University of São Paulo
“Luiz de Queiroz” College of Agriculture

Comparative analysis of the transcriptional profile of the sugarcane smut
pathogen during the interaction with resistant and susceptible genotypes

Renato Gustavo Hoffmann Bombardelli

Dissertation presented to obtain the degree of Master in
Science. Area: Genetics and Plant Breeding

Piracicaba
2020

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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With all my love and affection,
To my beloved mother Lilian,
And my dearest grandparents Armindo and Arnilda.

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EPIGRAPH

“Na vida nem tudo são flores, muitas vezes são chicotes”.

“What we know is a drop, what we don't know is an ocean.”

— *Isaac Newton*

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RESUMO

Análise comparativa do perfil transcricional do fungo causador do carvão da cana-de-açúcar durante a interação com plantas resistentes e suscetíveis à doença

O carvão da cana-de-açúcar, *Sporisorium scitamineum*, é um fungo dimórfico e biotrófico, dependente do hospedeiro para sua reprodução sexual. A doença está espalhada por todos os países produtores da cana-de-açúcar, com exceção de Fiji, um grupo de ilhas vulcânicas na ilha Sul Pacífica da Oceania, e causa perdas econômicas com a redução da produtividade. Durante os últimos anos, o Grupo de Genômica da ESALQ/USP vem estudado extensivamente a interação carvão-cana, considerando seus vários aspectos, incluindo os mecanismos de ataque do patógeno, e resposta de defesa da planta. Os patógenos secretam moléculas conhecidas como efetores a fim de modular a fisiologia da planta, contra-atacar e proteger a si mesmo contra as barreiras impostas pela planta hospedeira. No presente trabalho, nós predizemos o secretoma e candidatos a efetores do *S. scitamineum*. Nós usamos a técnica do RNA-Seq para determinar o perfil de expressão e comparar a colonização de dois genótipos de cana-de-açúcar com contrastantes níveis de resistência (resistente, SP80-3280 e suscetível, IAC66-6 ao carvão) 48 horas após a inoculação. No primeiro capítulo, apresentamos uma breve revisão sobre o carvão da cana-de-açúcar. O segundo capítulo contém o estudo fornecendo a análise dos dados do transcriptoma do *S. scitamineum* quando infectando dois genótipos contrastantes de cana-de-açúcar às 48 horas após a inoculação e a comparação com dados já publicados do crescimento do *S. scitamineum* em meio de cultura. Nós encontramos resultados convincentes considerando-se o perfil de expressão dos genes do fungo infectando genótipos resistentes e suscetíveis de cana-de-açúcar. O patógeno expressa mais peroxidases, incluindo as catalases KatE e KatG, quando infectando o genótipo resistente, consistentemente com as respostas iniciais de defesa envolvendo a produção de espécies reativas de oxigênio (ROS) pelo genótipo SP80-3280. Nós mostramos a expressão variável de candidatos a efetores em cada tratamento, identificando promissores candidatos para futuros estudos funcionais. Explorando os genes relacionados ao cruzamento e crescimento filamentosos, uma fase crucial nos estágios iniciais da infecção, levando a formação da hifa dicariótica infectiva, nós mostramos a expressão de genes da via cAMP/PKA em *S. scitamineum*. Juntamente com o equilíbrio REDOX (produção/detoxificação do peróxido de hidrogênio), o fungo necessita da expressão de genes relacionados ao cruzamento para a infecção com sucesso. Nesse sentido, nós providenciamos intuições na infecção diferencial de genótipos de cana-de-açúcar resistente e suscetível pelo patógeno do carvão, sugerindo alguns candidatos para futuras caracterizações funcionais a fim de incrementar a compreensão desse patossistema.

Palavras-chave: RNA-Seq, *Saccharum* spp., *Sporisorium scitamineum*

ABSTRACT

Comparative analysis of the transcriptional profile of the sugarcane smut pathogen during the interaction with resistant and susceptible genotypes

The sugarcane smut pathogen, *Sporisorium scitamineum*, is a dimorphic and biotrophic fungus, host-dependent to its sexual reproduction. The disease spread across all the sugarcane producing countries, except in Fiji, the group of volcanic islands in the South Pacific island in Oceania, causes economic losses reducing the yield. During the later years, the Genomics Group at ESALQ/USP has been extensively studying the sugarcane-smut interaction, considering its various aspects, including the pathogen attack mechanisms and plant defense responses. The pathogens secrete molecules known as effectors to modulate plant physiology, counterattack, and protect themselves against the plant host's defense barriers. In this work, we predicted the secretome and candidate effectors of the *S. scitamineum*. We used the RNA-Seq technique to determine the expression profile and compare the colonization of two sugarcane genotypes with contrasting resistance levels (smut-resistant, SP80-3280 and -susceptible, IAC66-6) 48 hours after inoculation. In the first chapter, we provided a brief review of the sugarcane smut disease. The second chapter contains the study providing data analysis of the transcriptome of *S. scitamineum* when infecting two contrasting sugarcane genotypes 48 hours after inoculation and a comparison with previous data of *S. scitamineum* growth in axenic culture. We found compelling results considering genes' expression profile of the fungus infecting resistant and susceptible sugarcane genotypes. The pathogen expressed more peroxidases, including the catalases KatE and KatG, when infecting the resistant genotype, consistent with the early defense response involving reactive oxygen species (ROS) production by the SP80-3280 genotype. We revealed the variant expression of candidate effectors genes in each of the treatments, identifying promising candidates for further functional studies. Exploring the genes related to mating/filamentation growth, a crucial phase in the early infection stage, leading to the formation of the infective dikaryotic hyphae, we unveil the expression of genes of the cAMP/PKA pathway in *S. scitamineum*. Together with the REDOX equilibrium (production/detoxification of hydrogen peroxide), the fungus requires the expression of mating-related genes for successful infection. Herein we provided insights into the differential infection of resistant and susceptible sugarcane genotypes by the smut pathogen, suggesting some candidate genes for further functional characterization studies to increase this pathosystem's comprehension.

Keywords: RNA-Seq, *Saccharum* spp., *Sporisorium scitamineum*

CHAPTER 1: Sugarcane smut: the state of art

1. Sugarcane

Sugarcane is one of the most important crops cultivated globally, being the third most-produced commodity. Currently, around 90 countries cultivate sugarcane. Brazil is the leading producer, with a crop equivalent to 39.3% of global stock, followed by India, China, and Thailand (FAO, 2018). The 2018/19 Brazilian harvest generated a total of 620.4 million tons cultivated on an area of 8.6 million hectares in the country (CONAB, 2019). Besides the production of sugar and biofuels such as ethanol, the most used alternative fuel, the sugarcane crop has potential to produce other renewable products such as bioplastics, bio-hydrocarbons, and bio-electricity (Waclawovsky *et al.*, 2010), which has increased the interest in sugarcane utilization by its various producing countries (Lam *et al.*, 2009).

Modern sugarcane varieties (*Saccharum* spp.) come from a complex hybridization among *Saccharum* species, with significant contributions from the *S. spontaneum* ($2n = 40$ to 128) and *S. officinarum* ($2n = 80$) (D'Hont, 2005; Amalraj and Balasundaram, 2006). As a result of this interspecific hybridization, the sugarcane has an elevated polyploidy and aneuploidy degree with chromosomes number varying from 100 to 130 ($2n$) (D'Hont *et al.*, 1996), and its genome size estimate to approximately 10 Gbp (D'Hont and Glaszman, 2001). Various techniques as the sequencing of expressed sequence tags (ESTs) (Vettore *et al.*, 2001, 2003), transcriptome based on RNA-Seq (Cardoso-Silva *et al.*, 2014; Schaker *et al.*, 2016), the construction of a monoploid genome sequence of a commercial variety (R570) using Bacterial artificial chromosomes (BACs) (Garsmeur *et al.*, 2018), the sequencing of another commercial variety (SP80-3280) predicting 373 thousands of genes (Souza *et al.*, 2019), the partial genome sequence based on selected inserts cloned in BACs (de Setta *et al.*, 2014) and the sequencing of a monoploid/tetraploid version from the parental species *S. spontaneum* (Zhang *et al.*, 2018) are improving the knowledge of the complex sugarcane genome, as well as helping to uncover the behavior of sets of genes involved in essential processes such as the accumulation of sugar and resistance to plagues and diseases.

2. Smut disease

The fungi of *Ustilaginomycetes* class in the *Basidiomycota* phylum, known as smuts, including more than 1,650 species (Toh and Perlin, 2016) are host-specific pathogens that affect

many angiosperm clades, being most common on the *Poaceae* family (Begerow *et al.*, 2014). The only other pathogenic class with a more significant number of species is that of rust pathogens.

Several species in the smut group cause disease in important cereal crops such as maize – *Ustilago maydis* / *Sporisorium reilianum*; sorghum – *S. reilianum*; oat and barley – *U. hordei*; wheat – *U. tritici* and sugarcane – *S. scitamineum*. The first sugarcane smut report dated from 1877 in South Africa, describing the disease in a *Saccharum sinense* clone, known as “China cane” (Luthea *et al.*, 1940; Waller, 1969; Lee-Lovick, 1978; Bailey, 1979). In India, problems with the reduction of sugarcane yield in commercial fields reported back to the 1930s, but probably smut was already there, colonizing susceptible genotypes of *S. barberi* and the wild parent of sugarcane *S. spontaneum* (Ferreira and Comstock, 1989; Croft and Braithwaite, 2006). In the 1940s, the disease reached Argentina and soon spread to Brazil, Paraguay, and Bolivia (Bergamin Filho *et al.*, 1987; Rago *et al.*, 2009). In Kenya, the first epidemic smut rose around the same time (Waller, 1969). Then other reports back to the 1970s described the observation of the disease in Hawaii and the Caribbean Islands (Byther *et al.*, 1971; Lee-Lovick, 1978). Sugarcane smut reached Western Australia in 1998 (Croft and Braithwaite, 2006; Magarey *et al.*, 2010). The origin center of sugarcane (Papua, New Guinea) was considered smut free until the recent 2016 report of the disease infecting commercial crop fields (Tom *et al.*, 2017). The sugarcane smut is, currently, present in almost every sugarcane-growing country, except for Fiji, an isolated island in Oceania (Croft and Braithwaite, 2006; Sundar *et al.*, 2012; Tom *et al.*, 2017).

The damage caused by the sugarcane smut varies widely, from negligible to severe losses, depending on the environmental conditions, the tolerance level of the sugarcane variety and the aggressiveness of the prevalence races of the pathogen (Whittle, 1982; Hoy, 1986; Sundar *et al.*, 2012). The decrease of yield is related to an increase of tillering, reduced diameters of the canes, reduced sucrose and increased fiber content (Martinez *et al.*, 2000; Wada *et al.*, 2016).

S. scitamineum is a biotrophic fungus from the *Basidiomycota* phylum, *Ustilaginales* order, *Ustilaginomycetes* class, and *Ustilaginaceae* family (Hawksworth *et al.*, 1995). Its life cycle comprises two distinct phases: a monokaryotic, with saprophytic growth of non-infective haploid cells (n) and an infective dikaryotic ($n + n$) phase, in which karyogamy results in teliospores ($2n$) formation (Figure 1 - A). The infective hyphae originate from the fusion of two mating compatible haploid cells (sporidial cells). Mating compatibility in smut fungi depends on the products of two loci, *a* and *b* (Bölker, 2001). The *a* locus comprises genes encoding a

pheromone (*mfa*) and a membrane receptor (*pra*) (Figure 1 - B). The compatible mating-types have alleles that encode complementary proteins of the pair receptor and pheromone. The *b* locus has genes that encode two subunits of a heterodimeric transcript factor (*bE* and *bW*) that must be encoded by different alleles (*bE1/bW2* and *bE2/bW1*) in order to produce a functional protein (Figure 1 - C) (Gillissen *et al.*, 1992; Kronstad and Staben, 1997; Bölker, 2001; Singh *et al.*, 2004). Compatible cells recognize each other and fuse resulting in the dikaryotic hyphae able to penetrate and infect the host tissues (Singh *et al.*, 2004; Peters *et al.*, 2017).

The sexual compatibility system of *S. scitamineum* is bipolar, regarding its linked mating loci *a* and *b* on chromosome 2 (Taniguti *et al.*, 2015). Bipolar systems, in which two alternative and distinct sets of genes and alleles at the mating loci, determine two mating-types, is the prevailing system in most fungi (Coelho *et al.*, 2010). However, in the *Basidiomycota* phylum, *Ustilaginomycotina* subphylum, most species of plant pathogens (for example, *U. maydis*) have a tetrapolar mating system, in which the two mating loci are unlinked (Coelho *et al.*, 2010). Some studies suggest the derivation of bipolar mating systems from the tetrapolar ones (Bakkeren and Kronstad, 1994; Lee *et al.*, 1999; Lengeler *et al.*, 2002; Hsueh *et al.*, 2008).

After penetrating the host surface, the fungus initiates an intense proliferation colonizing the meristematic tissues of the plant. In later stages of the infection, fungal sporogenesis results in the formation of a whip-like structure (the characteristic symptom of the disease). In the process of sporogenesis after karyogamy, the hyphae undergo fragmentation, followed by pigments deposition and cell wall reinforcement resulting in the release of billions of diploid teliospores (which ones have soot aspect and are the source of disease name). The dissemination of the teliospores occurs by wind, rain, or even during the harvest time, infecting other plants (Banuett and Herskowitz, 1996; Taniguti *et al.*, 2015).

Figure 1 – A) Developmental stages of *S. scitamineum* life cycle: diploid teliospores (2n); germination and reductive meiosis (R!); resulting into haploid yeast-like sporidia (n); and anastomosis forming a dikaryotic infective hyphae (n+n), adapted from Taniguti *et al.* (2015). B) and C) schematic representation of the mating-type, sexual and pathogenic development: pheromone production (mfa) and receptor (pra) recognition encoded by the *a* locus leading to anastomosis B); formation of the heterodimeric functional proteins by the two complementary transcription factor subunits bE/bW, leading to the pathogenic development C), adapted from Bölker (2001).

3. Secretome, effectors, and plant host defense responses

The secretome is a fraction of the total proteome encoded by an organism secreted by a group of cells, as well as the machinery responsible for the secretion of these molecules (Tjalsma *et al.*, 2000). Agrawal *et al.* (2010) have revised this definition to include in the secretome only those proteins secreted to the extracellular medium.

The processes of pathogen recognition and defense response by the plant host immune system may have two phases. The first one involves the recognition of pathogen-associated molecular patterns (PAMPs) described as PTI (PAMP-triggered immunity) and the second associated with effectors recognition known as ETI (effector-triggered immunity) (Jones and Dangl, 2006). Receptors identified as PRRs (PAMP-recognition receptor) localized in the cellular membrane recognize PAMPs and trigger a signal transduction cascade activating the plant defense response (Hogenhout *et al.*, 2009). PTI is the most common plant defense mechanism, allowing fast and efficient responses to a large variety of pathogens (Roux *et al.*, 2014).

In contrast to the plant host defense responses, the adapted pathogens encode an arsenal of secreted proteins that can suppress the PTI, resulting in the susceptibility triggered by effectors (ETS – effector-triggered susceptibility) (Birch *et al.*, 2008). However, the plant hosts may have genes encoding resistance proteins (R genes) that recognize the pathogen effectors, in order to suppress the ETS response, resulting in resistance (Howden and Huitema, 2012). The recognition of the effectors by the products of R genes can be direct (gene-for-gene model) (Oßwald *et al.*, 2014) or indirect (guard model) (Jones and Dangl, 2006). The development of new effectors by the pathogen, in order to manipulate the plant host defense responses, followed by the development of new R genes by the plants, in order to combat these effectors is known as “arms race” in the plant-pathogen interaction (Coll *et al.*, 2011).

After the recognition of PAMPs or effectors, various signals may activate the plant immune system. Among them signaling based on changes of Ca^{+2} levels in the cytoplasm, rapidly production of reactive oxygen species (ROS), and signaling cascade via MAP-kinases (Wu *et al.*, 2013; Que *et al.*, 2014; Sánchez-Elordi *et al.*, 2015; Peters *et al.*, 2017). Regulatory hormones, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) amplify the signs, resulting in the activation of transcription factors, defense genes, PR-proteins, phytoalexins, lignification of tissues, callose deposition and other cell wall reinforcement-related genes (Grant and Lamb, 2006).

In this context, understand the outcomes of critical components involved on the plant defense mechanisms, such as PAMPs, PRRs, effectors and R genes, can contribute to the development of new strategies for the disease management, avoiding significant yield and economic losses caused by diseases in the crops (Raffaele and Kamoun, 2012).

To study the function of the secreted effectors of smut fungi, usually, assays are conducted using the model for functional characterization of biotrophic fungi, *Ustilago maydis*. These effectors contribute to all steps of the disease development, since the early stages of the infection and penetration, and apoplastic, inter, and intracellular grown, until systemic dissemination. Therefore, there is a constant selection pressure from the host’s immune system over those effectors, making them the fastest evolving genes in the pathogen genome (van der Linde *et al.*, 2012). As a consequence, the accumulation of mutations during the coevolution with the host immune system results in species or even race-specific effectors (Zuo *et al.*, 2019). Comparative genome studies revealed that the cereal smuts (*U. maydis*, *S. reilianum*, *S. scitamineum*, *U. hordei*, *U. trichophora*, and *U. tritici*) have similar numbers of genes encoding for secreted proteins. Whereas, *Melanopsichium pennsylvanicum*, the causal agent of the gall smut on the dicot host *Persicaria* spp., have a reduced set of effectors, potentially related to

jumping host from monocot to dicot plants (Sharma *et al.*, 2014; Benevenuto *et al.*, 2018; Schuster *et al.*, 2018).

Various effectors genes were already functionally characterized in *U. maydis*. Some of the effectors common to all smuts contribute to counteracting the innate immune responses conserved among different host species and facilitates the pathogen infection and colonization (Zuo *et al.*, 2019). The protein encoded by the Pep1 gene is required for *U. maydis* to suppress the oxidative burst induced by the host apoplastic peroxidases. The function of Pep1 is essential for epidermal penetration and conserved among smut fungi infecting either monocot or dicot plant hosts (Doehlemann *et al.*, 2009; Hemetsberger *et al.*, 2015).

Moreover, *U. maydis* secretes several protective effectors that work as barriers from host-derived antifungal proteins (Zuo *et al.*, 2019). Among these effectors is the Rsp3 protein, which binds and shields the fungal cell wall, protecting the fungal mycelium from the antifungal activity of the maize mannose-binding proteins AFP1 and AFP2 (Ma *et al.*, 2018). Another protective effector is the UmFly1, which is a secreted fungal lysin metalloprotease and cleaves maize chitinase-A, reducing its catalytic activity (Ökmen *et al.*, 2018). UmFly1 is also required by *U. maydis* to activate endogenous chitinases by N-terminal processing necessary for the separation of cells in the yeast-like phase (Ökmen *et al.*, 2018). The opposite effects of UmFly1 on endogenous chitinases and host chitinases suggest a neofunctionalization of this effector toward virulence during evolutionary adaptation of *U. maydis* to maize (Zuo *et al.*, 2019). Pit2 is a secreted effector that inhibits maize papain-like cysteine proteases, which is crucial for the suppression of apoplastic host defense responses and successful infection and virulence of *U. maydis* (Doehlemann *et al.*, 2011; Mueller *et al.*, 2013).

Other effectors function reprogramming plant metabolic pathways when translocated into the host cell (Djamei *et al.*, 2011; Tanaka *et al.*, 2014; Redkar *et al.*, 2015a). An effector named Cmu1 prevents the production of the SA (the primary defense hormone) by sequestering its biosynthesis precursor chorismate (Djamei *et al.*, 2011; Djamei and Kahmann, 2012). The effector Tin2 redirects the lignin production pathway to anthocyanin production, through stabilization of the maize protein kinase ZmTTK1 (Tanaka *et al.*, 2014). It is known that the Tin2 effectors from *U. maydis* and *S. reilianum* target different kinase proteins paralogs in maize (Tanaka *et al.*, 2019). See1, another effector acting inside the host cell, prevents phosphorylation of the maize SGT1 protein involved in resistance and cell cycle regulation (Redkar *et al.*, 2015a). The sequence of See1 is conserved among the smut pathogens, however, the *U. hordei* See1 ortholog does not complement the *U. maydis* See1 knockout mutants

(Redkar *et al.*, 2015b), suggesting different host adaptation strategies of See1 orthologs in smut pathogens.

SAD1, functionally characterized in *S. reilianum*, is a species-specific effector causing suppression of apical dominance in maize plants and increase branching of female inflorescences and the production of *S. reilianum* teliospores (Ghareeb *et al.*, 2015). Effectors of *S. reilianum* contribute quantitatively to the virulence of the pathogen, as demonstrated recently by Ghareeb *et al.* (2019) in a study with deletion of a single gene and a gene cluster. Therefore, deleting a single gene may result in a slight reduction of virulence or no detectable phenotype (Ghareeb *et al.*, 2019). This fact complicates the studies for the functional characterization of a single effector gene.

4. Sugarcane-smut molecular interaction

Until now, there is no fungicide registered to control the sugarcane smut, only to prevent it in the form of bud treatment (Bayfidan EC – Triadimenol 250 g/L). The most efficient method to prevent the disease is the utilization of healthy seedlings free of pathogens and resistant varieties. However, the pathogen colonizes even resistant genotypes, occasionally producing the whip-like structure, but causing no economic losses in the field scale.

One of the best-characterized responses of sugarcane toward *S. scitamineum* is related to the oxidative burst. Genes encoding components of the reactive oxygen species (ROS) metabolism activated earlier after inoculation and have a different behavior according to the sugarcane genotypes (Menossi *et al.*, 2008; You-Xiong *et al.*, 2011; Schaker *et al.*, 2016; Peters *et al.*, 2017). Peters *et al.* (2017) demonstrated the accumulation of H₂O₂, and a reduction in antioxidant enzymes activities earlier in resistant sugarcane plants when compared to a susceptible genotype. The oxidative burst coincided with some phases of the fungal development: germination, formation of the appressorium, and colonization of the host tissues. Potentially, that would be the first layer in the sugarcane defense mechanism when the recognition of the pathogen occurs, leading to the activation of mechanisms related to the resistance.

Sugarcane plants resistant to the smut, accumulate glycoproteins during the infection (Fontaniella *et al.*, 2002) associated with the inhibition of the teliospores germination (Lloyd and Pillay, 1980; Lloyd and Naidoo, 1983; Martinez *et al.*, 2000; Blanch *et al.*, 2007). Detecting high levels of the polyamines conjugated with phenolic compounds in mature organs indicates the effect of the presence of the fungus (Legaz *et al.*, 1998; Piñon *et al.*, 1999). The phenolic

compounds produced in response to the infection inactivates the effect of polyamines through the conjugation inhibiting the germination of the fungus (Sundar *et al.*, 2012). The polyamines are essential for fungal growth and differentiation (Shapira *et al.*, 1989; Ruiz-Herrer, 1994).

The germination of the spores occurs on the base of new leaves, following hyphal growth and appressorium formation. In response to the infection, sugarcane produces β -1,3-glucanase and chitinase to attack components of the fungus cell wall (Blanch *et al.*, 2007). Also, the pathogen induces synthesis and activation of enzymes involved in the production and polymerization of monolignols involved in lignin synthesis, alters the expression pattern of transcription regulators, and that of genes related to ethylene and auxin pathways (Blanch *et al.*, 2007; LaO *et al.*, 2008; Menossi *et al.*, 2008; Schaker *et al.*, 2016; Peters *et al.*, 2017).

Studies involving the global responses of sugarcane to smut infection performed by different groups revealed a more comprehensive view of this particular plant-pathogen interaction. The authors used approaches such as transcriptomics (Thokoane and Rutherford, 2001; Borrás-Hidalgo *et al.*, 2005; LaO *et al.*, 2008; Wu *et al.*, 2013; Que *et al.*, 2014; Huang *et al.*, 2015; Taniguti *et al.*, 2015; Schaker *et al.*, 2016); proteomics (Que *et al.*, 2011; Barnabas *et al.*, 2016); and metabolomics (Schaker *et al.*, 2017). The first defense mechanism activated is based on PAMPs recognition by receptor proteins of the host. The resistant sugarcane variety N52/219 activates the expression of an RLK (S-receptor-like-kinase) protein involved in the perception of chitin, leading to the increase in expression levels of chitinases (Thokoane and Rutherford, 2001; Esh *et al.*, 2014; Que *et al.*, 2014). Other proteins associated with pathogenicity such as thaumatin, proteins with antifungal activity, are characteristic of plants resistant to smut (Heinze *et al.*, 2001; Que *et al.*, 2014; Su *et al.*, 2016). Resistant varieties also activate MAPK signaling pathways (Mitogen-Activated Protein Kinase), associated with defense mechanisms (Wu *et al.*, 2013; Que *et al.*, 2014; Sánchez-Elordi *et al.*, 2015). In susceptible genotypes, were observed an increase in the expression of an RGA (Resistant Gene Analog), homologous to the BAM1 (RGA482) (Schaker *et al.*, 2016). BAM1 is associated with meristematic identity suggesting an earlier association with whip development and meristematic functions. Genes encoding cytoplasmatic receptors NBS-LRR-like proteins (Nucleotide-Binding Site – Leucine-Rich Repeat) identified in both resistant (Borrás-Hidalgo *et al.*, 2005; LaO *et al.*, 2008; Que *et al.*, 2011) and susceptible varieties differentially expressed (Schaker *et al.*, 2016; Rody *et al.*, 2019).

Although various studies addressing sugarcane mechanisms in response to *S. scitamineum* infection are available, the mechanisms of fungal attack leading to disease have received little attention. Taniguti *et al.* (2015), using transcriptomic data, compared plants five

days after inoculation (dai) with ones after whip emission (200 dai). The authors identified approximately 13% of the *S. scitamineum* genes differentially expressed in the plant compared to *in vitro* growth. The predicted function of these genes suggested that the pathogen can overcome the plant defense through the detox of defense molecules (protease inhibitors, pisatin demethylase, benzoate 4-monooxygenase, chorismate mutase, superoxide dismutase, catalase) and the growing and colonization of the plant tissues using an arsenal of proteins related to nutrient absorption and cell wall degrading enzymes. Secreted proteins are of particular interest in understanding the molecular communication between the pathogen and its plant host. Using computational tools to predict the presence of signal peptide and absence of transmembrane domains and glycosylphosphatidylinositol (GPI) anchors, the authors proposed that *S. scitamineum* secretome comprise 305 proteins, including 54 carbohydrates active enzymes (CAZymes) and 70 effectors candidates (based on the predicted size of the proteins, and its cysteine content) (Taniguti *et al.*, 2015).

5. Dual transcriptomics in plant-fungal interaction: difficulties in the data collection and analysis

Even though the RNA-Seq technique provides more accurate detection of transcripts, including low-abundant ones, compared to other transcriptomics approaches, such as the microarrays chips, it still has a high cost when sequencing with high depth coverage (Kohler and Tisserant, 2014). Sequencing coverage reflects in the quantity and quality of the reads obtained, and consequently, the precision of detection. When conducting a dual transcriptomics assay aiming at the discovery of the plant and the pathogen components during the interaction, often the genome size and, consequently, the transcriptome size of the plant is significantly larger than that of the pathogen (Kohler and Tisserant, 2014). As plant growth is more expressive in the early stages of infection, the pathogen represents only a small percentage of the total RNA pool.

Unlike genome sequencing, predicting the number of sequences required to detect all transcripts is quite tricky, considering that the RNA concentration varies significantly between tissues and conditions (Wang *et al.*, 2009). For the *Escherichia coli* (4.6 – 5.3 Mb), Haas *et al.* (2012) estimated that sequencing two million reads cover about 80% of the transcriptome. Whereas, for covering 80% of the yeast (*Saccharomyces cerevisiae*, 12.1 Mb) transcriptome, it would be necessary to sequence four million reads (Nagalakshmi *et al.*, 2008; Sims *et al.*, 2014). On the plant side, for example, the model *Arabidopsis thaliana* (135 Mb), it would require a

sequence depth of 50 million reads to nearly saturate the coverage of expressed genes (Van Verk *et al.*, 2013).

Another crucial step into the dual transcriptome analysis is to correctly map the mixed sequenced reads to the respective reference genomes (if available). Generally, the genomes of plants and fungal are different enough to simply align the reads to the reference genomes without prefiltering (Kohler and Tisserant, 2014). When only one genome is available, usually the fungal, the separation can still be done, and the reads of the plant can be mapped to a set of transcripts, such as ESTs collections, or generated with high throughput sequencers.

For the mapping step, various methods and softwares are available, some “unspliced” that do not allow alignments within significant gaps, and consequently do not support mapping spliced reads to the reference genome. The most common of these mappers are the ones based on the Burrows-Wheeler transform method, because of its more computational and time efficiencies, such as BWA (Li and Durbin, 2010) and Bowtie2 (Langmead and Salzberg, 2012). To mapping spliced reads to a reference genome, the most popular softwares are TopHat2 (Kim *et al.*, 2013) and Hisat2 (Kim *et al.*, 2015). First, one uses an unspliced method to align the reads to a reference, and then, the split of unmapped reads into shorter segments allow an appropriated mapping. Subsequently, one searches for the spliced sites in the neighboring genomic regions.

As some genomes can contain repetitive regions and large multigene families, reads can align to multiple locations (Kohler and Tisserant, 2014). Thus, the suggestion is to remove these alignments or treat them in a random way to subsequent analysis. For this purpose, the recommended approach is to allocate these multi mapped reads in proportion to the number of uniquely mapped ones (Mortazavi *et al.*, 2008).

After the mapping step, it is necessary to attribute the mapped reads to a gene or transcript, using an annotation reference or *de novo* approach to generate a count table. Some softwares are available for that, such as the FeatureCounts implemented in the Subread package (Liao *et al.*, 2013, 2014), HTSeq (Anders *et al.*, 2015), the R package Limma (Ritchie *et al.*, 2015), RSEM (Li and Dewey, 2011) and Cufflinks & Cuffmerge (Roberts *et al.*, 2011a, 2011b).

Another problem when dealing with dual transcriptome studies is to normalize the data correctly. Various robust methods are available to deal with transcriptomics data normalization in order to allow compare different treatments and assays. One of the most popular normalization schemes for expression data is the RPKM (Reads Per Kilobase reads Mapped) (Mortazavi *et al.*, 2008) used for single-end reads, and FPKM (Fragments) for paired-end sequencing. Another type of normalization used when exploring expression data is DESeq2

implemented in the R package (Love *et al.*, 2014) and EdgeR (Trimmed mean of M values - TMM) (Robinson *et al.*, 2010; McCarthy *et al.*, 2012). These methods use a scaling factor estimated from the mean of the median of reads counts ratio across the samples, assuming that the majority of the expressed genes are not differentially expressed (Kohler and Tisserant, 2014).

Studies using the dual-transcriptomics RNA-Seq approach with plant-fungus interaction are still scarce. Schaker *et al.* (2016) used a dual-transcriptomics RNA-Seq of sugarcane plants inoculated with *S. scitamineum* at 5 days after inoculation (dai) and 200 dai (whip emission). In that study, the mapping results of aligned reads to the fungal reference CDS region reported only 2% mapped reads at 5 dai, and an increase up to 18% at 200 dai. In another study with the pathosystem rice-blast fungus (*Oryza sativa* vs *Magnaporthe oryzae*), Kawahara *et al.* (2012) reported that at 24 hai, only 0.2-0.3% of the mixed reads (plant + fungus mRNA) mapped on the fungal reference genome. These results come from the early stage of infection, in which the plant growth and development is faster compared with the fungal colonization, which difficult the detection of fungal mRNA unless considering a deep sequencing.

Other studies focus on the beneficial interactions between fungus and plants, exploring mycorrhizal interactions. Kohler and Tisserant (2014) produced a review exploring dual transcriptomics related to these interactions. In the review, the authors emphasize the importance of the experimental design in order to obtain sufficient depth sequencing to detect both organisms which interact. A study considering dual-transcriptomic issues and how to address the problems was conducted by Naidoo *et al.* (2018), the authors highlight how to analyze the data produced by a dual-transcriptomic assay, exploring the methods developed for such task and future perspectives with the lower cost brought by the Next Generation Sequence (NGS).

The lacking of funding support, or need to outscoring sequencing services in many cases, limits the number of reads that can be obtained per sequencing, implicating in the reduced accuracy of transcript detection. However, even in those cases, with a reduced number of reads, the analysis can be carried out, especially if a high-quality reference genome is available (Naidoo *et al.*, 2018), with some precautions and later validation of differential expressed genes using more accurate techniques such as RT-qPCR. Therefore, it can still guide functional characterization studies for a more comprehensive understanding of the plant-pathogen interaction.

6. Hypothesis

The use of dual transcriptome data analysis can reveal candidate genes associated with *S. scitamineum* expression profile differentiation when infecting smut-resistant vs. -susceptible plants.

7. Objectives

7.1 Sequence and analyze data of a dual transcriptome RNAseq data obtained from a previous experiment (Peters, 2016);

7.2 Select all reads of *S. scitamineum* from the total data set based on the reference genome (Taniguti *et al.*, 2015);

7.3 Perform statistical analysis of differentially expressed genes;

7.4 Use annotation strategies to compile data of homologs functional predictions and that of experimental approaches;

7.5 Differentiate expression profile of *S. scitamineum* genes infecting resistant and susceptible plants.

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CHAPTER 2: The smut pathogen *Sporisorium scitamineum* uses different strategies to infect resistant and susceptible sugarcane genotypes

ABSTRACT

Sporisorium scitamineum, the causal agent of sugarcane smut disease, is a dimorphic and biotrophic fungus, host-dependent to its sexual reproduction and teliospores formation. The Genomics Group has been extensively studying the sugarcane-smut interaction during the past few years, considering various aspects of the interaction, including pathogen attack mechanisms and plant defense responses. In this study, we present a transcriptomic profile of fungal genes expressed in sugarcane plants resistant and susceptible to smut 48 hours after inoculation, and a comparison with previously obtained data of *S. scitamineum* in axenic culture (Taniguti *et al.*, 2015). Furthermore, we predicted the putative fungal secretome, including candidate effectors, and in association with secreted proteases and hydrolases, revealed their expression patterns among the three treatments. We suggested that different strategies are used by the pathogen to colonize genotypes with different genetic backgrounds considering resistance to smut. *S. scitamineum* infecting resistant plants induced proteases that may be responsible for triggering the host immune system. The success of *S. scitamineum* in reproducing in sugarcane susceptible plants may rely on sets of effector candidates to ensure the biotrophic growth. We unveiled the expression of some genes that may help to develop strategies for the fungal control, for instance, the glucose oxidase, used in several studies as an agent of fungal control. Last, we present some of the antioxidant enzymes (KatE, KatG and Prx1) potentially involved in the colonization of resistant plants.

Keywords: RNA-Seq; *Saccharum* spp.; Transcriptome

1. Introduction

Sugarcane (*Saccharum* spp.) is a major crop used for sugar and biofuels production. The world production of sugarcane in 2018 was 1.9 billion tons, cultivated in an area of 26.3 million hectares (FAO, 2018). In Brazil, the 2018 harvest resulted in 746.8 million tons (which corresponds to 39.3% of the global production), cultivated in 10 million hectares (FAO, 2018). Considering the crescent usage of sugarcane and its subproducts, alongside the great concern in the rational use of land, it is now imperative to seek increased production without increasing the agricultural frontier. Climate changes and the green harvest practices recently implemented in Brazil may influence the incidence of pests and diseases in sugarcane fields (Zhao and Li, 2015). Thus, a possible alternative is the control of damages caused by pests and diseases. Higher temperatures (25-30 °C) and drier conditions usually favor the propagation and spread of diseases (Zekarias *et al.*, 2010), and for sugarcane, ratooning practices induce the

development of symptoms in infected plants (Akalach and Touil, 1996; Croft and Braithwaite, 2006).

Among the diseases that attack sugarcane crops, the smut is one of the most important as it can cause severe damage to the crop, reducing its yield and affecting the region's agriculture economy (Sundar *et al.*, 2012). This disease's causal agent is a biotrophic fungus from the *Ustilaginales* order, *Sporisorium scitamineum* (Syd.) M. Piepenbring, M. Stoll & Oberw (Piepenbring *et al.*, 2002). An elongated whip-like structure covered with spores resembling soot is the main symptom of the sugarcane smut disease. In this whip-like structure occurs fungal reproduction and teliospores production.

The selection of resistant genotypes is still the most efficient way to control smut disease. While there is no immunity to the fungus colonization, the number of whips developed in a given population of infected plants determine resistance or susceptibility. Sugarcane genotypes present different resistance levels to smut disease where less than 15% of whip formation, the genotype is considered resistant (Latiza *et al.*, 1980; Lemma *et al.*, 2015).

Understanding the transcriptional profile of the fungus *S. scitamineum* during the early infection of sugarcane can lead to the identification of fungal genes involved in modulating the plant metabolism and pathogenicity determinants. Among the strategies used by pathogens to penetrate and establish colonization is the secretion of enzymes and effectors. Herein, we investigated the fungal transcriptome 48 hours after inoculation (hai) of two contrasting sugarcane genotypes focusing on secreted proteins and candidate effectors.

5. Conclusions

The sugarcane smut pathogen (*S. scitamineum*) uses different strategies when infecting the two contrasting genotypes SP80-3280 (resistant) and IAC66-6 (susceptible). *S. scitamineum* colonizing resistant plants, which has an early response identified by an oxidative burst, induce antioxidant enzymes' expression to escape the ROS's toxic environment to colonize plant tissues. The host defense response is potentially associated with the perception of proteases produced by the fungus triggering the immune system. On the other hand, *S. scitamineum* infecting susceptible plants supposedly uses a set of effectors to inhibit plant defenses and colonizing the host tissues.

This work provided insights into the expression profile to differentiate colonization of resistant and susceptible genotypes by sugarcane smut pathogen – a guide of candidate genes for further functional characterization studies. Functional studies are essential to comprehend the pathosystem sugarcane-smut and the fungal mechanisms to successfully colonize both genotypes and only complete its cycle in susceptible plants. We propose studies related to the characterization of the gene g5302_chr16_Ss, which is related to glucose oxidase and may play a role in how the fungus can colonize the resistant genotype.

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