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Study of sugarcane metabolism modulation by the plant pathogenic fungus *Sporisorium scitamineum*

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Thesis presented to obtain the degree of Doctor in Science. Area: Agricultural Microbiology

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RESUMO

Estudo da modulação do metabolismo da cana-de-açúcar pelo fungo fitopatogênico *Sporisorium scitamineum*

Esta tese apresenta uma compreensão mais aprofundada da interação entre o fungo patogênico *Sporisorium scitamineum* e a cana-de-açúcar, doença conhecida como "carvão da cana". O desenvolvimento de uma longa estrutura similar a um "chicote" a partir do meristema de plantas infectadas é a principal característica da doença, permitindo a efetiva dispersão dos teliósporos no campo. As plantas doentes apresentam um teor reduzido de sacarose e qualidade do sumo, levando a perdas econômicas consideráveis. No primeiro capítulo, o perfil de expressão gênica do patógeno durante o seu desenvolvimento *in planta* – nos primeiros momentos da infecção e após a emissão do chicote - e *in vitro* foi avaliado utilizando a técnica RNA-Seq. Foram analisados os genes preferencialmente expressos em cada condição, diferencialmente expressos em relação ao crescimento em meio de cultura, ou expressos apenas durante a interação. Os resultados permitiram a elaboração de hipóteses sobre os mecanismos de patogenicidade, sobre os genes candidatos a efetores ativos e a identificação de agrupamentos de genes expressos apenas durante a interação. No segundo capítulo, para determinar o compartimento celular alvo de alguns dos efetores candidatos e estabelecer um protocolo viável para o estudo de proteínas de *S. scitamineum* foi utilizada a técnica de expressão transiente. Os quatro genes mais expressos durante os momentos iniciais da interação que fazem parte do secretoma do fungo foram fusionados ao gene que codifica a proteína verde fluorescente (Citrina) e expressos em *Nicotiana benthamiana*. Os resultados de microscopia confocal e *westernblots* indicaram um acúmulo de cada uma das proteínas candidatas na membrana, citosol e/ou núcleo, além da ocorrência de modificações pós-traducionais. Esses dados oferecem novas oportunidades de estudo para a identificação de proteínas vegetais que interagem com tais efetores. No terceiro capítulo, as respostas transcricionais da cana-de-açúcar nos primeiros momentos de uma interação compatível e após o desenvolvimento do chicote foram analisadas utilizando novamente os dados obtidos a partir do *dual* RNAseq cana-carvão. Entre as principais respostas da cana destacou-se um aumento da expressão de genes que codificam fatores de transcrição do tipo MADS, indicando que o desenvolvimento do chicote pode usar uma rota semelhante à do florescimento, cuja sinalização parece iniciar logo nos primeiros momentos de colonização. Além disso, o desenvolvimento do chicote é acompanhado pelo aumento da transcrição de genes envolvidos em vias energéticas, e vias de síntese e sinalização hormonal. Genes que codificam para RGAs foram diferencialmente expressos e podem estar relacionados ao reconhecimento de efetores. No quarto capítulo, foi avaliado o perfil metabólico da cana-de-açúcar durante a progressão da doença, confirmando que no meristema de plantas infectadas ocorre um aumento da alocação de carbono em vias energéticas, além da regulação de vários aminoácidos e mudanças em relação à composição da parede celular em resposta ao desenvolvimento do chicote. A abordagem metabólica também permitiu a identificação de uma provável micotoxina derivada de *S. scitamineum*. Os resultados obtidos neste estudo contribuíram para aumentar a compreensão da interação entre *S. scitamineum* e a cana-de-açúcar que se caracteriza pela alta complexidade e especialização ao hospedeiro, e poderão ser utilizados de forma a auxiliar a caracterização de variedades resistentes e contribuir para o melhoramento da cana-de-açúcar com resistência ao carvão.

Palavras-chave: 1. Carvão da cana-de-açúcar 2. Transcriptômica 3. Efetores 4. Metabolômica 5. Interação planta-patógeno

ABSTRACT

Study of sugarcane metabolism modulation by the plant pathogenic fungus Sporisorium scitamineum

This thesis presents a more in-depth understanding of the interaction between the pathogenic fungus *Sporisorium scitamineum* and sugarcane, a disease known as "cane smut". The development of a long structure like a "whip" from the meristem of infected plants is the main characteristic of the disease, allowing the effective dispersion of teliospores in the field. Infected plants have a reduced sucrose content and juice quality, leading to considerable economic losses. In the first chapter, the gene expression profile of the pathogen during its development *in planta* - in the first moments of infection and after the emission of the whip - and *in vitro* was evaluated using the RNAseq technique. Were analyzed genes preferentially expressed in each condition, differentially expressed in comparison to its growth *in vitro*, and expressed only during interaction. The results allowed the identification of some potential pathogenicity mechanisms, active effectors and gene clusters expressed only during interaction. In the second chapter, the transient expression technique was used to determine the target cell compartment of some of the candidate effectors and to establish a viable protocol for the study of *S. scitamineum* proteins. The four putatively secreted genes most expressed during the initial moments of the interaction were fused to the gene encoding the fluorescent green protein (Citrine) and expressed in *Nicotiana benthamiana*. The results of confocal microscopy and *westernblots* indicated an accumulation of each candidate protein in the membrane, cytosol and/or nucleus, in addition to the occurrence of post-translational modifications. These data offer new study opportunities for the identification of plant proteins that interact with such effectors. In the third chapter, the transcriptional responses of sugarcane in the first moments of a compatible interaction and after the development of the whip were analyzed using again the data obtained from the dual RNAseq cane-smut. Among the main responses, was identified an increase in MADS-type transcription factors expression, indicating that the whip development may use a route similar to flowering, whose signaling seems to start as early as the colonization. In addition, whip development is accompanied by increased transcription of genes involved in energetic pathways, and hormones synthesis and signaling pathways. Genes encoding RGAs were differentially expressed and may be related to pathogen effector's recognition. In the fourth chapter, the metabolic profile of sugarcane was evaluated during disease progression, confirming that in the meristem of infected plants carbon allocation is channeled to energetic pathways, besides the regulation of several amino acids and changes in plant cell composition in response to whip development. Metabolomics approach also allowed the identification of a probable mycotoxin derived from *S. scitamineum*. The results obtained in this study contributed to increase the understanding of the interaction between *S. scitamineum* and sugarcane that is characterized by high complexity and specialization to the host, and can be used in a way to help the characterization of resistant varieties and contribute to the improvement of sugarcane with resistance to smut.

Keywords: 1. Sugarcane smut 2. Transcriptomics 3. Effectors 4. Metabolomics 5. Plant-pathogen interaction

1. INTRODUCTION

Sugarcane is one of the most economically valuable crop worldwide, used as the main raw material to sugar and ethanol production. However, decrease in productivity due to biotic and abiotic stresses is a shared concern among producers. Regardless being a very rustic crop, sugarcane hosts several important pathogens that threatens every year productivity because the appearance of new pathogenic races. Sugarcane smut is one of the most harmful disease to the culture, causing losses in all sugarcane-producing countries due to a reduction in sugar content and juice quality. The disease is caused by the biotrophic basidiomycete *Sporisorium scitamineum*. The infection initiates with teliospore germination originating haploid cells of opposite mating types, which may combine to form the infective dikaryotic hyphae and colonize sugarcane tissues leading to teliospores differentiation. Disease cycle ends with the development of a structure like a whip, where billion of teliospores are formed and can easily be spread in the field by wind, achieving germinating buds and restarting the infection cycle.

This work brings new pieces to solve the "puzzle" of this unique interaction using omics approaches to evaluate both fungus and plant responses. The thesis was built on the hypothesis that *S. scitamineum* activates the expression of genes related to pathogenicity including those encoding a set of uncharacterized secreted proteins that may act as effectors inside different compartments of plant cells; whereas, sugarcane answers at transcriptional and metabolic levels to deal with *S. scitamineum* colonization. The understanding of this molecular cross-talking may bring new clues about genes and pathways contributing to plant susceptibility and disease symptoms development. To validate these hypotheses, RNAseq technique was used to evaluate *S. scitamineum* transcriptional profiles in early stages of the interaction and after whip development compared to its gene expression profile in culture medium growth, allowing the determination of pathogen set of genes preferentially expressed in each condition and genes expressed only during interaction. Predictions regarding effector proteins were depicted in a second step, by assessing plant cell compartment targeted by *S. scitamineum* candidates using *Nicotiana benthamiana* transient expression, as a first attempt to determine hereafter plant receptors. To access sugarcane responses to smut differential transcriptional profile of the susceptible sugarcane variety "RB925345" in the early stages of smut disease and after whip development was determined. Because of a strong metabolic change of sugarcane symptomatic plants, the metabolic profile of plants during the disease progression was compared to plants of normal growth. The results of this work provide valuable information about the sugarcane smut

disease, and represent a starting point for further research aiming the understanding of resistance mechanisms and factors involved in pathogen recognition.

1. FINAL CONSIDERATIONS

In the present study, it was determined global responses concerning sugarcane smut disease in both plant and pathogen sides. Transcriptome data analysis revealed genes from *S. scitamineum* that are good candidates acting as effector in early moments of the interaction or related to sporulation. Also, the repertoire of genes activated during growth *in planta* suggests several mechanisms that confer advantages to the pathogen, such as cell-wall degrading enzymes, nutrient transporters and detoxification enzymes. Genes encoding to putative effectors target several plant compartments, and their characterization in model systems will allow to determine more precisely mechanisms involved in pathogen recognition or signalization to susceptibility.

Sugarcane responses at transcriptional levels suggest a premature transcriptional reprogramming of the shoot meristem functions continuing until the emergence of the whip. The guidance of this altered pattern is potentially related primarily to auxin mobilization in addition to the involvement of other hormonal imbalances. Several MADS-type transcription factors were up-regulated, indicating that the development of the whip can use a route similar to flowering. Genes encoding RGAs were differentially expressed and may be related to pathogen effector's recognition.

Metabolomics data from sugarcane-smut interaction supported many hypotheses built on transcriptome data, such as the increased energy-related pathways, and the accumulation of starch to feed whip development. Additionally, disease progression was characterized by a shift in the primary metabolism between 65 and 100 DAI, especially of those metabolites related to cell wall biosynthesis, suggesting loosening of the cell wall to allow whip growth. However, increased levels of tyrosine in infected plants may be related of differential PTAL gene expression possibly leading to the synthesis of lignin to feed whip development. Metabolomics also allowed the identification of fungal metabolites, opening a new opportunity in the study of sugarcane smut pathogen, and provided some biomarkers worth to be exploited.

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