University of São Paulo
"Luiz de Queiroz" College of Agriculture

Arabidopsis thaliana as a model plant for the study of plant-pathogen interaction between *Sporisorium scitamineum* and sugarcane

Mariana Marrafon Lopes da Silva

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

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Mariana Marrafon Lopes da Silva
Biologist

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Advisor:
Prof. Dr. **CLAUDIA BARROS MONTEIRO-VITORELLO**

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To my parentes Cleusa and Geraldo

To my sister Clareana and to my aunt Cleide

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SUMMARY

RESUMO

Arabidopsis thaliana como planta modelo para o estudo da interação planta-patógeno entre *Sporisorium scitamineum* e cana de açúcar

Entre as doenças que impactam a produtividade da cana de açúcar está o carvão, causado pelo fungo basidiomiceto *Sporisorium scitamineum*. Os genótipos modernos da cana de açúcar são híbridos interespecíficos altamente poliploides. Essa característica restringe estudos moleculares e análises funcionais para a identificação de genes candidatos à resistência e a prova de conceitos. Nesse sentido, o presente trabalho avaliou o uso de *Arabidopsis thaliana* como planta modelo para a colonização de *S. scitamineum* por meio de análises morfológicas e moleculares. As análises morfológicas demonstraram que *S. scitamineum* reconhece *A. thaliana* como um hospedeiro e permite a germinação de telósporos, a formação de appressório e o crescimento de hifas do fungo nas folhas. As plantas infectadas apresentaram alguns sintomas como: clorose, acúmulo de antocianina no meristema e folhas, redução da biomassa e ramificação das raízes em estágios mais avançados na colonização. As análises moleculares detectaram expressão de genes relacionados à patogenicidade descrita para carvões, bem como para novos candidatos a efetores envolvidos na interação *S. scitamineum*-cana. *S. scitamineum* também orquestrou uma modulação da expressão de genes *A. thaliana* associados a alterações meristemáticas e à via do ácido jasmônico; as mesmas alterações também foram observadas em *S. scitamineum*-cana. Este é o primeiro relato do uso de planta modelo para estudo do carvão da cana de açúcar. Os resultados evidenciam que o fungo é capaz de causar alterações nas vias metabólicas do hospedeiro alternativo, semelhante ao seu hospedeiro natural. O estabelecimento do patossistema *S. scitamineum-A. thaliana* permitirá que várias aplicações futuras compreendam melhor a interação entre *S. scitamineum* e cana de açúcar.

Palavras-chave: Planta modelo; Carvão, Efetores; Hospedeiro não-natural

ABSTRACT

Arabidopsis thaliana as a model plant for the study of plant-pathogen interaction between *Sporisorium scitamineum* and sugarcane

Among the diseases that impact sugarcane productivity is smut, caused by the fungus basidiomycete *Sporisorium scitamineum*. Modern sugarcane genotypes are highly polyploid interspecific hybrids. This feature restricts molecular studies and functional analyses for the identification of resistance candidate genes and the proofs-of-concept. The present work evaluated the use of *Arabidopsis thaliana* as a model plant for *S. scitamineum colonization* through the use of morphological and molecular analyses. The morphological analyses demonstrated that *S. scitamineum* recognizes *A. thaliana* as a potential host allowing teliospore germination, appressorium formation and hyphal growth on leaves. The infected plants showed symptoms such as: chlorosis, accumulation of anthocyanin in the meristem and leaves, reduction in biomass, and roots ramification in advanced stages of the colonization. Molecular analyzes detected a transcription reprogramming of genes related to pathogenicity described for smuts, as well as for new effector candidates involved in *S.scitamineum*-sugarcane interaction. *S. scitamineum* also modulated the expression in *A. thaliana* gene associated with meristematic functions; changes also observed in *S. scitamineum*-sugarcane interaction. This is the first report of using a model plant to study sugarcane smut. The establishment of the pathosystem *S. scitamineum*- *A. thaliana* will allow several future applications to understand better *S. scitamineum*-sugarcane interaction

Keywords: Model plant; Smut; Effectors; Non-natural host

PREFACE

Sugarcane is one of the most important crops for agribusiness worldwide. Brazil is the world's largest sugar exporter and this crop is one of the agricultural commodities that most contributes to the Brazilian economy (FAO, 2019; MAPA, 2018). Sugarcane and its byproducts are raw material in various branches of industry. Considering the diverse and growing possibilities of their applications, it is of fundamental importance enhancing productivity without necessarily expanding agricultural frontiers. One way to achieve this goal is to control the damage caused by pests and diseases. Among the diseases that most affect sugarcane cropping is smut, caused by the biotrophic fungus *Sporisorium scitamineum*, and characterized by the formation of a black structure at the apex of the plant, popularly called smut whip. The disease impacts production mainly due to an increase in the fibrous content of stalks, decreasing the concentration of reducing sugars and sucrose. Currently, the primary disease control measures include seedling phytosanitary certification and the use of resistant varieties. However, *S. scitamineum* can also colonize resistant genotypes, only occasionally producing whips, a fact that could cause yield losses not yet estimated.

Therefore, it is relevant to obtain information about resistance and the modulation of host physiology that lead to the development of whip in susceptible plants. In other species infected by smut fungi, it was established a correlation between the sexual propagation of the pathogen and the modulation of the host reproductive pathways (Schuster et al., 2016). A similar type of response was also suggested for sugarcane smut disease (Schaker et al., 2016). However, in this case, an earlier transition from vegetative to reproductive stages was identified. The *S. scitamineum*–sugarcane transcriptome analysis revealed an enrichment of GO terms (Gene Ontology) related to plant reproductive pathways in differentially expressed genes since soon after inoculation until after whip emission. Limited information is available for sugarcane control of the transition from vegetative to reproductive development stages and flowering (Moore and Botha, 2013; Moore and Maretzki, 2017; Coleman, 1969). On the other hand, the scenario available for *Arabidopsis thaliana* regarding flowering is well-detailed and used as a model for various flowering plants, even for those distantly related.

The working hypothesis of this study is that *A. thaliana* serves as a model for smut-sugarcane interaction in the early stages, considering the pathogen colonization and its influence in meristematic functions.

CHAPTER 1: General Introduction

1. Sugarcane

Sugarcane (Saccharum sp.) is of economic importance in the modern world. The main byproducts originated from this crop are sugar and ethanol, but it can also be used as a substitute for petroleum derivatives (bioplastic and hydrocarbon production) or as a source of bioelectricity (Sugarcane.org, 2019). Brazil lost the first position of the largest sugar producer to India, but still is the world's largest sugar exporter (FAO, 2019). Sugarcane cultivation in Brazil occupies 8,589.2 million hectares, São Paulo contributing with more than 50% of the total production (Conab, 2019). It is estimated that 20% of the country's cities are directly or indirectly influenced by the sugar and alcohol industries, which generate almost 1 million direct jobs (IRENA, 2015; Moraes et al., 2016).

Belonging to the Poaceae family, as well as other economically important crops such as corn, wheat and rice, modern sugarcane varieties are complex interspecific hybrids of the genus Saccharum, with contributions mainly from the species *S. spontaneum* and *S. officinaru*m (Amalraj and Balasundaram et al., 2006; D'Hont et al., 2005). Genetic improvement was performed to solve disease-related problems as well as improve productivity by combining robustness and high sugar content attributes (Cheavegatti-Gianotto et al., 2011; Roach, 1972). Due to the numerous crossings carried out throughout the breeding process, sugarcane has become a crop of high genetic complexity (D'Hont et al., 1996). The sugarcane genome has a high level of aneuploidy, 2n = 108-118 (D'Hont et al., 1996) with an estimated size of approximately 10,000 Mpb (genotype " R570 ", 2n = 115) (D'Hont and Glaszmann 2001). Understanding the sugarcane genome as well as understanding the functioning of gene sets involved in essential processes such as sugar accumulation and disease resistance are being deciphered through the use of several strategies such as ESTs (Vettore et al., 2001; Vettore et al., 2003), RNAseq (Cardoso-Silva et al., 2014) and complete genome sequencing (de Setta et al., 2014; Garsmeur et al., 2018; Zhang et al., 2018; Souza et al., 2019)

2. *Sporisorium scitamineum* and the smut disease

The sustainability of sugarcane production in Brazil stands on the continuous supply of varieties resistant to pests, diseases, and climate variations. Among diseases affecting sugarcane productivity are rust, pineapple rot, leaf scald, ratoon stunting, and sugarcane smut. Smut disease, when uncontrolled, can cause mass reduction by about 40%, decreasing Brix, and consequently reducing the final sugar and alcohol production (Marchelo-d'Ragga, and

Bukhari, 2015). when infected, susceptible plants develop a long whip-like structure from the primary meristem formed by a central region of parenchymal cells and vascular tissues of the host plant surrounded by billions of fungal teliospores (Figure 1). Whip emission may take up to 6 weeks after infection in highly susceptible genotypes, whilst tolerant ones, the whip may develop only at the regrowth cycle.

Disease resistance is assigned based on the number of plants that emitted whips in a population artificially inoculated in a given period (Bailey et al.,1982). The scale of resistance ranges from 0 (no whip emitted in the population) to 9 (25.6 to 100% of plants emitted whips in the population). A highly susceptible genotype scores 9.

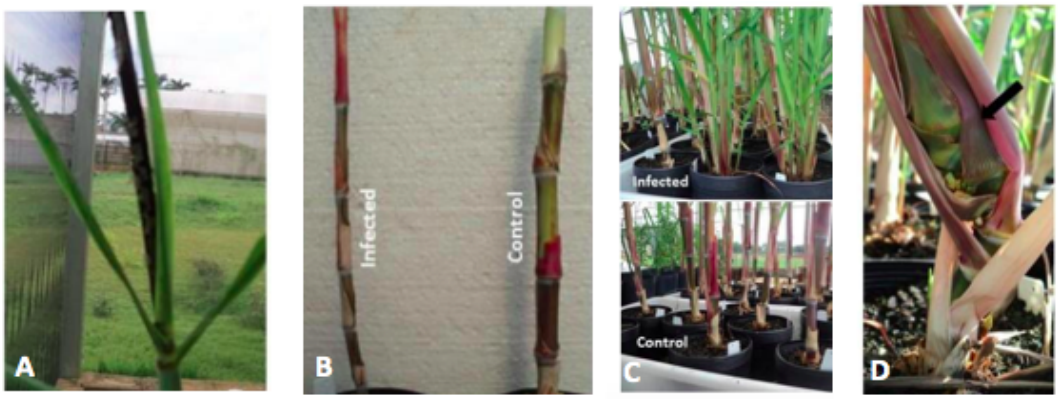

Figure 1. Symptoms of smut disease in sugarcane, cultivar RB925345 after whip development. (A)Whip development. (B) Stalks diameter reduction (C) Tillering. (D) Lump formation next to the buds (Schaker et al., 2016).

S. scitamineum is a basidiomycete, biotrophic, and dimorphic fungus (Singh et al., 2004). Sugarcane infection begins with the germination of teliospores (2n), especially in undifferentiated epithelial tissues such as those of the meristems in sprouting buds. The germination of teliospores produces probasidium that undergoes meiosis originating four haploid sporidial cells (n) (Waller, 1970). Haploid cells have two opposite and complementary (+ and -) sexual reaction types (Bakkeren and Schirawski, 2008; Waller, 1970). The genes of the sexual reaction *locus a* encode to a membrane receptor and a pheromone that are compatible with the opposite sex type membrane and pheromone receptor. The genes of *locus b* encode subunits of a heterodimeric transcription factor. The fusion of sexually compatible haploid cells allows the formation of the infectious dikaryotic hyphae (n + n) (Figure 2) (Sundar et al., 2012; Taniguti et al., 2015). The penetration of the dikaryotic hyphae into the plant tissue occurs through the appressorium, characterized by widening the end of the hyphae, which is capable of exerting a mechanical force to aid the fungus penetration.

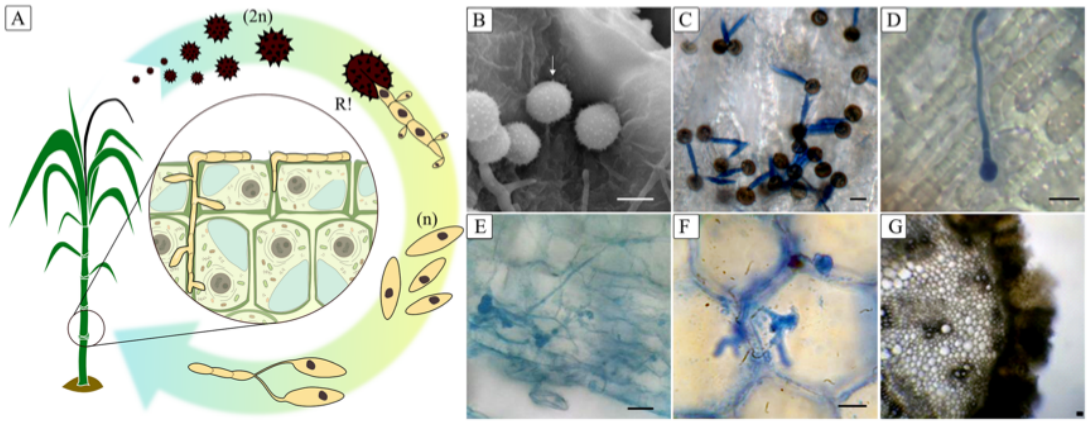

Figure 2. *Sporisorium scitamineum* life cycle. (A) Scheme of haploid, dikaryotic and diploid phases. (B) Teliospores. (C) Spores germination. (D) Apressorium. (E) Fungal growth in the buds. (F) Fungal growth in the parenchyma of the infected plant. (G) Mature teliospores. Scale 5 µm (Taniguti et al., 2015).

The spread of the disease in cultivated fields occurs through teliospores dispersion carried by rain and wind. Environmental conditions are crucial in the emergence of smut epidemics. Under conditions of drought and heat stress, the occurrence of the disease is favored even in varieties considered resistant (Lemma et al., 2015). Currently, the measures used to control the disease relies on seedling phytosanitary certificates, genetic diversity in sugarcane fields, and the use of resistant varieties (Lemma et al., 2015; Croft et al., 2008; Nalawade et al., 2013; Singh et al., 2005). The impact of the disease in Brazil has changed in recent years because of the new law of mandatory green harvest practices. Burn-before-harvest agricultural practices, although environmentally very damaging, had the benefit of controlling the inoculum of fungal diseases throughout ratoon cycles.

3. Plant-pathogen interaction

Plant-pathogen interaction is a result of a very complex co-evolution process. However, disease occurrence is always an exception in nature. The majority of the plants exhibit non-host resistance (NHR), which presents a sophisticated genetic control and involves several defense factors (Heath et al., 1985). NHR, as defined by Heath (2000), considers: “resistance shown by an entire plant species to a specific parasite or pathogen”. Unlike NHR, host resistance (HR) is characterized by parasite-specific restricted to a particular pathogen species. HR is often controlled by a single resistance (R) gene, which interacts with specific pathogen elicitors activating the defense response (Hammond-Kosack et al., 1997).

In both cases, the resistant phenotype relies on the activation of the innate immune system, which is formed by two distinct defense mechanisms: pattern recognition receptors (PRR) and

nucleotide binding site leucine rich repeat (NB-LRR) (Dangl and Jones, 2001). The first barrier requires the PRR (Pattern Recognition Receptors) that are transmembrane proteins able to identified molecular patterns associated with a microbial pathogen (PAMPs) such as flagellin or chitin (Zipfel and Felix, 2005). The second barrier includes the NB-LRR proteins, localized inside the host cell, able to recognize pathogen effector molecules and to activate the signaling for defense response mechanisms; the so-called effectors triggered immunity (ETI) (Glazebrook, 2005; Jones and Dangl, 2006)

Plants have a set of NB-LRR proteins able to recognize a diverse number of pathogen effectors. Jones and Dangl (2006) described the zig-zag model for resistance and effector proteins evolution. Mutations altering effector sequences scape recognition by the host cell and lead to susceptibility. New resistance genes selected throughout evolution turn host cells resistant again. The so-called arms-race system of evolution (Jones and Dangl, 2006)

4. Effectors and phytohormones

In order to successfully conquer host infection, the pathogens secrete molecules known as effectors capable of subverting the immune responses and facilitate the host colonization. (Cook et al., 2015; Rovenich et al*.,* 2014). According to the most conventional definition, effectors are small cysteine-rich proteins whose function is the subversion of plant immune responses. In general, these molecules block or inhibit pathways necessary to defense response, such as the activation of ROS (reactive oxygen species) metabolism, and biosynthesis of salicylic acid (SA) and jasmonic acid (JA).

One of the earliest plant cell responses is the oxidative burst; plants produce ROS immediately after pathogen recognition to restrain pathogen growth and to activate the immune response defense (Wojtaszek, 1997; Torres et al., 2006). However, the effectors produced by the pathogens can sometimes modulate this response, succeeding colonization. The effector PEP1, for instance, described as a core effector of smut fungi, blocks the activity of class III peroxidase, delaying the production of ROS and the activation of the defense response (Hemetsberger et al., 2012)

The host response defense also involves hormones such as SA and JA (Yang et al., 2015). The SA is produced from the phenylpropanoid metabolism and synthesized in plastids (Chen et al., 2009; Djamei et al., 2011). It is known that the external application of SA increases the expression of genes encoding pathogenesis-related proteins (PR), leading to resistance (Loake and Grant, 2007; Bertini et al., 2003). The genomes of smut fungi encode an effector

protein with chorismate mutase activity (CMU1). This protein acts by converting chorismate to prephenate, making chorismate unavailable for SA biosynthesis (Djamei et al., 2011). Other smut effectors also target SA, such as PIT2 (PROTEIN INVOLVED IN TUMORS 2) and STP1 (STOP AFTER PENETRATION 1) that are inhibitors of cysteine proteases localized in both the cytoplasm and apoplast of host cells (Doehlemann et al., 2011; Liang, 2013). The activity of cysteine proteases induces the synthesis of SA.

Interestingly different phytohormones could trigger the same defense response; for instance, SA and JA induce the anthocyanin accumulation (Shan et al., 2009; Lu et al., 2017; Di et al., 2017). However, the increase of anthocyanin content in a plant could also be intermediated by pathogen effectors. For example, the smut effectors RSP3 (REPETITIVE SECRETED PROTEIN 3) and TIN2 act in the host inducing the anthocyanin accumulation (Ma et al., 2018; Tanaka et al., 2019).

The JA also promotes resistance against pathogenic microbes trhough transcriptional responses triggered by jasmonyl-isoleucine that reprogram the metabolism to produce several defense compounds (Guo et al., 2018). The immune response is metabolically costly and associated with reduced growth. (Yan et al., 2007; Bömer et al., 2018).

Nevertheless, besides being a key immune signal, JA also affects developmental processes, including roots growth and flowering time (Guo et al., 2018) This regulation is mediate by the interaction of CORONATINE INSENSITIVE1 (COI1) / JAZMONATE ZIM DOMAIN proteins (JAZ). JAZ proteins are negative regulators of the JA-signaling pathway and target SCF^{COI1} complex, where COI1 is the F-box protein that functions as an E3 ubiquitin ligase (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The degradation of JAZ proteins mediated by the COI1 F-box, positively regulates the JA signaling (Thines et al., 2007). However, the repression of JAZ releases TARGET OF EAT1 (TOE1) and TOE2, both of which negatively regulate the transcription of FLOWERING LOCUS T (FT) and delay the flowering time of Arabidopsis (Zhai et al., 2015)

The flowering time is also regulated by auxin (Zhao, 2010). The activation of meristem differentiation is auxin concentration-dependent; nonetheless, this hormone is not homogeneously distributed at the shoot apical meristem (SAM) (Vernoux et al., 2010). In this regard, auxin transporters, such as PIN1(Auxin Efflux Carrier Component 1), set up the auxin concentration, being crucial regulators of flowering time (Vernoux et al., 2010). Besides the impact in the development process, auxin also acts in plant-pathogen crosstalking. Several pathogens synthesize the auxin indole-3-acetic acid (IAA) and/or virulence factors to

orchestrate host auxin signaling (Kunkel et al., 2017). Auxin can also affect the biology of some pathogens acting as a microbial signaling molecule (Kunkel et al., 2017).

5. Crosstalk between *S. scitamineum* and sugarcane

The plant immune response requires a significant resource investment from the plant, which may compromise its reproductive development. Therefore, the plant can either activate the immune system to fight the pathogen directly or accelerate its floral development so that the plant generates offspring before its death (Lyons et al., 2015). In the case of sugarcane - *S. scitamineum* pathosystem, Schaker et al. (2016) suggested that the pathogen influences the host reproductive pathways directing resources to the production of the whip where the fungal sporogenesis and spores dissemination take place.

More specifically, Schaker et al. (2016) suggested that *S. scitamineum* orchestrates transcriptional reprogramming of sugarcane meristematic functions. Orthologs of genes involved in the transition from vegetative to reproductive stages and those related to auxin and jasmonate signaling exhibited differential expression since early time points of the interaction when compared to mock plants (Schaker et al., 2016; Liu et al., 2017). These variant transcriptional patterns suggested that the events leading to the whip emission might involve shared pathways related to flowering and that the reprogramming of SAM initiates shortly after fungal contact (Schaker et al., 2016).

The pathogen infection influences the differential expression of genes such as CNA (CORONA) and BAM (BARELY ANY MERISTEM). CORONA belongs to the transcription factor (TF) family of the homeodomain-leucine zipper (HD-Zip) Class III (ATHB-15; CNA) (Green et al., 2005; Dengler, 2006) and BAM proteins are leucine-rich repeat receptor-like serine/threonine- RD kinases (LRR-RLKs) (Afzal et al., 2008). These proteins have similar roles in stem cells specification and organogenesis of the CLV (CLAVATA) proteins of Arabidopsis (Green et al., 2005; DeYoung et al., 2006; Dengler, 2006).

Also, two other genes COL6 and LNG were related to meristem functions. COL6 belongs to the CONSTANS family and encodes a putative zinc finger TF promoting the induction of flowering in Arabidopsis at long photoperiods through the activation of floral meristem-identity genes, such as LFY (Simon et al., 1996; Lagercrantz and Axelsson, 2000). LFY, in turn, controls the expression of genes coordinating reproductive development and disease response (Winter et al., 2011). This control ensures optimal allocation of plant resources for reproductive fitness and/or survival. For LNG, mutants overexpressing the LONGIFOLIA-

like gene in *A. thaliana* have long petioles, narrow but extremely long leaf blades with serrated margins, elongated floral organs, and elongated siliques, caused by polar cell elongation (Lee *et al.*, 2006).

These features resemble the whip developing following meristem modification in sugarcane. After whip emission the APETALA2/Ethylene RESPONSIVE FACTOR (AP2/ERF); a transcription factor GRF8 of *O. sativa* belonging to the GROWTH-REGULATING FACTOR family (GRF); a protein related to organ specification orthologous of VIN3- like proteins (VERNALIZATION INSENSITIVE protein); a bHLH transcriptional factor; a Myb-type transcription factor responsive to FLC; a trihelix transcription factor ASIL2; a LONGIFOLIA 2- like protein (LNG2); and an ARR12-like (*ARABIDOPSIS* RESPONSE REGULATOR) are all up-regulated, strengthening the hypothesis.

It is hypothesized that these genes may coordinate the pattern of whip development as an alternative route to flowering (Schaker et al., 2016)

6. *A. thaliana* as a model plant in the study of plant-pathogen interaction

From a temperate climate, Arabidopsis is a member of the mustard family (Cruciferae or Brassicaceae) (Meinke et al., 1998). Although considered a weed of small agricultural importance, Arabidopsis for over 40 years has been the focus of valuable research. The use of model organisms is extensive in the study of complex systems by similarity. Usually, they have a short life cycle; they are small in size; they have large offsprings, and simple genome. The entire life cycle of Arabidopsis, encompassing seed germination until the maturation of the first seeds, is completed in 6 weeks (Meinke et al., 1998).

The Arabidopsis complete genome sequence encompasses 135 million base pairs organized in five chromosomes. Approximately 30,000 are the number of genes encoding proteins in Arabidopsis (Cheng et al., 2017; Swarbreck et al., 2008). Arabidopsis has several metabolic pathways deciphered, including flowering (Dennis and Peacock, 2009; Fornara and Coupland, 2010; Arabidopsis Genome Initiative, 2000). The use of *A. thaliana* as a model plant is possible because of biological principles, such as metabolic and regulatory pathways, and the genes that encode them are conserved during evolution (Delatorre et al., 2008).

For the study of plant-pathogen interaction, the most widely used model organism is *A. thaliana*. *A. thaliana* has proven to be an excellent model for answering molecular questions of plant-pathogen interaction. For decades of studies using *A. thaliana* as a model, significant contributions helped to explain the evolution and the underlying mechanisms of disease resistance and susceptibility. Arabidopsis was essential for the genetic validation of the gene

by gene hypothesis (Flor, 1947). Currently, it is possible to find numerous studies using *A. thaliana* as a model plant for diseases caused by fungi such as *Blumeria graminis*, *Cladosporium fulvum*, *Magnaporthe oryzae* among others (van Esse et al., 2008; Schmidt et al., 2014, Park et al., 2009)

In the study of smuts, Arabidopsis served to reveal the expression of *U. maydis* genes. Méndez-Morán et al. (2005) conducted experiments showing fungal colonization of Arabidopsis tissues, and Martínez-Soto et. al. (2013) analyzed the transcriptome of the interaction. This later work showed that a similar set of fungal genes are responsive when Ustilago infects either maize or Arabidopsis. Even genes such as the effector of the fungus PEP1, specific to the process of plant infection, showed a higher level of expression in *A. thaliana.* For *Sporisorium reilianum*, a biotrophic fungus also causing smut in maize, Arabidopsis was used to reveal the function of the fungal effector SAD, responsible for the loss of apical dominance and the formation of multiple inflorescences in the subapical stem nodules (Drechsler et al., 2016).

Despite being a complex system, there are no reports in the literature of the use of *A. thaliana* as a model plant for interaction *S. scitamineum* and sugarcane.

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CHAPTER 2: *Sporisorium scitamineum* induces molecular and phenotypic modulation in non-natural host *Arabidopsis thaliana*

Abstract

Sugarcane is one of the most important crops in the world. This crop production is affected by several diseases, from which smut caused by the fungus *Sporisorium scitamineum* is one of the most relevant. The main sign of the disease is the whip emergence at the apical shoot. Toward whip formation, the fungus can modulate the expression of genes associated with hormones such auxin and jasmonic acid in sugarcane and also induces alterations in genes associated to meristematic functions. Nevertheless, the high complexity of the sugarcane genome, its long life cycle, and restriction to flowering in the subtropical area hamper a deeper study of this interaction. Therefore, we proposed the use of *A. thaliana* as a model plant to study tissue colonization by *S. scitamineum*. Our results showed that *S. scitamineum* could not only colonize but also orchestrated transcriptional changes in the tissues of the alternative host. Molecular analysis confirm a differential expression of genes related to acid jasmonic pathways (JAZ4) and to meristematic functions (BAM1). During colonization, anthocyanin accumulation at leaves and meristematic regions was detected besides an abnormal growth of lateral roots in the non-natural host. The outcome suggests that *A. thaliana* could be a good model to study early moments of smut interaction, accelerating the understanding of disease establishment.

Keywords: Plant model; Smut; Sugarcane; Effectors; Non-natural-host

1.Introduction

Sugarcane is one of the most important crops for modern world agribusiness. There are numerous possibilities of using sugarcane and its by-products as a raw material in diverse branches of industry, in which their high potential for generating clean energy is the most attractive nowadays (Ali et al., 2019). The efforts to increase productivity to meet future demands of biofuel require an association with the growing concern about land use, involving increasing productivity without necessarily expanding agricultural frontiers. Improve the control of damage caused by pests and diseases is one of the potential measures. Among the diseases affecting sugarcane, smut caused by the biotrophic fungus, *Sporisorium scitamineum* has reemerged in the international scenario potentially due to climate changes and the late demand for green harvest practices.

Sugarcane smut first documented in Natal, South Africa, in 1877, is currently affecting sugarcane growing fields worldwide (reviewed in Monteiro-Vitorello et al., 2018). Differently from other smut species, *S. scitamineum* infects through the germinating buds and colonizes the shoot apical meristem altering the reproductive development of the plant (Marques et al., 2016).

The host range of *S. scitamineum*, similar to other smut fungi, is very narrow (Antonovics et al., 2013). So far, it has been described infecting only Saccharum species (Benevenuto et al., 2018). The main sign of the disease is the emergence of a black whip-like structure at the apical shoot where the fungal sporogenesis takes place. The structure includes a mixture of teliospores and plant tissues. Rain and wind disperse teliospores for long distances contaminating the whole field and over successive ratoon crops.

Teliospores deposited on sprouting buds under suitable conditions of humidity and temperature germinate by budding. Sporidial cells of opposite mating-type fuse to produce the infectious dikaryotic hyphae essential for host penetration (Sundar et al., 2012). Swollen hyphal tips differentiate appressorium for fungal penetration, and inter- and intra-cellular colonization. *S. scitamineum* does not produce a typical haustorial structure but instead develops intracellular hyphae with a comparable role (Marques et al., 2016). The fungus can infect and colonize both susceptible and resistant genotypes; however, only occasionally, resistant plants develop whips (Carvalho et al., 2016). Resistant plants respond to the pathogen penetration by inducing an oxidative burst, likely to delay fungal growth and to signal for defense mechanisms (Peters et al., 2017).

Sugarcane responses to *S. scitamineum* are still poorly understood despite numerous investigations describing molecular events at transcriptomic, proteomic, and metabolomic levels (Schaker et al., 2016; Que et al., 2014; Barnabas et al., 2016; Su et al., 2016; Sigh et al., 2019; Peters et al., 2017; Schaker et al., 2017). The complete genome sequence of *S. scitamineum* is available with 26 chromosomes defined from telomere to telomere (Taniguti et al., 2015) and a repertoire of candidate effector proteins predicted (Benevenuto et al., 2018). Some studies stated that the *S. scitamineum* orchestrates a transcriptional reprogramming of sugarcane meristematic functions. Orthologues of genes involved in the transition from vegetative to reproductive stages and those related to auxin and jasmonate signaling exhibited differential expression since early time points of the interaction when compared to mock plants (Schaker et al., 2016; Liu et al., 2017). Although whips emerge in the late stages of the disease, the reprogramming of shoot apical meristem (SAM) initiates shortly after fungal contact (Schaker et al., 2016). These variant transcriptional patterns suggested that the events leading to the whip emission might involve shared pathways related to meristematic functions.

Modern sugarcane cultivars are highly polyploid and heterozygous interspecific hybrids, imposing difficulties in the identification of resistance candidate genes and proofs-of-concept in plant-pathogen interactions (Thirugnanasambandam et al., 2018). Besides, other

experimental constraints such as the long life cycle and the restrictions for flowering in subtropical regions impair obtaining a detailed genetic analysis for pathosystems involving sugarcane.

Several metabolic pathways held their first description in *Arabidopsis thaliana*, in which three out of four gene families identified have orthologs in other flowering plants (Woordward and Bartel et al., 2018). A model has been proposed for sugarcane flowering on previously published pathways observed in Arabidopsis (Glassop and Rae, 2019; Coelho et al., 2014). Arabidopsis is the prime model used in various branches of the plant science research offering essential advantages for basic research in genetics and molecular biology. *A. thaliana* is a small annual flowering plant with a short life-cycle that grows well in lab settings, besides having an entire genome sequenced freely available in various curated databases (Araport; Tair and ePlant) and many functionally characterized mutant lines (Woodward and Bartel et al., 2018).

Arabidopsis is also a model for plant-pathogen investigations, either considering closely or distantly-related plant species and infected with from necrotrophic to biotrophic fungi and oomycetes pathogens (Birkenbihl et al., 2011; Méndez-Morán et al., 2005; Huitema et al., 2003). It can produce responses such as those detected on a non-natural host, and also used in studies of non-host resistance (Méndez-Morán et al., 2005; Martínez-Soto et al., 2013; Pereira et al., 2019).

Considering the interaction with smut fungi, colonization of Arabidopsis plants results in symptoms such as leaf chlorosis, reduced plant size, anthocyanin accumulation and increased lateral roots when infected by *S. reilianum* (Martínez-Soto et al., 2019) or *U. maydis* (Méndez-Morán et al., 2005). Transcriptomic analysis revealed the expression of some effector genes, suggesting that smut fungi recognize Arabidopsis as a potential host (Martínez-Soto et al. 2013).

Given this scenario, we set out to investigate whether Arabidopsis can help study *S. scitamineum* developmental stages in plants and its influence in meristematic functions.

2. Material and Method

2.1 Biological material

We used *A. thaliana* strain Columbia-0 (Col-0), and teliospores of *S. scitamineum* Ssc39 isolate (Taniguti et al., 2015) maintained in the Genome Group at ESALQ / USP. No special permits were necessary for teliospores collection and use. This work does not involve endangered or protected species.

2.2 Arabidopsis cultivation and *S. scitamineum* inoculation

Arabidopsis seeds were surface sterilized by immersion in 70% alcohol for 5 minutes, then stirred for 20 minutes in 2% sodium hypochlorite solution and washed in sterile ultrapure water. The seeds were stored in the dark at 4ºC for four days for breaking dormancy. Then, placed in Petri dishes containing MS cultivation medium added of 1.5% sucrose (Rivero et al. 2014, with adaptions).

2.3 Time-course and microscopy analyses for Arabidopsis colonization by *S. scitamineum*

We established a time-course to analyze the events of Arabidopsis tissues colonization by *S. scitamineum*. These analyses were performed to identify teliospores germination, appressorium formation, and colonization of Arabidopsis tissues. The experiment consisted of 3 biological replicates, each containing a pool of 20 plants. They germinated and grew for 20 days in a non-inductive photoperiod (8 hours of light and 16 hours of dark), humidity above 70%, and an average temperature of 22ºC. Teliospores of *S. scitamineum* isolate Ssc39 suspended in saline solution (NaCl 0.85%) to a final concentration of 10^6 teliospores.mL^{-1} served as inoculum for Arabidopsis infection.

The viability and germination of the spores were verified in Petri dishes with agar-water medium (7.5%) (Taniguti et al., 2015). We used detached leaves of 20 days old plants growing as described above. Detached leaves kept in wet chambers were inoculated with teliospores of the Ssc39 isolated (Taniguti et al., 2015) using the protocol established by Méndez-Morán et al. (2005). The experiment was monitored using light microscopy (Figure 1A). Fungal hyphae were stained with a solution of lactophenol-cotton blue (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 0.02 g blue cotton, 10 mL deionized water) according to Marques et al. (2016).

The fungal growth were analysed at the plate dish. The inoculation site of the teliospores suspension (2 μL) was at the center of the plantlets (Figure 1B). After inoculation, plants developed in the same humidity and temperature conditions, however under long-day photoperiod (16 h light; 8 h dark)

Time points chosen were 24 hours after inoculation (hpi) (germination), 56 hpi (appressorium formation), and 6 days post-inoculation (dpi) (colonization). We also analyzed teliospores germination and appressorium formation, 24 and 56 hpi, respectively, in a scanning electron microscopy (SEM) Zeiss LEO 435VP equipment. Samples fixed in a 2% osmium

chamber for 24 hours were transferred to stubs, immobilized with carbon tape, then metalized and stored in silica.

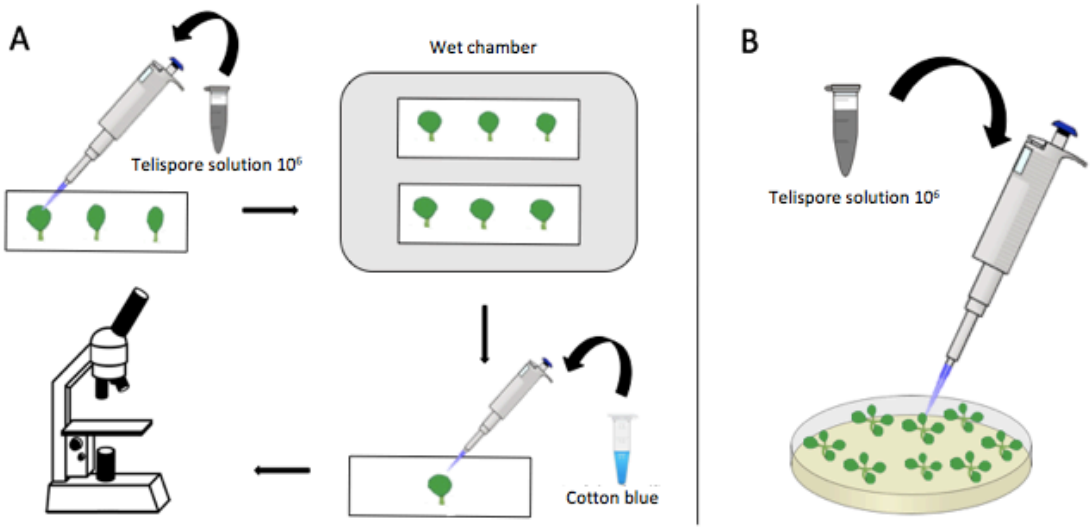

Figure 1. Arabidopsis inoculum. (A) Arabidopsis was inoculated with a teliospores suspension (2 µL) at the center of the plantlets (B) Detached leafs were kept in wet chambers and inoculated with teliospores of the Ssc39 isolated. The leaves were stained with a solution of lactophenol-cotton blue and monitored using light microscopy.

2.4 qPCR-assay for quantification of *S. scitamineum* growth in Arabidopsis

DNA extraction from infected plants was performed using the modified CTAB method (Porebski et al., 1997). The primer set selected for the standard curve targeted the IGS region of the *S. scitamineum* genome (F 5' CGGCTATTGTCGCACATCTC 3' and R 5' CCAAACGCAGGTCACAGTCT 3') (Peters et al., 2017). DNA quantification was performed on a Fast Real-Time 7300 PCR System (Applied Biosystems) using the GoTaq® qPCR Kit and RT-qPCR Systems - Promega ®. The conditions of qPCR cycles comprised 2 minutes at 95 °C, 95 °C for 15 seconds, and 1 minute at 60ºC (40 times).

The initial input of target DNA (infected plant) in each reaction was 100ng / µL. We estimated the concentration of fungal DNA in infected plants at 0 hpi, 24 hpi, 56 hpi and 6 dpi. The resulting standard curve showed a linear correlation between Ct values (cycle threshold) and *S. scitamineum* DNA amounts, described by the equation y = -3.0179x + 28.54 and R = 0.99276.

2.5 Late development of *S. scitamineum*

Arabidopsis seeds cultivated and inoculated in Petri dishes following the same methodology described in the topic 2.2 and 2.3 were cultivated for a further 30 days under long-

day photoperiod conditions (16 h light; 8 h dark). We counted the number of plants that reached flowering stage 12 (Alvarez-Buylla et al., 2010), noted root and shoot growth, and estimated the biomass, including both fresh and dry weights. The fresh mass samples were immediately determined, and the dry mass subsequently obtained after 48h at 50°C. The experiment included five biological samples, each consisting of a collection of 10 plants. We calculated the statistical significance among the mock and inoculated treatment using the T-test ($p < 0.05$).

2.6 Anthocyanin content

The same experimental conditions above-described were used to determine the Arabidopsis anthocyanin content 30 days after *S. scitamineum* inoculation (Martínez-Soto et al., 2013). Samples immediately frozen in liquid nitrogen were macerated to a fine powder. Each sample collected in microtubes and mixed with 300 µL of methanol/1% HCl was incubated overnight at 4°C in the dark. Next, we used chloroform (500 µL) and ultrapure water (200 µL) to separate the organic elements of the cell from the pigments with chloroform (500 µL) and ultrapure water (200 µL) by centrifuging for 5 min at 13,000 rpm. It was collected 400 µL of supernant, added 400µL of a 60% Methanol 1% HCl : 40% Milli-Q H_20 solution to each tube. The final solution was submitted to a spectrophotometer, and the absorbance measured at 530 nm for anthocyanin and 657 nm for chlorophyll (Chen et al., 2013). The anthocyanin concentration was calculated following the formula A530 nm - (0.25 × A657 nm) / g fresh weight (Chen et al., 2013). Each sample was compesed by 100mg of tissue and the statistical significance was calculated between the mock and inoculated treatment using the T-test ($p < 0.05$).

2.7 Gene expression analysis of *S. scitamineum* colonizing Arabidopsis tissues

We selected ten genes of *S. scitamineum* related to virulence and pathogen development in the plant. They encode for orthologs of the previously described effectors PEP1, STP1, CMU1, RSP3, TIN2, PIT2 (Hemetsberger et al., 2012; Liang, 2013; Djamei et al., 2011; Ma et al., 2018; Doehlemann et al., 2011, Benevenuto et al., 2018), candidate effectors g6610 and g5159, identified in our own studies (Teixeira-Silva, 2019); the orthologs of the transcription factor YAP1, activated by reactive oxygen species of host defense response (Maeta et al., 2004); and the clathrin precursor gene AP18 involved in vesicular transport (Žársky, 2016).

The primers were designed based on the genome sequence of *S. scitamineum* (Taniguti et al., 2015) and tested using genomic DNA as a template in conventional PCR reactions. The samples collected during the experiment were separated into 50 mg portions and macerated in liquid nitrogen. RNA extractions were performed using the PureLink ™ RNA Mini Kit (Invitrogen ™), and the samples treated with DNAse (Sigma Aldrich®). To verify the quality and quantity of the RNA samples obtained, they were loaded onto a 1.5% agarose gel and measured by a NanoDrop ™ spectrophotometer, taking into account the absorbance ratios of 260/280 and 260/230 nm. cDNA was synthesized from 200 ng / µL RNA using the GoScript ™ Reverse Transcriptase Kit (Promega).

All amplification reactions were performed using Fast Real-Time 7300 PCR System (Applied Biosystems). The reaction mixture consisted of 6.5 µL GoTaq® qPCR Master Mix, 0.2 µM of each primer, 0.25 µL of GoScript ™ RT Mix, 2 µL cDNA and nuclease-free water to complete the final volume of 12.5 µL. The amplification cycle protocol was: 95 ° C for 2 minutes; 40 cycles of 95 ° C for 15 seconds, 60 ° C for 1 minute.

Primer specificity was confirmed by melting curve analysis of each reaction. PCR efficiency and CT values were obtained using LinReg PCR software (Ramakers et al., 2003). We considered only primers with efficiency higher than 80% for expression analysis. Changes in gene expression were calculated by the ΔCT method using the gene encoding for β-tubulin as the endogenous control in the quantitative analysis (Livak and Schmittgen, 2001; with adaptations). Statistical tests used T-test and p-value <0.05 in RStudio software.

Table 1. Primers designed for *S. scitamineum* analysis. The genes accession numbers (ID) are according Taniguti (2015).

Gene	I.D	Forward sequence (5' - 3')	Reverse sequence(5' - 3')	Reference
CMU1	g6307_chr21_Ss	GCAGTGGAGCGAATACAAGG	GTTGGAGGTGAGGATGTTGC	this work
PEP1	g1816_chr03_Ss	CACACTGACTCAAGCCATCC	TGTAGCACACACCGAGTTCC	this work
STP1	g674_chr01_Ss	CTTCCTCAACACGTTCATGC	TGGTGTCGAACTTGATAGGC	this work
TIN2	g4911_chr14_Ss	CATGTTCTTTGGCACTACCG	AGCGTAGAAAAGCGTCTTGC	this work
PIT2	g2337_chr05_Ss	TCACACACAACGACGATGC	TTCCAATTAGGGTGCTGACG	this work
RSP3	g3970_chr10_Ss	GCCGGAGGGATACGACAACA	TCCTTCTCCTGCTCCTTGCG	Teixeira-Silva, 2019
g6610	g6610_chr24Ss	CGACGAGTCTGGCTCTCATTC	GAGAAGCGATGATGCCACC	Teixeira-Silva, 2019
g5159	g5159_ch15_Ss	CTCATCGGCAAGCACTCCA	GTTCAAAAGCGGCGTAGGTC	Teixeira-Silva, 2019
YAP1	g4200_chr11_Ss	CGAACGCAAGCAATCTTACCTC	CGCTCAATGTGGGCAAACTT	this work
AP18	g3636_chr09_Ss	TTTGGCGGTATGGGTATGGG	CAGTCGCTTGTGGCTGAATG	this work
β -tubulin	g1237_chr02_Ss	ACTTCCGTGGTAAGGTGTCG	GCTCCGAGATACGCTTGAAC	Teixeira-Silva, 2019

2.8 Expression analysis of Arabidopsis genes induced in response to *S. scitamineum* colonization

We selected Arabidopsis genes orthologs of those previously identified in sugarcane infected with *S. scitamineum* (Schaker et al., 2016). They were relevant for meristematic functions (BAM1; COL3; FT), auxin (PIN1) and jasmonic acid metabolisms (JAZ4; TOE2); and known to be involved in recognition of biotrophic pathogens in Arabidopsis (PR4; PAD4) (Bertini et al., 2003; Ren et al., 2008).

The same samples and conditions (topic 2.6) used for fungal genes studies were used to analyze the expression of Arabidopsis genes. PCR efficiencies and Cq values were obtained using the LinReg PCR (Ramakers et al., 2003). We used REST software (Pfaffl et al., 2002) to

determine expression values and fold changes comparing inoculated/mock plants. Actin_2 and At4g26410 genes were endogenous controls.

Table 2. Primer designer for *A. thaliana* analysis.

Gene	I.D - *A. thaliana*	Forward sequence (5'- 3')	Reverse sequence (5'- 3')	Reference
FT	AT1G65480.1	CAACCCTCACCTCCGAGAATA	CGAGTGTTGAAGTTCTGGCG	this work
BAM1	AT5G65700.1	TGTTGCTGACTTTGGTCTCG	TCTCCGACAGGTTTTCTTCC	this work
COL3	AT2G24790.1	TCAAGACTTTGCGATTCGTG	CATAGCCAAACTCGTTCGTG	this work
TOE2	AT5G60120	GATGGATGAATCGGTGACG	CTTGACCGTTCGTGTAGAGC	this work
PIN1	AT1G73590.1	TCCGAGACCTTCCAACTACG	CTTGTCTTTTCCCACCAACC	this work
PR4	AT3G04720.1	CGACCAACAACTGTCAGAGC	TGGAGCAATAAGCACTCACG	this work
PAD4	AT3G52430	TAAAGACTGGCGGGCATTAC	CTTTTCTCGCCTCATCCAAC	this work
JAZ4	AT1G48500.1	CCTTCTCTATGGCTCCAACAGTGG	CTCTAAGAACCGAGCCAAGGATGC	Oblessuc et al., 2019
ACTIN2	AT3G18780	TATGTCGCCATCCAAGCTGTTCTC	TCACCAGAATCCAGCACAATACCG	Oblessuc et al., 2019
AT4G26410	AT4G26410	GAGCTGAAGTGGCTTCCATGAC	GGATCATGGGTATGTCGGACC	Ross and Somssich, 2016

3. Results

3.1 *S. scitamineum* colonizes Arabidopsis plants

We established a time course for *S. scitamineum* teliospores germination and colonization over detached leaves of Arabidopsis 3-week old plants. We defined 3-time points as relevant for our study using 2 µL of the teliospores suspension (10^6.mL^{-1} saline solution) drop-inoculated over the leaf surface. We observed germination and appressorium development 24 hpi and 56 hpi, respectively (Figure 2). The fungus produced the aerial white mycelium and sporidia on the surface of leaves 6 dpi.

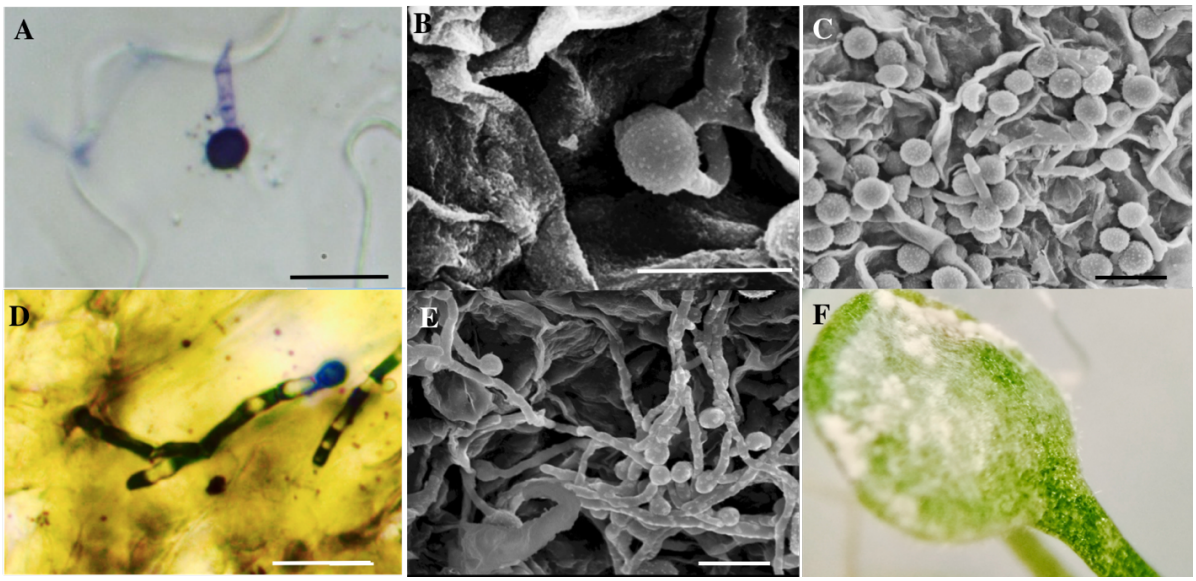

Figure 2. (A) and (B) germination of *S. scitamineum* at 24 hpi in leaf adaxial surface of *A. thaliana*. (C) Overview of *S. scitamineum* germination on *A. thaliana* at 24 hpi. (D) Fusion of two opposite yeast matching types originated a hyphae and appressorium formation on leaf surface (E) Overview of appressorium formation at 56 hpi. (F) Colonization of *S. scitamineum* in *A. thaliana* at 6 dpi. Scale 10µm.

We quantified the amount of *S. scitamineum* DNA in samples of total DNA extracted from infected Arabidopsis plants 24, 56 hpi, and 6 dpi. The qPCR runs of three biological and three technical replicates compared to the standard curve values confirmed *S. scitamineum* colonization of Arabidopsis tissues. Fungal initial concentration duplicated from the inoculation to 24 hpi, reaching approximately 35 ng 6 dpi (a 900-fold increase of the initial concentration) (Table 3) (Figure 3). These results were consistent with the visualization of fungal growth detailed in the previous images (Figure 2).

Table 3. Quantification of *S. scitamineum* during *A. thaliana* infection process by qPCR. Values of Cq during the time-course.

Time	Cq	Average	ng DNA
	23.75		
0 hpi	24.09	24.09	0.0385
	24.42		
	22.54		
24 hpi	21.58	22.00	0.0975
	21.88		
	18.68		
56 hpi	18.60	18.81	1.8490
	19.15		
	14.84		
6 dpi	15.03	14.87	34.6843
	14.75		

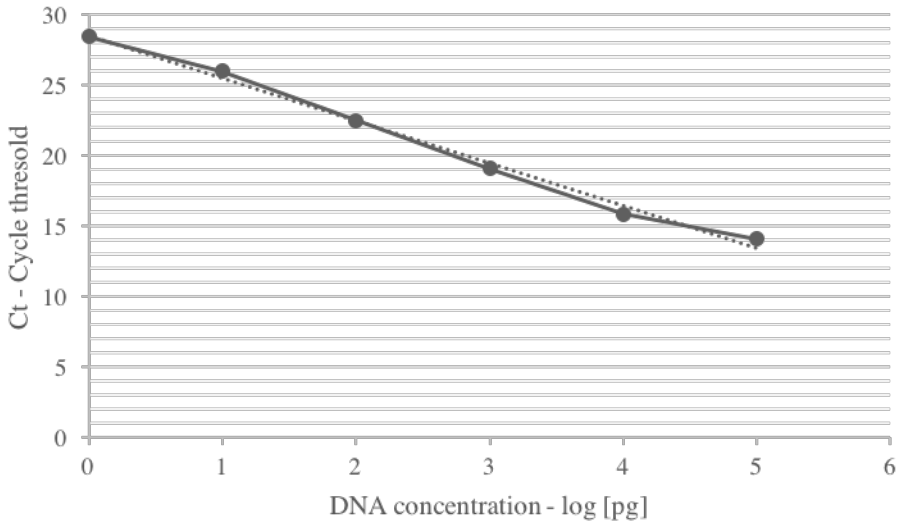

Figure 3. Standard curve of a linear correlation between Ct values and *S. scitamineum* quantification. The equation of the standard curve is y = -3.0179x + 28.54 and R = 0.99276

3.2 Late development in plant

The plants were collected 30 dpi (51 days after germination) for biomass (Figure 5) and anthocyanin assessments (Figure 4). Inoculated plants presented a smaller overall size, chlorosis, and signs of anthocyanin accumulation in the meristematic region and leaves. Roots were shorter and thicker, besides presenting more ramifications (root hairs) (Figure 5E). Most of the plants flowered regardless of the treatment; however, the number of branches per plant and size differed (Figure 5D).

The fungus spread over the leaves away from the inoculation center point producing the white aerial hyphae and sporidia (Figure 2F). Eventually, the fungus reached the medium. In an attempt to protect the roots, we also planted the seeds protected by parafilm. The results were the same, showing clear stunting of the roots and reduced aerial shoot length. We did not detect necrotic lesions in any of the replicates.

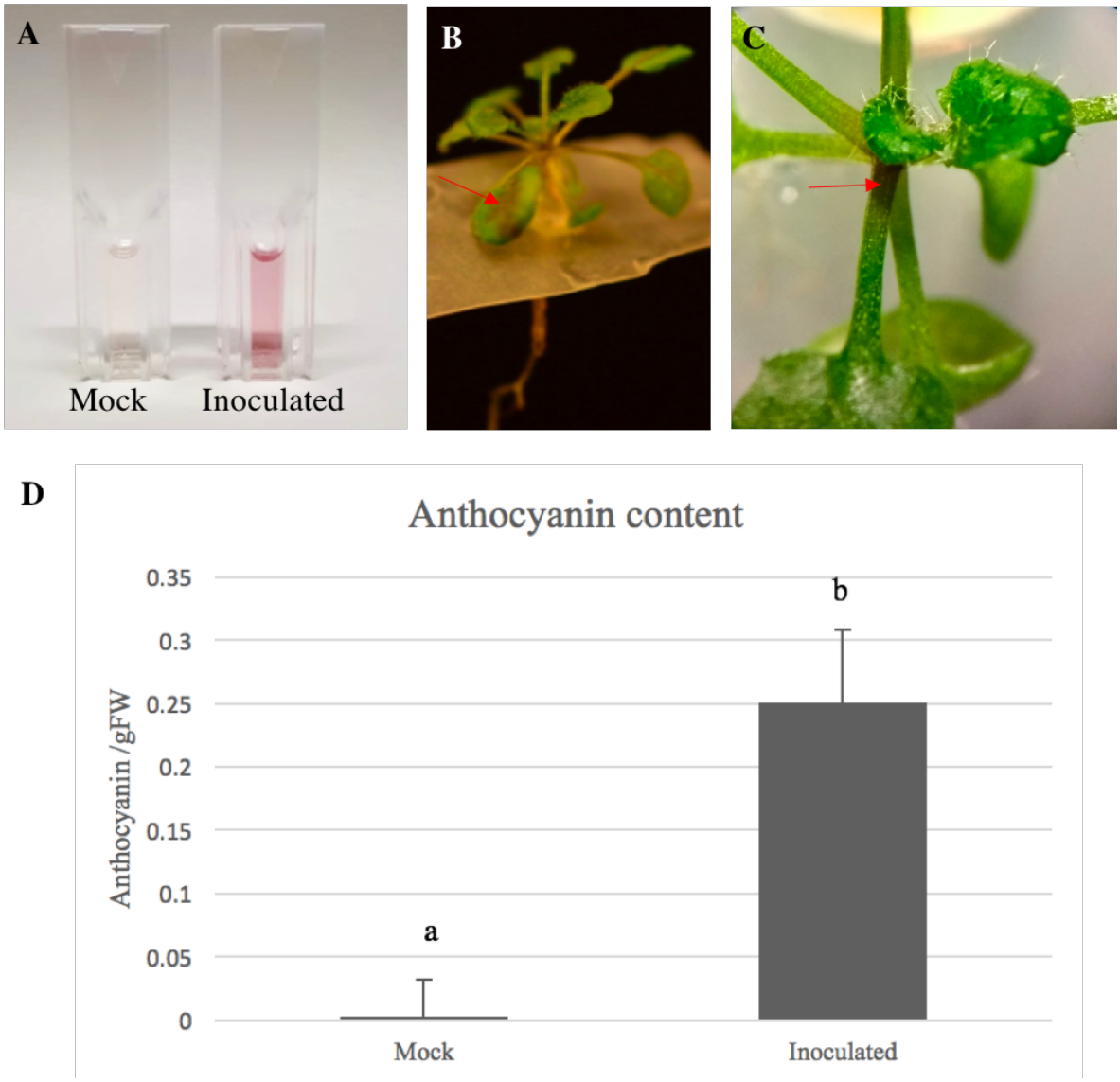

Figure 4. (A) Anthocyanin extract from mock and inoculated plants. (B, C) Meristematic region and leaves of inoculated plants showing purple color. (D) Quantification of anthocyanin by fresh weight in plants for statistical analysis was used p-value <0.05.

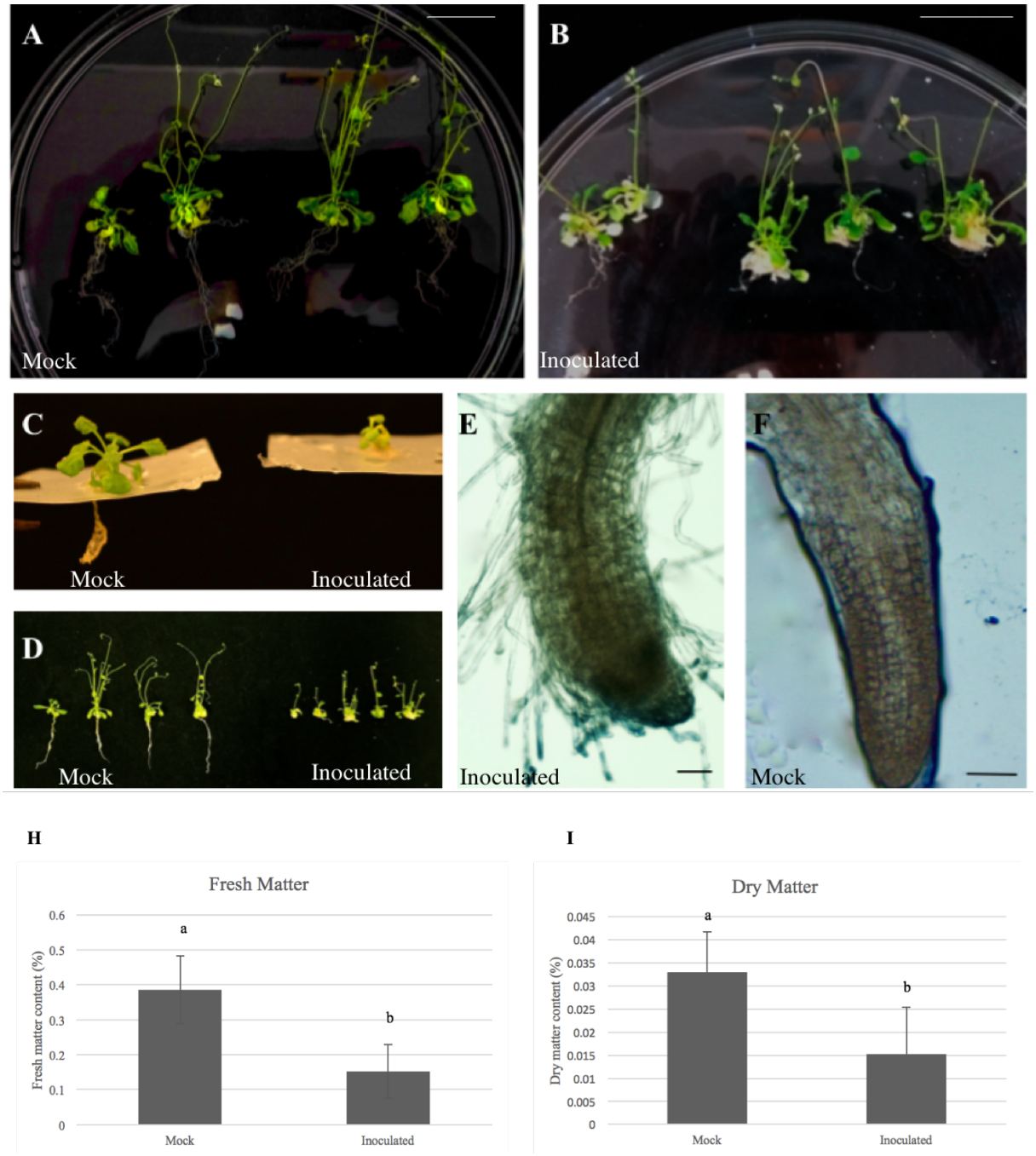

Figure 5. Phenotypic evaluation of the interaction between *S. scitamineum* and *A. thaliana* at 30 dpi. (A) and (B) Mock-treated and *S. scitamineum*-infected *A. thaliana* plants, respectively, from which seeds were protected by parafilm. (C) Roots of an infected *A. thaliana* plantlet, protected by parafilm. (D) Light microscopy of a mock plantlet root. (E) Light microscopy of an inoculated plantlet root. (F) Comparison between mock and infected plants. (H, I) Graphics of biomass dry and fresh matters.

3.3 Gene expression analysis in *S. scitamineum*

Ten *S. scitamineum* genes had their expression analyzed using RT-PCR in different phases of the fungus development. We used teliospores, the fungus growing in vitro both as yeast-like cells (haploid cells) and forming hyphae in the dikaryotic stage, and growing in plant tissues 24, 56 hpi and 6 dpi. Six of these genes were candidate effectors of *S. scitamineum*, six

previously described in other smut interactions interactions (Benevenuto et al., 2018), and two associated with fungal growth and defense responses (Teixeira-Silva, 2019).

The genes expression patterns differed substantially between the assays of the fungus growing *in vitro* and *in planta* and also among the sampled genes (Figure 3). The absence of expression in teliospores was a single common feature among the examinations. Eight of all the genes analyzed expressed solely *in planta*. One of them, the effectors STP1, was no significantly induced in germination (24 hpi), however increased the levels of expression after appressorium formation (56 hpi) and colonization of Arabidopsis tissues (6 dpi). Otherwise, for g6610, the expression reached its maximum during germination (24 hpi), decreasing toward colonization (6 dpi).

The induction of the other two, PEP1 and g5159, occurred exclusively after Arabidopsis colonization. The effector gene CMU1 expressed *in planta* showed its highest level of expression during appressorium formation 56 hpi and also when growing *in vitro* as a dikaryon. Curiously, gene expression of the candidate effectors TIN2 and PIT2 could not be detected *in planta* during the time points analyzed in the present study.

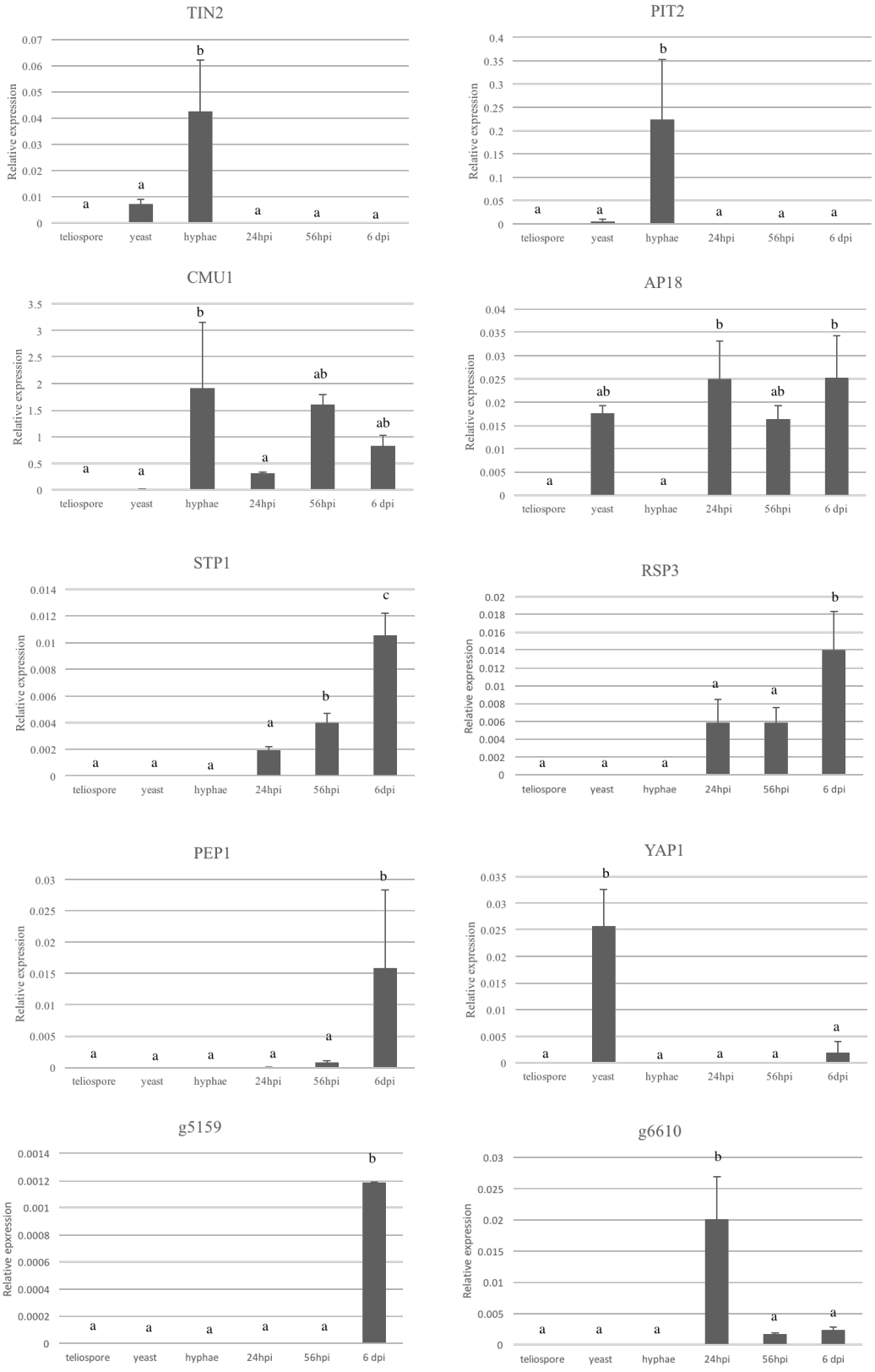

Figure 6. Relative expression of *S. scitamineum* genes during the initial moments of interaction with *A. thaliana*. Times analyzed were 24 hpi (germination), 56 hpi (appressorium formation), 6 dpi (colonization). The value of relative expression was calculated with delta Cq. Tukey test were performed ($p < 0.05$) to compare the expression among the time points.

3.4 Analysis of gene expression in *A. thaliana*

We analyzed the expression of eight Arabidopsis genes. We chose genes encoding proteins commonly associated with defense responses in Arabidopsis (PAD4 and PR4) and proteins related to meristematic functions and the transition from vegetative to reproductive stages (BAM1, JAZ4, TOE2, FT, COL3, PIN1).

The genes encoding for FT, JAZ4, and TOE2 exhibited the same behavior of repression 6dpi with advanced fungal growth. COL3 had a strong induction shortly after inoculation (24 hpi) followed by repression during fungal penetration (appressorium formation) that extended until colonization (6dpi). Otherwise, BAM1 showed induction after fungal penetration progressing until colonization. For PIN1, gene expression did not alter comparing both inoculated and mock plants (Figure 7).

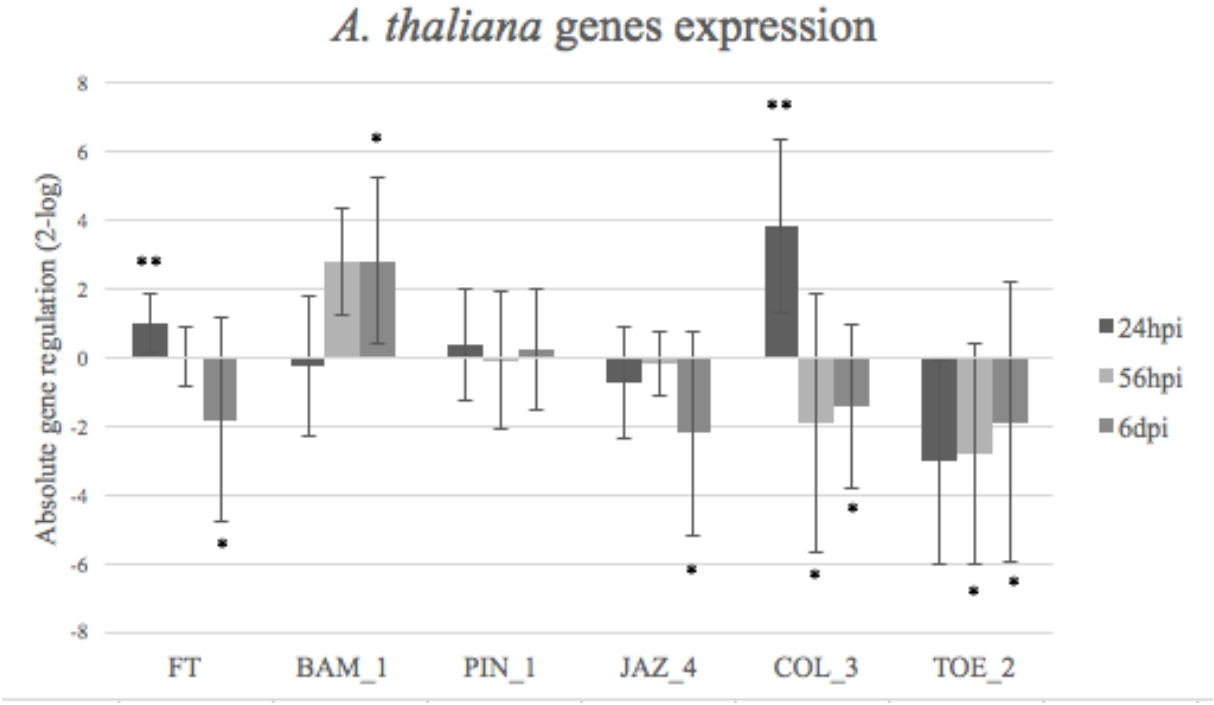

Figure 7. Fold change of *A. thaliana* genes expression was obtained thorugh the formula R= (E target) $^{\Delta cp\ target\ (mean\ control\ -\ mean\ sample)}$/ (E ref) $^{\Delta cp\ target\ (mean\ control\ -\ mean\ sample)}$. The statistical significance, p-value < 0.05 =*; p-value <0,01 = **. The calculation was performed using the software REST (Pfaffl et al., 2002)

4. Discussion

Méndez-Morán et al. (2015) and Martínez-Soto et al. (2019) described the use of Arabidopsis for studying other smut fungi infections, considering the development of symptoms and the induction of fungal genes during the interaction. In an attempt to use Arabidopsis to examine the events leading to altered meristematic functions, as previously described for sugarcane, we designed an experiment to verify colonization and evaluate gene

expression of fungal and Arabidopsis genes. Our results showed similarities in pathogen development compared to sugarcane (Peters et al., 2017). As observed for other smut fungi-inoculated Arabidopsis (Méndez-Morán et al., 2005; Martínez-Soto et al., 2013), teliospores germinated, developed appressorium over the leaves surface and colonized the tissues forming white aerial mycelium and sporidia.

Various plant pathogens can extend host specificity under axenic experimental conditions (León-Ramírez et al., 2004). We observed comparable results for *S. scitamineum* infecting Arabidopsis, an entirely unrelated species to sugarcane, its natural host. However, germination and penetration in Arabidopsis dawdled about 18 hours when compared to susceptible sugarcane plants in similar conditions (Peters et al., 2017). Sugarcane resistant plants show signs of oxidative burst 48 and 72 hpi, not detected in Arabidopsis. It seems that Arabidopsis imposed no obstacles to impair the initial development of the fungus. Electron microscopy images detailed spores germination, hyphae, and sporidia formation (n) covering Arabidopsis inoculated detached leaves.

Differently from the infection of *U. maydis* in maize, in which penetration occurs via stomata aperture (Méndez-Morán et al., 2005), we identified *S. scitamineum* penetrating via appressorium formation. Appressorium is a specialized structure used by fungal pathogens to infect host plants. Its formation relies on the perception of physical and biochemical signs at the leaf surface coupled with the cell cycle control and growth of the dikaryotic hyphae to penetrate the plant tissue (Tucker and Talbot, 2001; Lanver et al., 2014). The presence of the appressorium suggests that *S. scitamineum* recognized Arabidopsis as a potential host.

Thirty days after inoculation, *S. scitamineum* caused symptoms of chlorosis, induced anthocyanin production, reduced plant size, and altered root morphology. We used the long-day photoperiod to test whether smut would compete for the same pathways to impair flower development in advanced stages of the infection. However, the timing and number of flowers developed were not significantly affected (data not shown). Usually, the smut whip only develops in susceptible sugarcane plants lacking flower-like structures (Marques et al., 2016).

Nonetheless, the root development was affected, exhibiting stunting and numerous hairs. This increase of hairs usually occurs to compensate for the reduced root surface area required for nutrient and water uptake (Bao et al., 2014). Contrarily, non-host monocot and dicots plants inoculated with *U. maydis* increased root development and de number of adventitious roots (León-Ramírez et al., 2004; Méndez-Morán et al., 2015). So far, we have no descriptions of *S. scitamineum* affecting the development of the root system in infected sugarcane plants. However, Singh et al. (2019) showed that *S. scitamineum* modulates cytokinin

and ethylene when infecting resistant sugarcane genotypes in both the shoot and roots 30 days after inoculation.

Comparative analysis of various smut species reported a set of effector candidate genes common to all genomes sequenced to date (Benevenuto et al., 2018). Some of these genes were functionally characterized in other smut and demonstrated previously to be expressed in *S. scitamineum* infecting sugarcane (Hemetsberger et al., 2012; Liang, 2013; Djamei et al., 2011; Ma et al., 2018; Drechsler et al., 2016; Doehlemann et al., 2011; Peters et al., 2017). We selected six of these genes, showing different functions to analyze their expression during colonization of Arabidopsis. CMU1, for instance, encodes a chorismate mutase localized in the plant cell cytosol to convert chorismate in prephenate. The conversion of chorismate results in the decrease of SA biosynthesis, and consequently, depression of the cell defense mechanism (Djamei et al., 2011). The ortholog of *S. scitamineum* CMU1 expressed equally in vitro in the infective dikaryotic stage of the fungus and in-plant in all moments of the interaction with Arabidopsis.

In Arabidopsis, the triggering of SA pathways resulting from a fungal pathogen infection induces the expression of various pathogenesis-related genes, including PR4 and PAD4 (Bertini et al., 2003; Ren et al., 2008; Baccelli et al., 2014). The expression of these two genes of Arabidopsis analyzed in our experiments were no detected.

S. scitamineum infecting sugarcane resistant genotypes induces oxidative burst (Peters et al., 2017). We did not see signs of necrosis in Arabidopsis. Fungal cells exposed to ROS react by inducing the expression of YAP1, which is a transcriptional factor involved in activating genes of the antioxidant system (Maeta et al., 2004). *S. scitamineum* activated the YAP1 expression at 6dpi, however it was not detected a statistical differential when compared against others time of infection. This result is in agreement with the late expression (6dpi) of the effector PEP1. PEP1 is a member of the smut effectors core, induced during the interaction to deal with class III peroxidases responsible for increasing levels of toxic oxygen peroxides in the apoplast shortly after inoculation (Hemetsberger et al., 2012; Peters et al., 2017).

S. scitamineum, however, induced the expression of the other two effectors olny expressed *in plant*: STP1 (Liang, 2013) and RSP3 (Ma et al., 2018). The expression of STP1 is essential for the initial establishment of hyphae in the epidermal cell layer by encoding an inhibitor of a cysteine protease (SIP3) (Liang, 2013). The RSP3 gene encodes for a chitin disguise protein to impair the fungal recognition by the host receptors (Ma et al., 2018). Smut

fungi require both proteins for a successful compatible interaction (Liang, 2013; Ma et al., 2018).

Differently from STP1, PIT2 encoding a papain-like protease inhibitor was not induced in Arabidopsis infection. Although Arabidopsis showed anthocyanin accumulation in the late stages of the infection, TIN2, an effector involved in the induction of the pigment, was not responsive in the early stages of the interaction.

We also evaluated the expression of two other candidate effectors specific to *S. scitamineum*, revealed in our previous studies, g6610 and g5159. In sugarcane, their expression could be detected (Teixeira-Silva, 2019). When infecting Arabidopsis, they expressed 24 hpi and 6 dpi, respectively.

Therefore, we concluded that, indeed, Arabidopsis is susceptible to the infection of *S. scitamineum*, potentially using a combination of effectors to establish colonization and repress the plant immune system, partially common to what was found for sugarcane.

Our second and most important goal was to investigate the application of Arabidopsis to understand the influence of *S. scitamineum* infection upon meristematic functions. A feature described in *S. scitamineum*-infected sugarcane (Schaker et al., 2016, Marques et al., 2016, Sundar et al., 2012; Carvalho et al., 2016)

S. scitamineum alters the expression of auxin-related genes since early after inoculation in sugarcane, extending throughout the development of the whip (Schaker et al., 2016). In an attempt to identify auxin involvement in Arabidopsis during *S. scitamineum* infection, we used the marker PIN1. PIN1 is an auxin efflux carrier acting to mediate the formation of an auxin gradient required to ensure correct organogenesis (Benková et al., 2003). However, PIN1 expression was not affected by the *S. scitamineum* infection in Arabidopsis (Figure 8).

In sugarcane, *S. scitamineum* induced the expression of the Arabidopsis orthologs of BAM1, with COL3, TOE2 and FT 5 dpi (Schaker et al., 2016). In Arabidopsis, BAM1 is a receptor-like kinase involved in several developmental pathways, most notably in the meristem. BAM mutants (bam1, bam2, bam3) in Arabidopsis lose meristem functions at the shoot and flower meristems by reducing stem cell maintenance (DeYoung et al., 2006). In our experiment, pathogen colonization induced the expression of BAM1 from 6 dpi onwards (Figure 8).

Arabidopsis flowers earlier in long days than in short days, in coordination with the circadian clock and photoperiod. Various mechanisms are involved in the perception of light from which the most studied is the circadian regulation of CONSTANS (CO) gene expression. After inoculation, our experiment was conducted in long-day conditions to induce flowering.

In Arabidopsis, the *col3* mutant positively regulates the light-dependent development and formation of lateral roots, inhibits shoot elongation, flowers early and shows a reduced number of lateral branches specifically in short-day conditions (Datta et al., 2006). In our work, the fungus strongly induced COL3 expression 24 hpi, following by a repression 56 hpi onwards. COL3 targets the florigen FT in the presence of the transcription factor BBX32 to regulate the flowering pathway (Tripathi et al., 2017). Following the repression of COL3, *S. scitamineum* also repressed the expression of FT 6 dpi (Figure 8).

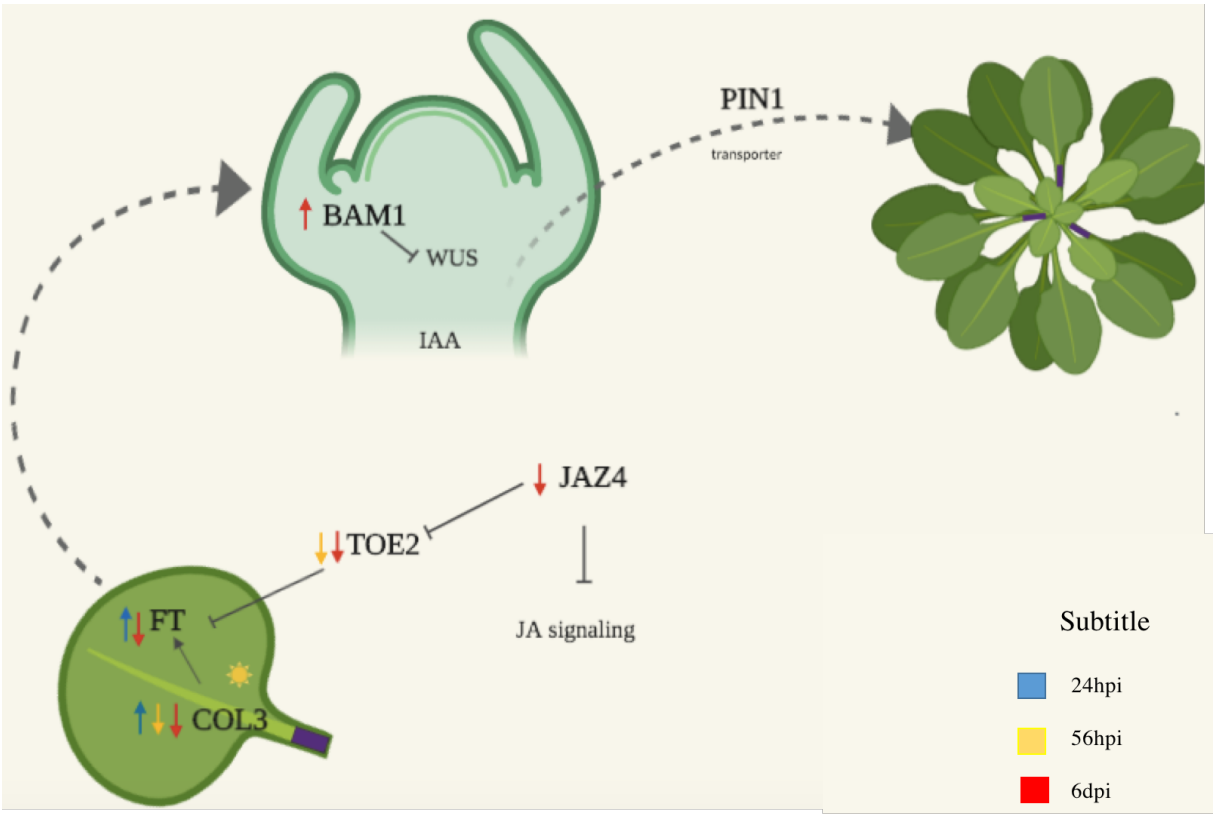

Figure 8. Scheme of *A. thaliana* gene expression profile associated with meristematic changes, jasmonic acid signaling and auxin transport during an interaction with *S. scitamineum*. The arrows orientation and colors indicated the differential expression of the genes during the time course. Figure made with BioRender™ software.

The last set of genes investigated considered *S. scitamineum* influencing jasmonate functions. Liu et al. (2017) and McNeil et al. (2018) observed the activation of JA signaling in sugarcane infected with *S. scitamineum.* Also, *S. scitamineum* affected the expression of TOE2 (Schaker et al., 2017). Arabidopsis TOE1 and TOE2 regulate JA-mediated flowering time by interacting with a subset of JAZ proteins to repress the transcription of FT (Zhai et al., 2015). Among the JAZ proteins, TOE2 interacts with JAZ4. Under biotic stress, JAZ4 operates through the canonical JA pathway to repress JA responses, enhancing Arabidopsis resistance

to a bacterial pathogen (Oblessuc et al., 2019). Also, the authors described that JAZ4 inhibits aging-associated processes such as the accumulation of anthocyanin and transition to flowering. *S. scitamineum* repressed JAZ4, TOE2, and FT 6 dpi in long-day conditions (Figure 8).

The differential expression of genes related to metabolic pathways of defense and meristematic differentiation suggests that *S. scitamineum* modulated the expression of genes associated with meristematic functions through flowering pathways. Therefore, the results suggest that *S. scitamineum* can recognize the model plant as a potential host, activating pathogenicity genes, and effectively colonize *A. thaliana* tissues. When infecting sugarcane, the smut fungus induces transcriptional changes in genes associated with meristematic functions, as it does in the model plant. Besides, later, in advanced stages of colonization, the fungus induced morphological changes such as anthocyanin accumulation.

Thus, we conclude that *A. thaliana* could bring new insights as a model for the study of morphological and molecular aspects at the early moments in the *S. scitamineum* interaction.

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