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Insights on effector candidates of *Austropuccinia psidii*: identification, characterization and comparative genomic analysis

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Thesis presented to obtain the degree of Doctor in Science.
Area: Genetics and Plant Breeding

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Bsc in Biology Sciences

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1. CHAPTER 1 - FUNCTIONAL STUDY OF EFFECTOR CANDIDATE PROTEINS FROM *Austropuccinia psidii*

ABSTRACT

Austropuccinia psidii is a biotrophic fungus and the causal agent of myrtle rust, first described in Brazil and spread quickly around the world. Besides its economic importance to Brazil and around the world, studies that approach its interaction with its hosts are a challenge due to its biotrophic lifestyle. Regarding *A. psidii – Eucalyptus* spp. interaction, the study of effector highlights, in general, effectors are molecules secreted by the pathogen that modulates the host's physiology to allow the pathogen infection. The identification of effector candidates may support the understanding of the mechanisms involved during the host infection. In our research, we identified and characterized *in silico* effector candidates of *A. psidii* MF-1 biotype, isolated from *E. grandis* in Brazil. We obtained a profile of 255 effector candidates predicted by two methods: a manual pipeline based on effector features, and by the software EffectorP 2.0. The effector expression validation was performed under cuticular wax extracts of eucalypt leaves due to the challenge to deal with *A. psidii* biotrophic lifestyle. Seven effector candidates selected and validated showed different patterns of expression under the stimulus of cuticular species resistant and susceptible, which may indicate that the host influences the gene expression of effector candidates during the infection. To investigate the subcellular localization of effector candidates, we transiently expressed two effector candidates tagged with GFP proteins in leaves of *Nicotiana benthamiana*. We observed the accumulation of the effector candidate Ap28303, described as inhibitor I9 domain-containing protein, in the nucleus by microscopy. To the best of our knowledge, Ap28303 is the first effector candidate from *A. psidii* that was identified, validated its expression, and had its subcellular localization characterized. Our findings open new perspectives to the study of effector and interaction of *A. psidii* – host, as the validation of other effector candidates. A deep investigation about the effector candidate Ap28303 and the comparison to other biotypes of *A. psidii* may be performed in the future.

Keywords: Myrtle rust; Agroinfiltration; Plant-pathogen interaction; Effectoromic; EffectorP

1.1. Introduction

Austropuccinia psidii is a biotrophic fungus, the causal agent of the myrtle rust (Beenken, 2017), that was described infecting approximately 480 species of Myrtaceae (Soewarto et al., 2019). Currently, the myrtle rust is spread out around the world, including Australia, a country that hosts around 2250 Myrtaceae species, being a threat to the ecosystem (Berthon et al., 2018; Pegg et al., 2017). *A. psidii* is native from South America (Coutinho et al., 1998; Glen et al., 2007) since first reported infecting guava (*Psidium guajava* L.) in Brazil (Winter, 1884). Then, it was reported infecting *Eucalyptus* species also in Brazil in 1912 (Joffily, 1944). The disease causes economic losses in the forestry industry, causing damages in nurseries and young plants, hence reducing productivity and sometimes causing death of susceptible plants (Dianese et al., 1984; Masson et al., 2013). Despite the wide range of myrtle species infected by *A. psidii* and its economic and ecological importance, little is

known about the molecular mechanisms involved in the host infection, including the role of effector proteins on the *A. psidii* pathogenesis.

Dalio et al. (2017) defined effectors as secreted molecules associated with an organism that may modify the physiology, structure, and function of another organism. During the plant infection, pathogenic fungi use these molecules to modulate the plant physiology and colonize the host (Dodds & Rathjen, 2010). The plants also use their mechanisms to counteract the fungi infection, as described in the *zig-zag* model proposed by Jones & Dangl (2006). Briefly, after the pathogen contact with the plant, Pathogen-Associated Molecular Pattern (PAMP) or Microbe-Associated Molecular Pattern (MAMP) can be recognized by Pattern Recognition Receptors (PRRs), which trigger PAMP Triggered Immunity (PTI). The non-recognized pathogens use effector proteins to infect the plant, resulting in Effector Triggered Susceptibility (ETS). The effectors can be recognized by *R* genes, it may lead to Effector Triggered Immunity (ETI). In last phase, the pathogen evolves to avoid ETI, and successfully infects the host, as well the pathogen and host are under co-evolution (Jones & Dangl, 2006).

The study of effectors is crucial to comprehend the mechanisms involved in plant-pathogen interactions (Hogenhout et al., 2009). To identify and characterize effectors, several methods have been used, as computational tools to predict candidate effectors from genome or transcriptome (Kunjeti et al., 2016; Petre et al., 2015) and RT-qPCR to effector expression validation (Duplessis et al., 2011; Zhang et al., 2017). It is also common to employ transient expression mediated by *Agrobacterium tumefaciens* to functionally characterize effectors from obligate pathogens, since they are not liable to genetic transformation (Caillaud et al., 2012; Lorrain et al., 2018), as the case of *A. psidii*.

A. psidii MF-1 biotype was isolated from a single pustule of *Eucalyptus grandis* (Leite, 2012) and successfully infects *Eucalyptus* species (personal communication). Bini (2016) described the viability to cultivate *A. psidii* MF-1 *in vitro* until 48 hours after inoculation, using culture medium agar-water amended with olive oil and dialysis membrane, which offered the necessary stimulus to the development of penetration hyphae and appressorium. From this method it was performed an RNA-seq to obtain the *A. psidii* transcripts during its germination *in vitro* conditions. The author found transcripts differentially expressed which probably codified to effector proteins. Lopes (2017) identified and validated by RT-qPCR the expression of effector candidates during the early pathogen infections *in vitro*, based on the method proposed by Bini (2016). The experiment was performed using urediniospores from two biotypes, one isolated from *Eucalyptus* (MF-1) and another from *Syzigium jambos* (GM-J1). The results suggested a modulation from the host in the effector candidate's expression. Moreover, Santos et al. (2019) described the use of cuticular wax extract from *Eucalyptus* leaves *in vitro* conditions as an important pre-formed mechanism against the pathogen. It was showed a relationship between the cuticular wax composition of resistant and susceptible species and the *in vitro* germination pattern of *A. psidii*, thus the cuticular wax may influence the effectors expression.

Based on these previous studies, we used the genome of *A. psidii* MF-1 biotype, deposited at NCBI (National Center for Biotechnology Information, Accession Number: AVOT00000000), to

identify effector candidates. Among the *in silico* predicted effector candidates, 7 were selected to further investigation. We used cuticular wax from *E. grandis* (susceptible species) and *E. urophylla* (resistant species) leaves to validated *in vitro* the expression of effector candidates along the early stages of *A. psidii* MF-1 infection. Our results suggested that the effector candidate's expression was modulated by the cuticular wax extract source. We observed the candidate G3GFP-tagged Ap28303 accumulated in the nucleus by *A. tumefaciens* transient expression using binary plasmid, the protein was detected by Western blot, probing with an anti-HA antibody. In summary, we obtained a profile of effector candidates from *A. psidii* and the expression profile of effector candidates *in vitro* conditions according to cuticular wax host source. We also confirmed the nuclear localization of Ap28303, firstly described as inhibitor I9 domain-containing protein. For the first time, it was carried out an investigation and characterization of effector candidates of *A. psidii*.

1.2. Conclusion

In a complex pathosystem as the *A. psidii* and *Eucalyptus*, the *in vitro* assays allowed simulate nearest the conditions *in planta* and enable to elucidate slightly the molecular mechanisms involved during the host infection. Our results showed that there is a differential expression of the candidate effectors comparing cuticular waxes from species resistant and susceptible. The suppression or increasing of candidate effector expression depends on the host susceptibility or resistance, and cuticular wax as a stimulus to fungi growth rises as an alternative to the *in vivo* assays, in which the challenge is access fungi material genetic. The candidate Ap28303 accumulated in the nucleus of epidermic cells of *N. benthamiana*. Although this candidate was predicted as a protease inhibitor, our findings showed that probably this candidate develops another function during the infection. This is the first study of localization subcellular of effector in *A. psidii* and a step forward in the study of genes associated with infection of myrtle rust. However, further studies are necessary to characterize the Ap28303 as an effector of *A. psidii*, and its function.

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2. CHAPTER 2 – COMPARISON OF EFFECTOR CANDIDATE PROTEINS FROM THREE BIOTYPES OF *Austropuccinia psidii*

ABSTRACT

Austropuccinia psidii is a biotrophic rust fungus that infects plants of the Myrtaceae family. First reported on guava in Brazil in 1884, it has since been reported to infect many other myrtaceous species and has spread globally. Several biotypes of *A. psidii* have been identified that differ in host range. In 2010, myrtle rust was detected in Australia, the centre of origin for the majority of Myrtaceae species. A Brazilian biotype (MF-1) is known to successfully infect *Eucalyptus species*, while the Australian pandemic (Au) and the South African (SA) biotypes infect distinct Myrtaceae species. Little is known about the complex pathogen-host interactions underlying myrtle rust. Proteins secreted into hosts by rust pathogens, termed effectors, are acknowledged to be fundamental for their pathogenicity, thus investigate these proteins may help understand mechanism involved in pathogen-host interaction. We predicted the effector candidates for each biotype and identified polymorphisms to be validated by PCR. We computationally predicted the effector candidates of three *A. psidii* biotypes: Au (di-haploid), SA (diploid), and MF-1 (diploid), estimated at 671, 365, and 282 candidates for each, respectively. We found 269 effector candidates with the strongest evidence of homology among the three biotypes, Au and MF-1 biotypes shared 351 of effector candidates, Au and SA biotypes shared 397, and MF-1 and SA shared 161. We also performed an orthology analysis and identified the SA and MF-1 biotypes closest, concerning effector candidates. The Au biotype showed a greater number of unique protein clusters compared to other biotypes. Our findings showed differences related to the number of effector candidates, however, no differences concerning effector localizations, and the most effector candidates have no predicted functional annotation. The localization prediction *in silico* showed a percentage similar to effector candidates from the biotypes targeting apoplast, nucleus, mitochondria, and chloroplasts. The effector candidates showed polymorphisms by PCR validation, however, supplementary studies are necessary to comprehend the implication of that to host variability.

Keywords: Secreted proteins; Myrtle rust; Effectoromic; Comparative genomic analyses; Pandemic biotype; *Eucalyptus* rust; Obligate pathogens

2.1 Introduction

Austropuccinia psidii (G. Winter) Beenken comb. nov. (Beenken, 2017) is a biotrophic fungus, the causal agent of myrtle rust, considered native of South America (Coutinho et al., 1998; Glen et al., 2007). The first report of this pathogen was in Brazil infecting guava (*Psidium guajava* L.) and named *Puccinia psidii* in 1884 (Winter, 1884). Just in 1912 *A. psidii* was detected in *Eucalyptus* (Joffily, 1944). Since first reported in Brazil, *A. psidii* spread quickly to neighbouring countries Paraguay and Uruguay (Carnegie & Pegg, 2018; Spegazzini, 1884), and is now worldwide spread (Carnegie & Pegg, 2018). Despite efforts to minimise the incursion of myrtle rust into Australia, in

2010 the pandemic biotype was detected in New South Wales (Carnegie et al., 2010). It was reported a single genotype in Australia (Sandhu et al., 2016), the pandemic biotype, and appears to be the same found in Hawaii and New Caledonia (Machado et al., 2015; Sandhu et al., 2016; Stewart et al., 2018). In 2013, *A. psidii* was first reported in South Africa (Roux et al., 2013) and by 2016 was considered widespread, and pieces of evidence indicate only one genotype affecting native and non-native genera and species of Myrtaceae (Roux et al., 2016). The pathogen is a major threat to biodiversity in Australia since the country hosts 2,280 myrtaceous species (Berthon et al., 2018) among the 5,950 globally described (Christenhusz & Byng, 2016). In Australia, 232 species from natural infection and 115 from artificial inoculation were known to be affected by the pandemic biotype (Carnegie et al., 2016). In South Africa, the genotype infects non-native species (*Myrtus communis* and *Backhousia citriodora*) and native species (*Eugenia erythrophyllum*, *E. verdoornii*, *Heteropyxis canescens*, *H. natalanesis*, *Syzygium cordatum*, *S. legatii*) with different degrees of susceptibility (Roux et al., 2016). The South African biotype apparently is genetically unrelated to currently genotyped biotypes (Roux et al., 2016). In Brazil, there is the highest genetic variability among the *A. psidii* populations (Quecine et al., 2014; Stewart et al., 2018). The MF-1 biotype was collected from eucalypt plants in Brazil, and it appears to be more selective to *Eucalyptus* species (Leite, 2012; unpublished data).

Despite its global impact and wide host range, *A. psidii* genome was only recently available, to knowledge biotrophic fungi in general have a large genome, with high repeat regions, and limitations related to DNA extraction, therefore genome sequencing and assembly are a challenge (McTaggart et al., 2018). Initially, it was estimated size of 103-145 Mb to *A. psidii* genome using paired-end sequences of 250 bp from Illumina MiSeq (Tan et al., 2014). McTaggart et al. (2018) used Chromium 10X Library to sequence and assembly the South African biotype genome, to overcome the biotrophic fungi limitations. This technology was efficient to sequence 1.2 Gb of this pathogen using a small quantity of DNA. Tobias et al. (2020) estimated the genome size 1.02 Gb of the pandemic biotype from Australia, sequenced by PacBio and scaffolds assembly by Hi-C technology. The authors observed more repetitive regions (>90%) when compared to other Pucciniales species genomes, and the large genome may be associated with expansion due to transposable elements from Gypsy family (Tobias et al., 2020). It was sequenced and assembled a 630 Mb genome of MF-1 biotype from Brazil, using a combination of sequence data from 454 Plataform, MiSeq (Illumina), SMRT (PacBio), and HiSeq 2.5 (Illumina) (unpublished data – deposited at NCBI, Accession Number: AVOT00000000). The size differences among the biotypes are probably associated with sequencing techniques used for each, and their limitations.

Beyond the information about the genomes and their variability, some biotypes have apparent host biological specificity, for example, the MF-1 strain appears to successful infect *Eucalyptus* species but not *Syzygium jambos* (unpublished data), also in Brazil, guava isolates did not infect successfully eucalyptus and *vice-versa* (Ferreira, 1983; Quecine et al., 2016). Quecine et al. (2014) based on physiological variability among *A. psidii* populations investigated the genetic diversity in populations

isolated from guava, syzygium, jaboticaba and eucalypt. The results showed a higher level of diversity in guava, jaboticaba and syzygium than eucalypt. These physiologic and genetic variabilities suggest an evolution of host-specific genotypes (Quecine et al., 2014).

To study the variability among pathogenic strains, information from the genome is useful to identify sequence variations, since there is selective pressure to avoid host recognition (Tsushima et al., 2019). To successfully infect the plant, phytopathogens must overcome the plant's defenses, one alternative is the use of effectors to avoid recognition (Jaswal et al., 2020), which is particularly essential to biotrophic pathogens because of their lifestyle. Effectors are described as molecules secreted by an organism, which modulate the functions and physiology of another organism (Dalio et al., 2017). Thus, the study of effector candidate genes has been useful to understand the interaction of rust-host (Lorrain, Petre, et al., 2018). Schwessinger et al. (2020) compared the whole genomic of two isolates from *Puccinia striiformis* f.sp. *tritici*, the causal agent of stripe rust of wheat. The authors observed that 30% of the predicted candidate effector genes were variable between the two isolates, which may explain host adaptation and different phenotypes. It was hypothesised the variations of effector candidates may be due to de novo evolution, horizontal gene transfer or gene loss after divergence (Tsushima et al., 2019) or mutations caused by small insertions and deletions, structural variations, and the move of transposable elements (Schwessinger et al., 2020). Moreover, for rust pathogens, the set of effectors used by *Puccinia* group may not be the same as *Melampsora* group, suggesting that the difference is associated with their ability to infect a distinct set of host species (Bruce et al., 2014).

Here, we hypothesized that the comparative analysis of those three biotypes may show a variation among candidate genes to effectors. The biotypes might request a wide and variable repertoire of effector candidates to overcome defense responses from different hosts. Therefore, in this study, we identified the effector candidates from the three biotypes, the South African (SA), the Brazilian (MF-1), and the Australian pandemic (Au). Then, we predicted their subcellular localization and functions. Some effectors were searched for polymorphisms (indels and SNPs), using bioinformatic tools to elucidate if the differences among the effectors would be connected to the several hosts affected by *A. psidii*. This is the first study comparing effector candidates from biotypes of *A. psidii*, and also the first detailed report of effector candidates from South African and Australian biotypes.

2.1. Conclusion

There are several biotypes of *A. psidii*, though the evolutionary mechanisms involved to explain the several hosts of *A. psidii* are still unclear. The effector's study may provide information about the genetic variability among the biotypes. Our findings in Chapter 2, showed effector candidates conserved and non-conserved among the biotypes Au, MF-1, and SA, as well unique and shared protein clusters to the biotypes. This work provided evidence of variability on effector candidates of *A. psidii*

biotypes, however, it is necessary to solve the limitation about genome information from the biotypes. Therefore, our findings lay a strong foundation for further studies to characterize the variability among the effector candidates of *A. psidii* biotypes.

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