresponding to the switch from asymptomatic growth to necrotrophy for *Z. tritici*) compared to axenic culture was associated with a decrease in the level of H3K9me3 and/or H3K27me3 in their genomic environment (Soyer et al. 2019), as also observed for other effector genes of *Z. tritici* by Meile et al. (2020). Similar observations were made in *M. oryzae* (Zhang et al. 2021). Altogether, these analyses of distantly related fungi and displaying various interaction mode with their host confirmed that, during host interaction, specific induction of secondary metabolites / effector genes, located within heterochromatin domains during axenic growth, is associated with a dynamic re-organization of the histone modifications at their loci. The next challenge to be tackled now is to assess the chromatin dynamics *in planta* to fully decipher influence of histone modifications in the regulation of (effector) gene expression. So far, and as discussed before, genome-wide scale epigenomic analyses of plant-interacting fungi have only been performed during axenic growth. This lack of a global comprehensive view of the chromatin dynamics of the fungal

genome during plant interaction is currently due to technical limitations. Indeed, the success of performing such epigenomic analyses is jeopardized by the low amount of fungal biomass during plant infection combined with the fact that fungal genomes are usually small compared to massive host genomes. The next step to move forward would be to analyze the fungal epigenomic dynamics during host interaction through a deep-sequencing or by developing biochemical approaches to isolate the fungus from infected tissues and conduct the necessary epigenomic analysis approaches.

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