NATÁLIA RISSO FONSECA

Etiology and *de novo* transcriptome analysis of the powdery mildew pathogen on *Eucalyptus* in Brazil

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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(Coorientador)

Acelino Couto Alfenas
(Orientador)

À minha família e amigos

Eu dedico essa tese

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BIOGRAFIA

Natália Risso Fonseca nasceu na cidade de São José dos Campos, no Estado de São Paulo, no dia 19 de junho de 1986.

No ano de 2005 ingressou no curso de Engenharia Florestal na Universidade Federal de Viçosa, na cidade de Viçosa, Minas Gerais, sendo o mesmo concluído ao final do ano de 2009. No período de 2007 a 2009, foi bolsista de Iniciação Científica sob orientação do Professor Acelino Couto Alfenas e em agosto de 2010 iniciou o curso de mestrado em Fitopatologia sob mesma orientação no Laboratório de Patologia Florestal do Departamento de Fitopatologia/UFV, no qual trabalhou com bactérias fitopatogênicas ao eucalipto, em especial *Ralstonia solanacearum*, causadora da murcha-bacteriana do eucalipto. Finalizando o mestrado em agosto de 2012, deu início ao doutorado em Fitopatologia na mesma instituição ainda sob a orientação do Prof. Acelino C. Alfenas com o projeto sobre a etiologia da doença oídio do eucalipto. Durante 2015 realizou doutorado-sanduíche no Rocky Mountain Research Station do USDA Forest Service, localizado em Moscow, Idaho, EUA, sob a orientação do pesquisador Dr. Ned B. Klopfenstein.

SUMÁRIO

RESUMO

FONSECA, Natália Risso, D.Sc., Universidade Federal de Viçosa, agosto de 2016. **Etiologia e análise *de novo* do transcriptoma do patógeno causador de oídio em *Eucalyptus* no Brasil.** Orientador: Acelino Couto Alfenas. Coorientador: Lúcio Mauro da Silva Guimarães.

Oídio do eucalipto é uma importante doença que ocorre principalmente em casas de vegetação e minijardins clonais protegidos de eucalipto (*Eucalyptus* spp.) no Brasil. O fungo infecta folhas jovens e brotações. Sobre o tecido afetado, observam-se colônias superficiais isoladas ou agrupadas do fungo de coloração branca, que podem atingir toda superfície foliar e induzir malformação dos órgãos afetados e resultar em redução do crescimento e da produção de brotos para estaquia. Devido ao aumento da incidência e importância dessa doença nos últimos anos e também à falta de pesquisas relacionadas a esse patossistema, esse estudo objetivou: i) determinar a etiologia do oídio do eucalipto por meio do sequenciamento da região ITS e 28S do rDNA e de análises morfológicas de isolados de oídio coletados em diferentes regiões geográficas do Brasil; e ii) analisar o transcriptoma do patógeno durante a infecção em *Eucalyptus urophylla* gerado pelo sequenciamento do transcriptoma (RNA-Seq) e montagem *de novo*. Baseado nos resultados de análises filogenéticas e caracterização morfológica, todos os 42 isolados coletados foram identificados como *Podosphaera pannosa*, também conhecido como agente etiológico do oídio das roseiras. Inoculações cruzadas com isolados de *P. pannosa* de roseira e eucalipto demonstraram que *P. pannosa* pode infectar ambas as espécies. O sequenciamento do transcriptoma de *P. pannosa* pela plataforma Illumina resultou em 12.107 transcritos. Entre os 10 transcritos mais abundantes, encontram-se os genes codificadores de enzimas envolvidas no estabelecimento e crescimento do fungo. A predição do secretoma do fungo resultou em 217 proteínas, das quais 14 foram consideradas como candidatas a efetores. Além disso, 242 pares de *primers* foram desenhados a partir das sequências do transcriptoma com potencial para amplificar regiões microssatélites (*Simple Sequence Repeats* - SSR) de *P. pannosa*. Os resultados gerados neste trabalho demonstram que apenas a espécie *P. pannosa* causa doença no eucalipto. Além disso, fornece informações úteis para novos avanços nos estudos sobre a doença por oferecer uma base para a melhor compreensão sobre o patossistema *P. pannosa*- eucalipto.

ABSTRACT

FONSECA, Natália Risso, D.Sc., Universidade Federal de Viçosa, August, 2016. **Etiology and *de novo* transcriptome analysis of the powdery mildew pathogen on *Eucalyptus* in Brazil**. Adviser: Acelino Couto Alfenas. Co-adviser: Lúcio Mauro da Silva Guimarães.

Eucalypt powdery mildew is an important disease that occurs mainly in greenhouses and protected clonal hedges of eucalypt (*Eucalyptus* spp.) in Brazil. The fungal pathogen infects new leaves and shoots. White superficial colonies isolated or grouped that grow over the affected plant tissue are observed, which can subsequently spread to all leaf surface, causing leaf malformation and reduction on growth and production of shoots for mini-cutting. Because this disease has increased in incidence and importance in recent years, and research on this pathosystem is largely lacking, the objectives of this study were to i) determine the etiology of the disease through ITS and 28S rDNA sequencing and morphological analyses of powdery mildew pathogens isolates collected in different regions in Brazil; and ii) analyze the transcriptome of the powdery mildew pathogen during infection on *Eucalyptus urophylla* generated by RNA sequencing (RNA-Seq) and *de novo* assembly. Based on the results of phylogenetic analyses and morphological characteristics, all 42 pathogen isolates collected were identified as *Podosphaera pannosa*, also known to cause rose powdery mildew. Cross inoculations with pathogen isolates from rose (*Rosa* spp.) and eucalypt demonstrated that *P. pannosa* can infect both host species. The transcriptome sequencing by Illumina platform resulted in 12,107 transcripts. Among the 10 most abundant transcripts included genes encoding enzymes involved in fungal establishment and growth. The secretome prediction resulted in 217 proteins, of which 14 were considered as candidate effectors. In addition, 242 primer pairs were designed from the transcriptome sequences with potential to amplify *P. pannosa* microsatellites (Simple Sequence Repeats – SSR) regions. The results demonstrate that *P. pannosa* is the only causal agent found for eucalypt powdery mildew. In addition, this study provides technological advances in the study of this disease that will provide a basis for a better understanding of the *P. pannosa*- eucalypt pathosystem.

GENERAL INTRODUCTION

Eucalypt plantations in Brazil covers an area of 5.6 million hectares with a mean productivity of 36 m³/ha/year in 2015 (IBÁ, 2016). This area has been increasing over the past few years to supply the growing demand of raw material for the production of pulp and paper, charcoal, essential oils, timber, utility poles, fence posts, and wood for construction (Alfenas et al., 2009). To achieve this high productivity forestry-based Brazilian companies have maintained significant investments in research and development, seeking for improvements in genetic and management techniques. Great technological advances in eucalypt production have occurred over the years, especially since the introduction of clonal propagation and improvement of the nursery facilities (Alfenas et al., 2009). Unfortunately, typical environmental conditions in nurseries, such as free water, high humidity, high temperatures, tender plant tissue, high density of plants, and continuous cultivation of the same clones, are also conducive for disease outbreaks (Grigoletti Junior et al., 2001). Drip irrigation on sand beds of clonal hedges, established under fixed or retractable translucent plastic roof disfavors infection of most foliar pathogens, with the exception of powdery mildew pathogen, that requires high relative humidity, but absence of free water (Silva et al., 2003). As a result, powdery mildew has become one of the most important diseases in eucalypt clonal hedges in Brazil.

Powdery mildew, which infects nearly 10,000 host species, is easily recognizable by the superficial white colonies produced by anamorphic mycelia, conidiophores, and conidia developed on the surface of affected leaves and shoots (Braun and Cook, 2012). Although powdery mildew rarely kills eucalypt plants, it can cause severe leaf and shoot distortion, shoot discoloration, and growth reduction that results in losses for production (Ferreira, 1989; Keane et al., 2000). It may occur under nurseries, greenhouses, and eventually in the field (Santos et al., 2001).

Powdery mildew is caused by a group of biotrophic plant pathogens, which belongs to the order Erysiphales. Five species of powdery mildew pathogens that infect eucalypt in nurseries have been identified worldwide: *Golovinomyces cichoracearum* (=*Erysiphe cichoracearum*) in United Kingdom, New Zealand, and USA (Stone, 1972; Gardner and Yarwood, 1974; Boesewinkel, 1979; Matheron and Matejka, 1992), *Golovinomyces orontii* (=*Erysiphe orontii*) in New Zealand

(Boesewinkel, 1981), *Podosphaera aphanis* (=*Sphaerotheca aphanis*) in New Zealand, Australia, and Japan (Boesewinkel, 1981; Cunnington et al., 2003; Tanda and Hirose, 2003), *Podosphaera macularis* (=*Sphaerotheca macularis*) in Germany (Brandenburger, 1961), and *Podosphaera pannosa* (=*Sphaerotheca pannosa*) in Italy, United Kingdom, Denmark, Poland, Portugal, Argentina, New Zealand, Australia, South Africa, and recently, in Korea (Grasso, 1948; Glasscock and Rosser, 1958; Spaulding, 1961; Gibson, 1975; Boesewinkel, 1981; Crous et al., 1989; Cunnington et al., 2003; Delhey et al., 2003; Cho et al., 2016).

In Brazil, powdery mildew on *Eucalyptus* was first reported in 1936 by Grillo, and subsequent reports determined that *Oidium* sp. or *Oidium eucalypti* Rostrup. was the causal agent, based on the asexual structures of the fungal pathogen (Mucci et al., 1980). The classification and identification based on anamorph morphology of Erysiphales has been useful; however, accordingly to Braun (1987) some species are not easily identified based solely on anamorphic characteristics, and the identification is provided at only the genus level (*Oidium* sp.). Based on anamorph morphology, Silva et al. (2001) concluded that eucalypt powdery mildew pathogen in Brazil is similar to rose powdery mildew pathogen, described as *P. pannosa*, which requires definitive confirmation. With the advent of molecular techniques, substantial DNA sequencing data of Eysiphales have been generated (Mori et al., 2000; Limkaisang et al., 2006). DNA sequence comparisons allow Erysiphales anamorphs to be linked with their respective teleomorph, even when the teleomorph is not observed (Cunnington et al., 2003; Wingfield et al., 2012).

Despite the advances in generating molecular data, powdery mildew pathogen databases are still in their infancy compared to other fungal taxa. Whole transcriptome sequencing using next-generation sequencing technologies or RNA Sequencing (RNA-Seq) provides a particularly useful alternative for obtaining high-quality sequence information from diverse organisms. RNA-Seq has increased the quality and utility of transcriptome analysis through the sequencing of entire transcriptome of an organism under a given condition. This technique is a powerful and relatively cost-effective, high-throughput sequencing method that uses deep sequencing to produce millions of short-sequence reads. RNA-Seq is a practical approach, because a reference genome is not required and provides a starting point for functional genetic characterization of non-model organisms (Parchman et al., 2010).

RNA-Seq provides a method to find and identify protein-coding genes in the transcriptome that act in important metabolic pathways necessary for successful pathogen infection, host resistance, or other host-pathogen interactions (Zheng et al., 2009; Weßling et al., 2012; Weng et al., 2014). RNA-Seq also provides fundamental information for comparative genomic studies (Hurtado Páez et al., 2015; Wang et al., 2015), secretome analysis, and prediction of fungal candidate secreted effectors proteins (Bruce et al., 2014; Meinhardt et al., 2014; Liu et al., 2015). Furthermore, RNA-Seq is a useful tool for discovering genetic markers that can be applied in population genetics studies (Frenkel et al., 2012; Tollenaere et al., 2012).

This thesis was divided in two articles. In article 1, the objective was to identify the causal agent of powdery mildew pathogen on eucalypt through ITS and 28S rDNA sequencing and morphological studies of isolates collected from eucalypt nurseries in different regions of Brazil. In addition, article 1 determines if the eucalypt powdery mildew pathogen is the same pathogen that causes powdery mildew on roses. Article 2 provides analyses of the powdery mildew pathogen transcriptome during infection on *E. urophylla*, which was generated by next-generation sequencing and *de novo* assembly.

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ARTICLE 1 - Eucalypt powdery mildew caused by *Podosphaera pannosa* in Brazil

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ABSTRACT

Eucalypt powdery mildew is an important disease in greenhouses and clonal hedges in Brazil, which can cause leaf and shoot distortion, shoot discoloration, and growth reduction that results in production losses. Because reliable information regarding the causal agent of the disease is lacking, this study used ITS and 28S rDNA sequencing and morphological analyses to identify the powdery mildew pathogen that occurred in eucalypt nurseries within different regions in Brazil. Based on the results of morphological characteristics and phylogenetic analyses, the pathogen isolates were identified as *Podosphaera pannosa*, also known as rose powdery mildew. Cross inoculations with pathogen isolates from rose and eucalypt demonstrated that *P. pannosa* can infect both host species. The ITS sequence-based phylogeny showed that 42 sequences generated in this study were comprised within a single clade containing *P. pannosa*, which was supported by posterior probability of 88%. Identical ITS sequences were obtained from all 42 pathogen isolates, which suggests the potential of a clonal population.

Key words: ITS rDNA, 28S rDNA, Bayesian analysis, *Eucalyptus*, *Rosa*, cross inoculation.

1 INTRODUCTION

The production system of eucalypt (*Eucalyptus* spp.) cuttings in Brazil has been evolving over recent decades with the introduction of improved clonal mini-cuttings and enhanced nursery facilities (Alfenas et al., 2009). The current environmental conditions in nurseries, such as nursery coverage and drip irrigation, are disadvantageous to most diseases except powdery mildew, which is favored under these conditions (Silva et al., 2003). Although powdery mildew rarely occurs in eucalypt plantations, it is commonly encountered in greenhouses and clonal hedges where it can cause severe leaf and shoot distortion, shoot discoloration, growth reduction, and production losses (Keane et al., 2000). As a result, powdery mildew has become one of the most important diseases in eucalypt clonal hedges in Brazil.

Powdery mildew diseases are caused by biotrophic fungi in the Erysiphales. This fungal pathogen is readily observed as conspicuous external mycelium, typically forming white patches that may cover the entire leaf surface (Figure 1) (Braun and Cook, 2012). Several eucalypt species are infected by powdery mildew pathogens (Old et al., 2003). Five species of powdery mildew pathogens that infect eucalypt in nurseries have been identified worldwide: *Golovinomyces cichoracearum* (=*Erysiphe cichoracearum*) in United Kingdom, New Zealand, and USA (Stone, 1972; Gardner and Yarwood, 1974; Boesewinkel, 1979; Matheron and Matejka, 1992), *Golovinomyces orontii* (=*Erysiphe orontii*) in New Zealand (Boesewinkel, 1981), *Podosphaera aphanis* (=*Sphaerotheca aphanis*) in New Zealand, Australia, and Japan (Boesewinkel, 1981; Cunnington et al., 2003; Tanda and Hirose, 2003), *Podosphaera macularis* (=*Sphaerotheca macularis*) in Germany (Brandenburger, 1961), and *Podosphaera pannosa* (=*Sphaerotheca pannosa*) in Italy, United Kingdom, Denmark, Poland, Portugal, Argentina, New Zealand, Australia, South Africa, and Korea, which was recently reported (Grasso, 1948; Glasscock and Rosser, 1958; Spaulding, 1961; Gibson, 1975; Boesewinkel, 1981; Crous et al., 1989; Cunnington et al., 2003; Delhey et al., 2003; Cho et al., 2016). Powdery mildew has also been reported on field-grown *Corymbia citriodora* in Brazil, with leaf deformation and loss of apical dominance observed in young plants (Ferreira, 1997).

Figure 1. A) *Eucalyptus* nursery in State of Minas Gerais, Brazil, with plants showings powdery mildew signs; B) plants highly infected with powdery mildew pathogen; and C) eucalypt leaf with mycelia and conidia of powdery mildew pathogen.

In Brazil, powdery mildew on *Eucalyptus* was first reported in 1936 by Grillo, and subsequent reports determined that *Oidium* sp. or *Oidium eucalypti* Rostrup. was the causal agent, based on the anamorphic stage of the fungal pathogen (Mucci et al., 1980). Most powdery mildew pathogen anamorphs are poorly differentiated at the species level based on morphology (Braun and Cook, 2012). Furthermore, accurate identification of the eucalypt powdery mildew pathogen in Brazil is hampered because sexual reproductive structures are lacking (Bedendo, 2011). Previous artificial inoculation studies indicated that powdery mildew pathogen isolates from *Rosa* sp. and *Dhalia* sp., which were classified as *P. pannosa* and *G. cichoracearum*, respectively, were also pathogenic to *E. pellita* (Silva et al., 2001). Based on morphological features of the anamorph, Silva et al. (2001) concluded that the eucalypt powdery mildew pathogen found in Brazil was similar to rose powdery mildew pathogen.

Currently, the widely accepted concept of one fungus = one name proposes to end the dual nomenclature of pleomorphic fungi, and provide one species name that comprises the teleomorphic and anamorphic stages of the same fungus (Taylor, 2011; Wingfield et al., 2012). With the advent of molecular techniques, substantial DNA sequencing data of Eysiphales have been generated (Mori et al., 2000; Limkaisang et al., 2006). DNA sequence comparisons allow Erysiphales anamorphs to be linked with their respective teleomorph, even when the teleomorph is not observed (Cunnington et al., 2003; Wingfield et al., 2012). Despite the available sequence data, the eucalypt powdery mildew pathogen in Brazil remains known

solely by the anamorphic species *Oidium eucalypti*, which is not well characterized (Braun and Cook, 2012), or *Oidium* sp., which can be attributed to several Erysiphales teleomorphs.

Studies that help develop technologies for managing the powdery mildew are an urgent need. For disease management, it is essential to know precisely which powdery mildews pathogen species is/are causing disease on eucalypt in Brazil. Identification of powdery mildew pathogens is needed to 1) help breeding programs aimed at developing resistant plants, 2) contribute to more effective chemical and cultural control, which can be influenced by pathogen species or races, and 3) determine if the pathogen is native or introduced. Thus, the objective of this study was to identify the powdery mildew pathogens infecting eucalypt in different regions of Brazil using phylogenetic analyses and morphological characteristics.

2 MATERIAL AND METHODS

2.1 Sample sources

Eucalypt powdery mildew pathogen isolates were collected during March to September 2014 from mini-clonal hedges in greenhouses from several clonal *Eucalyptus* spp. nurseries in five states of Brazil (Table 1). Six isolates of rose powdery mildew pathogen (*Podosphaera pannosa*) from nurseries near Viçosa, MG, Brazil were also included in this study. Specimens (mycelia and conidia) collected by scraping a leaf from one diseased plant or clone were considered as an isolate.

2.2 DNA extraction

Total DNA was extracted from conidia and mycelia by the Chelex method (Walsh et al., 1991; Hirata and Takamatsu, 1996). Conidia were added to 50 µL of 5% Chelex® (Bio-Rad) in a 1.5-mL microcentrifuge tube and incubated at 56 °C for 2 h. After mixing vigorously, the extract was incubated in boiling water for 8 min. The extract was mixed again and centrifuged at 15,000 x g for 5 min. The supernatant was transferred to another tube and used as DNA template.

2.3 PCR amplification

The nuclear rDNA region including the ITS regions were amplified by nested- PCR using primers ITS5 (5'-GAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990) and P3 (5'-GCCGCTTCACTCGCCGTTAC-3') (Kusaba and Tsuge, 1995) for the first amplification. The first PCR product was used as a template for the second PCR using the primers ITS5 and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). For PCR amplification of the 28S rDNA gene, the primer set PM3 (5'-GKGCTYTMCGCGTAGT-3') (Takamatsu and Kano, 2001) and TW14 (5'-GCTATCCTGAGGGAAACTTC-3') (Mori et al., 2000) were used. The reaction was performed in a final volume of 25 µL. The amplification program consisted of an initial step of denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing from 52 °C, for 30 sec, and extension at 72 °C for 30 sec, with a final extension at 72 °C for 7 min. The PCR products were analyzed by electrophoresis in 1.4% (w/v) agarose gels, stained with ethidium bromide (1.0 µg mL^{-1}), and photodocumented.

2.4 DNA sequencing and data analysis

Both amplicon strands were sequenced using ITS5/ITS4 primers for ITS rDNA sequencing, and primers PM3/TW14 for 28S rDNA sequencing. PCR-amplified regions were sequenced using an ABI PRISM 3100 sequencer (Applied Biosystems). Sequences were edited and used in similarity searches on GenBank database using BLASTN (Basic Logical Alignment Search Tool) (Altschul et al., 1990) at NCBI (http://www.ncbi.nlm.nih.gov) to confirm them as Erysiphales. The sequences were aligned with homologous sequences available on GenBank. A total of 37 ITS sequences of *Podosphaera* spp. were aligned and used for analyses. For 28S rDNA-sequence alignment, 20 sequences of *Podosphaera* spp. were used, which are available on GenBank (Table 1). Sequences of *Cystotheca wrightii* and *Cystotheca lanestris* were used as outgroups, based on Mori et al. (2000). Analyses were performed in Muscle program (Edgar, 2004) built in MEGA v. 5 software (Tamura et al., 2007), followed by manual adjustments. The Bayesian inference method was used to construct phylogenetic trees using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The substitution model was chosen based on the Akaike information criterion (AIC). The posterior probability in the distribution of the trees

was calculated using the MCMC algorithm (Metropolis-coupled Markov chain Monte Carlo), with two chains from a random tree and 1×10^6 generations executed, discarding the first 25% of the trees. The phylogenetic trees were viewed and edited in the program FigTree v. 1.3.1. (http://tree.bio.ed.ac.uk/software/). Some ambiguous bases was coded using IUPAC (International Union of Pure and Applied Chemistry) codes.

2.5 Morphological study

Eucalypt powdery mildew pathogen isolate LPF 615 was selected for morphological studies. In addition, rose powdery mildew isolates LPF 659 and LPF 660 were also analyzed. Mycelia, conidiophores, and conidia were scraped from the infected surface of a fresh leaf and placed into a drop of lactic acid on a glass slide for light microscopy. Alternatively, mycelia, conidiophores, and conidia were stripped off the leaf surfaces with clear adhesive tape, mounted on a microscope slide, and examined in water using light microscope at 40x magnification. Germination tests were performed by lightly tapping an infected leaf upon a glass slide. The slide was placed in a plastic germination box 'gerbox' containing a wet paper towel, and the box was closed to constitute a moist chamber. The slide was maintained in the gerbox for 24 h. After that period, the slide was examined in water using light microscope at 40x magnification. Morphological characters, such as size and shape of conidia; presence or absence of fibrosin bodies in fresh materials; characteristics of the conidiophore; nature of conidiogenesis; hyphal morphology; position of conidial germ tubes; and shape of germ tube-derived appressoria were observed and recorded.

2.6 Cross inoculations

To evaluate the capability of eucalypt powdery mildew pathogens to infect rose plants, 10 healthy rose cuttings (*Rosa* sp. L. var. 'Ambiance') were kept in growth chamber at 19 ± 2 °C with a 12-h photoperiod and light intensity of 40 $\mu mol/s/m^2$, which was free of powdery mildew inoculum. Cuttings were monitored for possible latent infections for a period of 10 days. Eucalypt powdery mildew pathogen isolate LPF 615, collected in Viçosa, MG, Brazil and maintained on eucalypt cuttings in a growth chamber free of other inoculum sources, was used for

inoculation. Inoculations were performed with a small soft brush by dusting conidia from an infected eucalypt leaf onto young leaves of rose. The substrate of inoculated rose plants were then watered, covered separately with a plastic bag, and closed on the bottom with rubber band to constitute a moist chamber. The inoculated plants were covered for 24 h. After that period, they were uncovered and placed interspersed among five *Eucalyptus* plants with powdery mildew. Rose plants were evaluated daily for powdery mildew. The same procedure was used to test whether eucalypt cuttings could be infected by powdery mildew pathogens from rose. Ten healthy *E. urophylla* cuttings were kept in a growth chamber at 19 °C with a 12-h photoperiod and light intensity of 40 μmol/s/m^2, free of powdery mildew inoculum. Rose plants with powdery mildew were used for inoculation as described previously. Eucalypt plants were evaluated daily for powdery mildew signs and symptoms. To reconfirm the identity of the pathogen on inoculated eucalypt and rose plants, ITS rDNA sequencing was performed using ITS5/ITS4 primers and both amplicon strands were sequenced.

3 RESULTS

3.1 DNA extraction, PCR amplification, and DNA sequencing

Of 82 samples of powdery mildew pathogens collected from eucalypt plants, 49 samples yielded sufficient DNA for PCR, which resulted in 42 sequences of ITS rDNA and 49 sequences of 28S rDNA. The sequences were 417 bases in length for ITS rDNA and 768 bases for 28S rDNA. All 42 ITS sequences were identical without insertions, deletions, or substitutions. For this reason, only one sequence was deposited in GenBank (accession #KX185528), and the same number was assigned to ITS sequences of all eucalypt powdery mildew pathogen isolates. Among the ITS sequences of rose powdery mildew pathogens, the isolates LPF 659 and LPF 663 differed from other rose isolates by one base, forming a subclade within the *P. pannosa* clade. The rose isolates LPF 660, LPF 661, LPF 662, and LPF 664 were identical to ITS sequences of the eucalypt powdery mildew pathogen isolates. For the 28S rDNA sequence analysis, the partial sequence of the 28S rDNA gene including the D1/D2 region was determined. The 28S rDNA sequence alignment showed that sequences of eucalypt isolates LPF622 and LPF657 differed from other

isolates by two and one base(s), respectively. ITS sequencing of isolate LPF657 was unsuccessful. The 28S rDNA sequences of rose isolates did not differ and were identical to the 28S sequences of eucalypt isolates. The 28S rDNA sequences were also deposited on GenBank (Table 1).

Searches on BLASTN revealed that all sequences were very similar to species within the genus *Podosphaera* for both ITS and 28S rDNA regions. The best evolutionary model selected by MrModeltest 2.3 for Bayesian analysis of ITS alignment data by AIC was the model GTR + I + G, with parameters I = 0.5848 and G = 1.3165. The model general time reversible (GTR) takes into account the rate of substitution for each pair of nucleotides and considers the frequencies of the four nucleotides. Accordingly with the phylogenetic tree obtained based on Bayesian analyses with the sequences of ITS rDNA all sequences generated in this study were comprised within a single clade that contained *Podosphaera pannosa*, supported by 88% of posterior probability (Figure 2).

For 28S rDNA sequence data, the best evolutionary model selected by AIC was GTR + I, with parameter I = 0.8710. The resulting 28S rDNA-based phylogenetic tree did not result in a well-defined separation among species of *Podosphaera* (Figure 3), in contrast to the ITS sequence-based tree. The 49 sequences of eucalypt powdery mildew pathogens and six rose powdery mildew pathogens generated in this study were contained within a polytomy comprising *P. pannosa*, *P. clandestina*, *P. fugax*, *P. lini*, *P. spiraeae*, and *P. negeri* in a clade supported by 100% of posterior probability. An additional subclade comprising other *Podosphaera* species is also evident.

Table 1. Powdery mildew pathogen identity (ID), host, origin, and GenBank accession number.

Isolate ID	Clone/Variety	Host	Origin	GPS coordinates	GenBank accession number		Collector/ Reference
					ITS	28S	
LPF 610	6061	*Eucalyptus* sp.	Viçosa – MG	20°46'48.59"S/42°49'29.63"W	KX185528	KX185529	N. R. Fonseca
LPF 611	C219	*Eucalyptus* sp.	Viçosa – MG	20°46'48.59"S/42°49'29.63"W	KX185528	KX185529	N. R. Fonseca
LPF 612	VM3	*Eucalyptus* sp.	Viçosa – MG	20°46'48.59"S/42°49'29.63"W	KX185528	KX185529	N. R. Fonseca
LPF 613	10	*Eucalyptus* sp.	Belo Oriente - MG	19°18'48.59"S/42°23'30.09"W	KX185528	KX185529	A. G. B. Medeiros
LPF 614	37036	*Eucalyptus* sp.	Aracruz – ES	19°50'32.17"S/40°04'47.76"W	KX185528	KX185529	R. G. Mafia
LPF 615	1183	*Eucalyptus urophylla*	Viçosa – MG	20°46'48.59"S/42°49'29.63"W	KX185528	KX185529	L. M. S. Guimarães
LPF 616	1183	*Eucalyptus urophylla*	Viçosa – MG	20°46'27.7"S/42°52'35.75"W	KX185528	KX185529	N. R. Fonseca
LPF 617	BA 2004	*Eucalyptus* sp.	Itabatã – BA	18°02'34.17"S/39°55'25.77"W	KX185528	KX185529	E. A. V. Zauza
LPF 618	BA 1922	*Eucalyptus* sp.	Itabatã – BA	18°02'34.17"S/39°55'25.77"W	KX185528	KX185529	E. A. V. Zauza
LPF 619	BA 1922	*Eucalyptus* sp.	Itabatã – BA	18°02'34.17"S/39°55'25.77"W	KX185528	KX185529	E. A. V. Zauza
LPF 620	144	*E. grandis* X *E. urophylla*	Brás Pires - MG	20°49'10.3"S/43°15'22.4"W	KX185528	KX185529	N. R. Fonseca
LPF 621	1528	*E. grandis* X *E. urophylla*	Brás Pires - MG	20°49'10.3"S/43°15'22.4"W	KX185528	KX185529	N. R. Fonseca
LPF 622	144	*E. grandis* X *E. urophylla*	Lima Duarte - MG	21°45'57.7"S/43°37'22.3"W	KX185528	KX355455	N. R. Fonseca
LPF 623	144	*E. grandis* X *E. urophylla*	Guaíba - RS	30°07'54.61"S/51°19'07.56"W	KX185528	KX185529	N. Borges Junior
LPF 624	32864	*E. saligna*	Guaíba - RS	30°07'54.61"S/51°19'07.56"W	-	KX185529	N. Borges Junior
LPF 625	BA2004	*Eucalyptus* sp.	Itabatã - BA	18°02'34.17"S/39°55'25.77"W	KX185528	KX185529	E. A. V. Zauza
LPF 626	BA2004	*Eucalyptus* sp.	Itabatã - BA	18°02'34.17"S/39°55'25.77"W	KX185528	KX185529	E. A. V. Zauza
LPF 627	3367	*Eucalyptus* sp.	Curvelo - MG	18°50'43.4"S/44°35'13.0"W	-	KX185529	N. R. Fonseca
LPF 628	3335	*Eucalyptus* sp.	Curvelo - MG	18°50'43.4"S/44°35'13.0"W	-	KX185529	N. R. Fonseca
LPF 629	2682	*Eucalyptus* sp.	Curvelo - MG	18°50'43.4"S/44°35'13.0"W	KX185528	KX185529	N. R. Fonseca
LPF 630	PEM 02598	*Eucalyptus* sp.	Paraopeba - MG	19°17'15.9"S/44°29'24.4"W	-	KX185529	N. R. Fonseca
LPF 631	VM3	*Eucalyptus* sp.	Paraopeba - MG	19°17'15.9"S/44°29'24.4"W	-	KX185529	N. R. Fonseca
LPF 632	PEM 04098	*Eucalyptus* sp.	Paraopeba - MG	19°17'15.9"S/44°29'24.4"W	KX185528	KX185529	N. R. Fonseca
LPF 633	BSCDT 47702	*Eucalyptus* sp.	Paraopeba - MG	19°17'15.9"S/44°29'24.4"W	KX185528	KX185529	N. R. Fonseca
LPF 634	A08 (3301)	*Eucalyptus* sp.	Jequitibá - MG	19°09'21.8"S/43°58'25.6"W	-	KX185529	N. R. Fonseca
LPF 635	144	*E. grandis* X *E. urophylla*	Jequitibá - MG	19°09'21.8"S/43°58'25.6"W	KX185528	KX185529	N. R. Fonseca
LPF 636	1528	*E. grandis* X *E. urophylla*	Jequitibá - MG	19°09'21.8"S/43°58'25.6"W	KX185528	KX185529	N. R. Fonseca
LPF 637	224	Híbrido de *E. urophylla*	Caetanópolis - MG	19°19'47.8"S/44°21'43.3"W	KX185528	KX185529	N. R. Fonseca
LPF 638	144	*E. grandis* X *E. urophylla*	Caetanópolis - MG	19°19'47.8"S/44°21'43.3"W	KX185528	KX185529	N. R. Fonseca
LPF 639	2034	(*E. camaldulensis* X *E. grandis*) X *E. urophylla*	Caetanópolis - MG	19°19'47.8"S/44°21'43.3"W	KX185528	KX185529	N. R. Fonseca
LPF 640	224	Híbrido de *E. urophylla*	Inimutaba - MG	18°41'30.0"S/44°15'07.2"W	KX185528	KX185529	N. R. Fonseca
LPF 641	1528	*E. grandis* X *E. urophylla*	Inimutaba - MG	18°41'30.0"S/44°15'07.2"W	KX185528	KX185529	N. R. Fonseca

LPF 642	144	*E. grandis* X *E. urophylla*	Inimutaba - MG	18°41'30.0"S/44°15'07.2"W	KX185528	KX185529	N. R. Fonseca
LPF 643	144	*E. grandis* X *E. urophylla*	Três Marias - MG	18°14'59.0"S/45°10'54.0"W	KX185528	KX185529	N. R. Fonseca
LPF 644	1528	*E. grandis* X *E. urophylla*	Três Marias - MG	18°14'59.0"S/45°10'54.0"W	KX185528	KX185529	N. R. Fonseca
LPF 645	42864	*Eucalyptus* sp.	Viçosa – MG	20°46'27.7"S/42°52'35.75"W	KX185528	KX185529	N. R. Fonseca
LPF 646	D18	*E. dunnii*	Viçosa – MG	20°46'27.7"S/42°52'35.75"W	KX185528	KX185529	N. R. Fonseca
LPF 647	BA2004	*Eucalyptus* sp.	Itabatã – BA	18°02'34.17"S/39°55'25.77"W	KX185528	KX185529	E. A. V. Zauza
LPF 648	E13	*E. grandis* X *E. urophylla*	Três Lagoas – MS	20°59'30.61"S/51°47'39.78"W	KX185528	KX185529	J. F da Silva
LPF 649	E17	*Eucalyptus* sp.	Três Lagoas – MS	20°59'30.61"S/51°47'39.78"W	KX185528	KX185529	J. F da Silva
LPF 650	CNB 005	*E. grandis* X *E. urophylla*	Belo Oriente – MG	19°18'48.59"S/42°23'30.09"W	KX185528	KX185529	A. G. B. Medeiros
LPF 651	CNB 010	*E. grandis* X *E. urophylla*	Belo Oriente – MG	19°18'48.59"S/42°23'30.09"W	KX185528	KX185529	A. G. B. Medeiros
LPF 652	CNB 011	*E. grandis* X *E. urophylla*	Belo Oriente – MG	19°18'48.59"S/42°23'30.09"W	KX185528	KX185529	A. G. B. Medeiros
LPF 653	G26	*Eucalyptus* sp.	Viçosa – MG	20°46'27.7"S/42°52'35.75"W	KX185528	KX185529	N. R. Fonseca
LPF 654	57	*Eucalyptus* sp.	Viçosa – MG	20°46'27.7"S/42°52'35.75"W	KX185528	KX185529	N. R. Fonseca
LPF 655	D25	*E. dunnii*	Guaíba – RS	30°07'54.61"S/51°19'07.56"W	KX185528	KX185529	N. Borges Junior
LPF 656	D18	*E. dunnii*	Guaíba – RS	30°07'54.61"S/51°19'07.56"W	KX185528	KX185529	N. Borges Junior
LPF 657	BE 314	*E. benthamii*	Guaíba – RS	30°07'54.61"S/51°19'07.56"W	-	KX185530	N. Borges Junior
LPF 658	37036	*Eucalyptus* sp.	Guaíba – RS	30°07'54.61"S/51°19'07.56"W	KX185528	KX185529	N. Borges Junior
LPF 659	Nórdia	*Rosa* sp. var. Nórdia	Viçosa – MG	20°45'24.85"S/42°50'37.83"W	KX355453	KX355456	N. R. Fonseca
LPF 660	Greta	*Rosa* sp. var. Greta	Viçosa – MG	20°45'24.85"S/42°50'37.83"W	KX355454	KX355456	N. R. Fonseca
LPF 661	Grand Gala	*Rosa* sp. var. Grand Gala	Viçosa – MG	20°45'24.85"S/42°50'37.83"W	KX355454	KX355456	N. R. Fonseca
LPF 662	Karola	*Rosa* sp. var. Karola	Viçosa – MG	20°45'24.85"S/42°50'37.83"W	KX355454	KX355456	N. R. Fonseca
LPF 663	Not identified 1	*Rosa* sp.	Viçosa – MG	20°45'24.85"S/42°50'37.83"W	KX355453	KX355456	N. R. Fonseca
LPF 664	Not identified 2	*Rosa* sp.	Teixeiras – MG	20°38'47.20"S/42°50'50.64"W	KX355454	KX355456	P. S. Hermenegildo
P. negeri	-	*Escalloniaceae - Escallonia rubra*	Argentina	-	AB525919	AB525919	Takamatsu et al., 2010
P. negeri	-	*Escalloniaceae - Escallonia rubra*	Argentina	-	AB525920	AB525920	Takamatsu et al., 2010
P. fugax	-	*Geraniaceae - Geranium thunbergii*	Japan	-	AB525922	AB525922	Takamatsu et al., 2010
P. fugax	-	*Geraniaceae – Geranium nepalense*	-	-	AB026134	-	Takamatsu et al., 2000
P. lini	-	*Linaceae - Linum usitatissimum*	Switzerland	-	AB525925	AB525925	Takamatsu et al., 2010
P. clandestina	-	*Rosaceae - Amelanchier laevis*	Germany	-	AB525927	AB525927	Takamatsu et al., 2010
P. clandestina	-	*Rosaceae - Crataegus oxyacantha*	Argentina	-	AB525931	AB525931	Takamatsu et al., 2010
P. clandestina	-	*Rosaceae - Crataegus* sp.	Argentina	-	AB525932	AB525932	Takamatsu et al., 2010
P. pannosa	-	*Rosaceae - Rosa rubiginosa*	Argentina	-	AB525937	AB525937	Takamatsu et al., 2010
P. pannosa	-	*Rosaceae - Rosa* sp.	-	-	AB022348	AB022347	Mori et al., 2000
P. pannosa	-	*Rosaceae - Rosa* sp.	Mexico	-	KM001666	-	Felix Gastelum et al., 2014
P. pannosa	-	*Rosaceae - Rosa* sp.	Mexico	-	KM001669	-	Felix Gastelum et al., 2014
P. pannosa	-	*Rosaceae - Catharanthus roseus*	USA	-	KF703448	-	Romberg et al., 2014
P. pannosa	-	*Rosaceae - Prunus cerasus*	France	-	JN654341	-	Hubert et al., 2012

P. pannosa	-	*Rosaceae - Rosa* sp.	France	-	DQ139421	-	Leus et al., 2006
P. pannosa	-	*Rosaceae - Rosa* sp.	Germany	-	DQ139425	-	Linde & Debene, 2003
P. pannosa	-	*Rosaceae - Rosa* sp.	Germany	-	DQ139427	-	Leus et al., 2006
P. pannosa	-	*Rosaceae – Prunus* sp.	Belgium	-	DQ139429	-	Leus et al., 2006
P. pannosa	-	*Myrtaceae – Eucalyptus* sp.	Australia	-	AF298543	-	Cunnington et al., 2003
P. pannosa	-	*Rosaceae – Rosa maltiflora*	Japan	-	AB525939	-	Takamatsu et al., 2010
P. spiraeae	-	*Rosaceae – Filipnedula purpurea* var. *purpurea*	-	-	AB022385	AB022384	Mori et al., 2000
P. xanthii	-	*Asteraceae - Calendula officinalis*	Argentina	-	AB525914	AB525914	Takamatsu et al., 2010
P. xanthii	-	*Asteraceae – Helianthus annuus*	Japan	-	AB040311	AB462774	Ito and Takamatsu, 2010
P. xanthii	-	*Asteraceae – Lactuca raddeana* var. *elata*	Japan	-	AB040352	AB462776	Ito and Takamatsu, 2010
P. xanthii	-	*Verbenaceae – Verbena* x *hybrida*	Japan	-	AB040347	AB462780	Ito and Takamatsu, 2010
P. xanthii	-	*Lamiaceae – Lycopus lucidus*	Japan	-	AB040343	AB462778	Ito and Takamatsu, 2010
P. astericola	-	*Asteraceae – Zinnia elegans*	Japan	-	AB040335	AB462779	Ito and Takamatsu, 2010
P. balsaminae	-	*Balsaminaceae - Impatiens balsamina*	Japan	-	AB462803	AB462788	Ito and Takamatsu, 2010
P. balsaminae	-	*Balsaminaceae - Impatiens noli-tangere*	Japan	-	AB462805	AB462789	Ito and Takamatsu, 2010
P. aphanis	-	*Rosaceae - Agrimonia pilosa*	-	-	AB000938	-	Takamatsu et al., 1998
P. aphanis	-	*Rosaceae - Agrimonia pilosa* var. *japonica*	-	-	AB026141	-	Takamatsu et al., 2000
P. aphanis	-	*Myrtaceae – Eucalyptus* sp.	Australia	-	AF073355	-	Cunnington et al., 2003
P. aphanis	-	*Rosaceae - Fragaria chiloensis*	Argentina	-	AB525933	-	Takamatsu et al., 2010
P. tridactyla	-	*Rosaceae - Prunus laurocerasus*	Switzerland	-	AY833654	-	Cunnington et al., 2005
P. tridactyla	-	*Rosaceae - Prunus persica*	Australia	-	AY833653	-	Cunnington et al., 2005
P. tridactyla	-	*Rosaceae - Prunus japonica*	-	-	-	AB022393	Mori et al., 2000
P. tridactyla	-	*Rosaceae - Prunus* sp.	-	-	AB000943	-	Takamatsu et al., 2000
P. longiseta	-	*Rosaceae - Prunus grayana*	-	-	AB000945	AB022423	Takamatsu et al., 2000
C. wrightii	-	*Fagaceae - Quercus glauca*	-	-	AB000932	AB022355	Takamatsu et al., 2000
C. lanestris	-	*Fagaceae - Quercus agrifolia*	-	-	AB000933	AB022353	Takamatsu et al., 2000

2

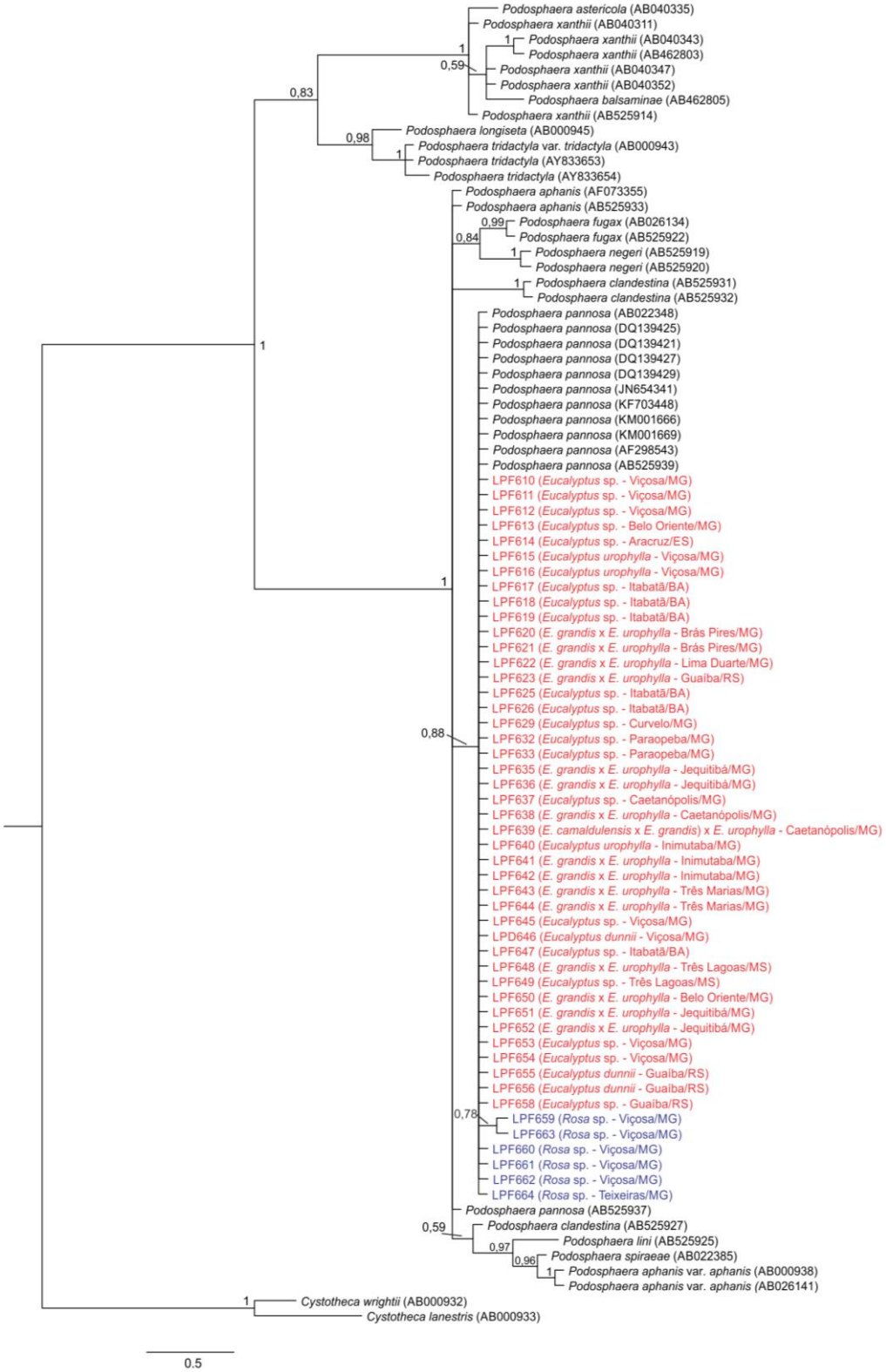

Figure 2. ITS sequence-based Bayesian phylogenetic analysis of powdery mildew pathogens of *Eucalyptus* spp. (depicted in red) and *Rosa* sp. (in blue). Posterior probability support percentages are indicated at the branch nodes.

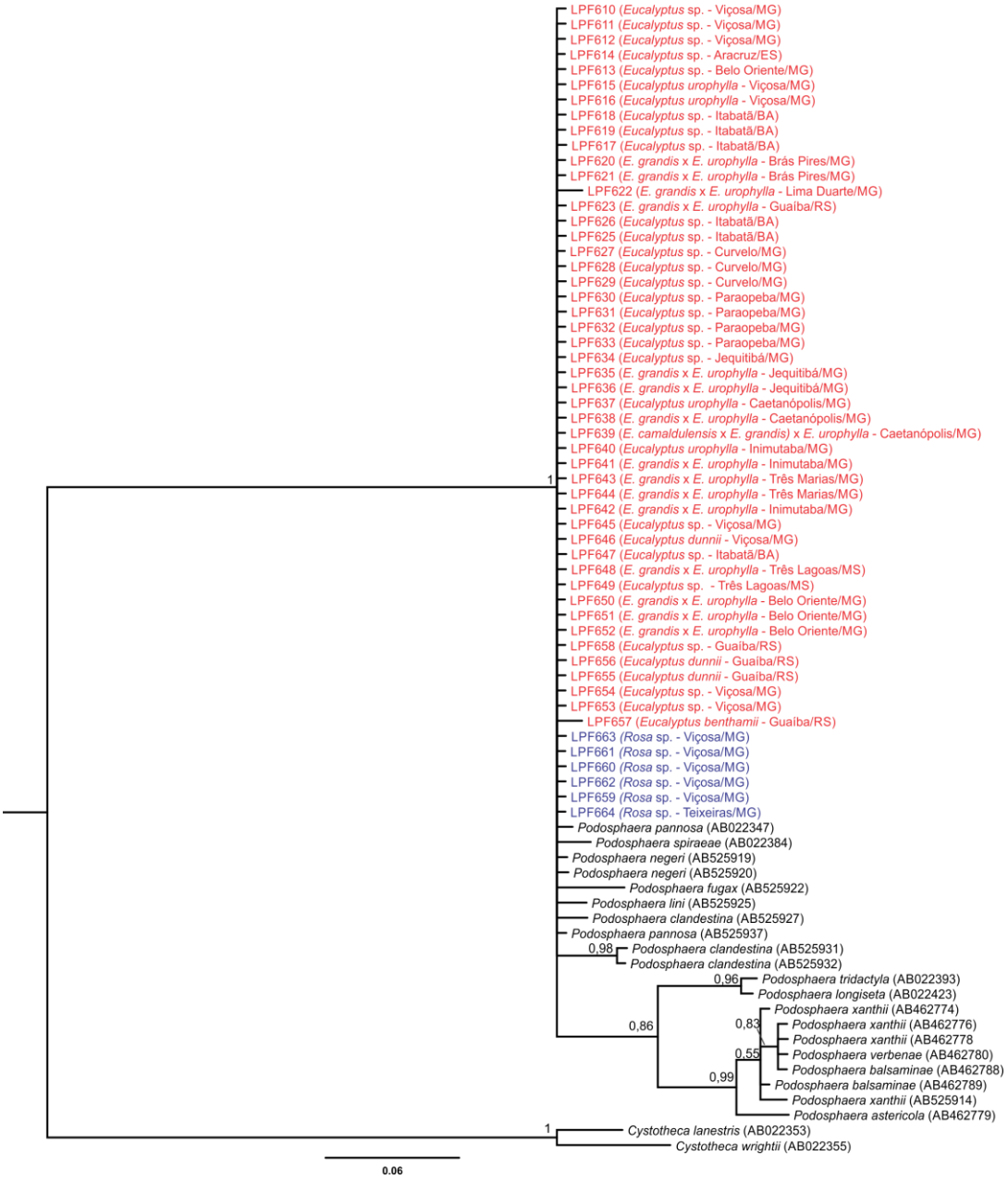

Figure 3. 28S rDNA sequence-based Bayesian phylogenetic analysis of powdery mildew pathogens of *Eucalyptus* spp. (depicted in red) and *Rosa* sp. (depicted in blue). Posterior probability support percentages are indicated at the branch nodes.

3.2 Morphological study

The isolates studied presented morphological characters typical of *Podosphaera*, having conidia formed in chains and presence of fibrosin bodies. Hyphae were septate, branched and hyaline with nipple-shaped appressoria; conidiophores were epiphytic, septate, hyaline; foot-cells were cylindrical, 42 – 68 × 7.3 – 9.5 µm; conidia were produced in chains at the apex of the conidiophores, ellipsoid-ovoid to doliiform, 21.6 – 32.5 × 10.9 –19 µm, aseptate, hyaline with germ tubes terminal to lateral *Fibroidium* type, orthotubus subtype (Figure 4). Although *P. aphanis* and *P. macularis* have been described on *Eucalyptus*, *P. macularis* has a characteristic symptom of numerous limited patches on leaves (Braun and Cook, 2012), while *P. aphanis* presents long foot-cells (Braun 1987), which were not observed on infected eucalypt and during morphological analyses.

3.3 Pathogen identification

Based on comparisons of DNA sequences and morphological analyses, it is concluded that all eucalypt powdery mildew pathogens isolates collected from different regions of Brazil belong to the same species, *Podosphaera pannosa.*

3.4 Cross inoculations

After rose plants were inoculated with eucalypt powdery mildew pathogen isolate LPF 615, mycelial signs of powdery mildew were first observed at 10 days post-inoculation (Figure 4). Thus, the eucalypt powdery mildew pathogen is capable of infecting roses. For eucalypt inoculation with rose powdery mildew pathogens, the first signs of powdery mildew were observed at 7 days post-inoculation (Figure 4). Thus, the rose powdery mildew pathogen is capable of infecting eucalypt. ITS sequencing reconfirmed the identity of powdery mildew pathogen isolates collected from eucalypt and rose infected plants. Generated sequences were aligned with previous ITS sequences of this study and resulted in 100% homology among then.

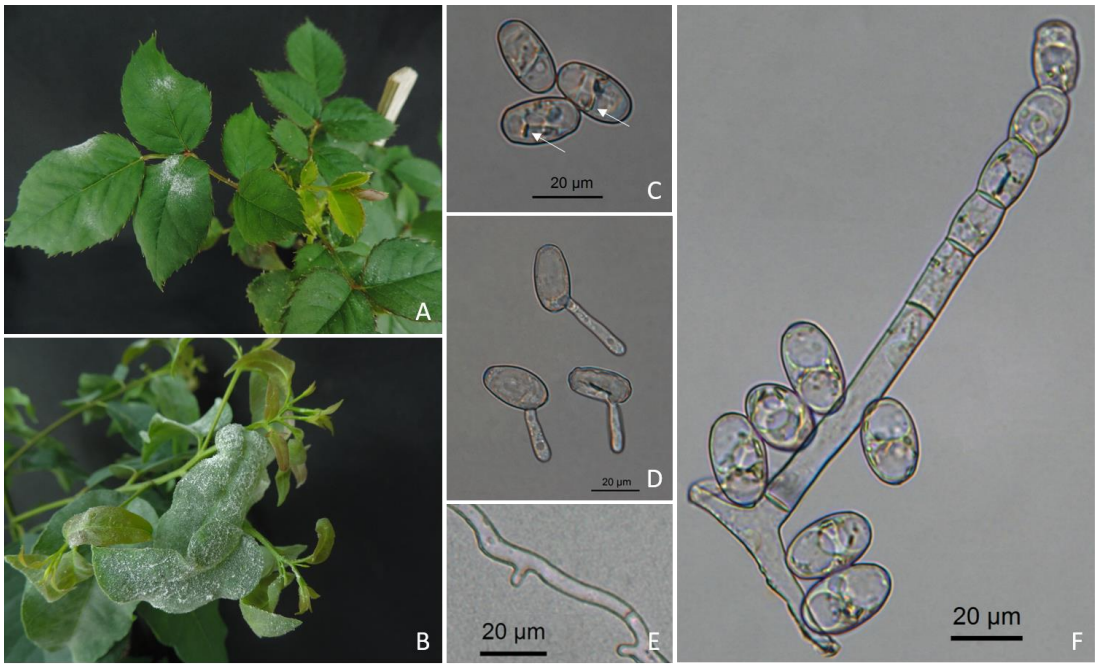

Figure 4. Infected leaves exhibiting powdery mildew signs of *Podosphaera pannosa* on A) rose (*Rosa* sp.), and B) eucalypt (*Eucalyptus urophylla*) after cross inoculation; C) conidia with fibrosin bodies (arrows); D) germinating conidia; E) hyphal appressoria; and F) conidiophore and conidia.

4 DISCUSSION

Based on comparisons of DNA sequences and anamorph morphology, all isolates of eucalypt powdery mildew pathogen collected from different regions of Brazil in this study, were identified as *P. pannosa*. These results confirm the hypothesis raised by Silva et al. (2001), who previously suggested that the eucalypt powdery mildew pathogen in Brazil was similar to the rose powdery mildew pathogen, *P. pannosa*, on the basis of morphological analyses of the anamorph and cross-inoculation studies. The results generated in this study also corroborate with the results found by Cho et al. (2016) who reported *P. pannosa* as the causal agent of powdery mildew on *Corymbia citriodora* in Korea. This is a significant finding because it now confirms *P. pannosa* as the causal agent of a very important disease on eucalypt in Brazil, instead of *O. eucalypti*, a species that is not well characterized. In addition, we determined that all isolates collected in this study belong to a single species with identical ITS sequences among the isolates. Because the identity of

eucalypt powdery mildew pathogen species in Brazil has been confirmed, disease management activities can now focus on the appropriate causal pathogen.

Until the 1990s, taxonomy within Erysiphales was fundamentally based on fungal morphology and biology, with a focus on the sexual structures. In many tropical regions where chasmothecia (= cleistothecia) are rarely formed, studies on disease etiology were often compromised (Bedendo et al., 2011). With the introduction of a new generic concept by Braun et al. (2002), which includes morphological, biological, molecular information of the organism, and the use of phylogenetic analysis of ITS and 28S rDNA sequences, it is now possible to connect a majority of anamorphic species with their teleomorphic species, even when only the anamorph is found (Cunnington et al., 2003). This study further corroborates the utility of these tools to better classify species within Erysiphales.

The ITS sequences provided a more robust phylogenetic tree than the phylogenetic tree generated with 28S rDNA sequences. The addition of more sequence data could perhaps resolve the politomy found with phylogenetic analyses of 28S rDNA sequences; however, more 28S rDNA sequences of diverse *Podosphaera* spp. are needed to obtain more robust and reliable analyses. Although some authors discourage the use of ITS region for phylogenetic analyses in fungi (e.g., Harrington et al., 2014), the ITS and 28S rDNA regions were selected for phylogenetic analyses in this study because they are the most employed and well elucidated genetic regions for Erysiphales (Glawe, 2008; Braun and Cook, 2012), while also providing separation among taxa. In a preliminary study, beta-tubulin and translation elongation factor primers were tested to assess diversity among isolates, but amplifications were unsuccessful (unpublished). Perhaps low-quantity DNA contributes to reduced amplification, or perhaps more precise sequence information for these genetic regions is needed to develop more suitable primers. In addition, the lack of available information about these genetic regions in species of Erysiphales limits reliable classification based on comparisons of these sequences.

Podosphaera pannosa is a cosmopolitan species occurring on several species of different families as *Rosa* spp. and *Prunus* spp. (Rosaceae), *Cotinus coggygria* (Anacardiaceae), *Forsythia* spp. (Oleaceae), *Eucalyptus* spp. (Myrtaceae), and *Corymbia citriodora* (Myrtaceae) (Braun and Cook, 2012; Cho et al., 2016). Studies revealed a close evolutionary relationship between *Podosphaera* spp. and

Rosaceae, suggesting the Rosaceae may have been the first host for *Podosphaera*, and host jumps from the Rosaceae to other plant families may have occurred spontaneously during the evolution of *Podosphaera* (Takamatsu et al., 2010). The cross-inoculation studies support the hypothesis that eucalypt powdery mildew may be the result of a host jump from *Rosa* sp.; however, more focused population genetic studies are needed to confirm this hypothesis.

The identical ITS sequences of eucalypt powdery mildew pathogen isolates obtained in this study could be an indicative of a clonal population structure attributable to the lack of sexual reproduction in tropical regions, perhaps due the absence of cold environmental conditions or maybe due the presence of only one mating-type in the population. This powdery mildew pathogen was reported from Brazil in 1936 (Grillo); thus, it can be surmised that this pathogen has been in Brazil for several decades. Because sexual reproduction is lacking, several decades is perhaps too short of a time period to allow the evolution of significant genetic differences, which could also explain the low sequence diversity found in powdery mildew pathogen isolates from different regions of Brazil.

Improvement of disease management practices, such as resistance breeding/screening programs and cultural practices, depend on a precise understanding of the pathogens that cause disease. The identification of *P. pannosa* as the causal agent of eucalypt powdery mildew allows us to transfer management techniques used for other hosts, such as roses and *Prunus* spp., and test them with eucalypt. Recently, studies using eco-friendly disease control measures have been effective for rose powdery mildew. A silicon treatment reduced powdery mildew development by inducing host defense responses (Shetty et al., 2012), and ultraviolet irradiance exposure suppressed powdery mildew via reduction of spore germination, disease severity, and sporulation of surviving colonies (Suthaparan et al., 2012). In addition, correct pathogen identification can enhance chemical control methods, because different species may respond differently to various fungicides with specific modes of action.

This is the first unequivocal report of *P. pannosa* on *Eucalyptus* spp. in Brazil, based on detailed analyses of morphology, DNA-sequence data, and pathogenicity tests. The identification of *P. pannosa* as a cause of eucalypt powdery

mildew provides a baseline for continued studies to better understand the pathosystem for which information is largely lacking.

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ARTICLE 2 - Transcriptome analysis of *Podosphaera pannosa* infecting *Eucalyptus urophylla*: *de novo* assembly, expression profiling, secretome prediction, and discovery of SSRs

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ABSTRACT

Podosphaera pannosa is the causal agent of powdery mildew on eucalypt in Brazil, which is an important nursery disease causing leaf and shoot distortion, shoot discoloration, and reduction in growth, which causes losses in mini-cutting production. The advent of RNA sequencing (RNA-Seq) technology has allowed increased knowledge about the transcriptome of several pathogens and hosts, enabling a better understanding of their interaction at the gene level. In this study, we analyzed the transcriptome of *P. pannosa* during leaf infection on *Eucalyptus urophylla* plants by RNA-Seq and *de novo* transcriptome assembly. The transcriptome was sequenced by Illumina platform and assembled *de novo*, generating over 178 million RNA-Seq reads assembled onto 200,473 contigs. After filtering steps, the resulting 12,107 (8.2%) transcripts were identified as the *P. pannosa* transcriptome dataset. Among the 10 most abundant transcripts included genes encoding enzymes likely involved in fungal establishment and growth, such as dihydrofolate reductase, putative methyltransferases, acyl-desaturase, glycoside hydrolase, and dehydrogenase. Besides, genes putatively encoding an aquaporin and

an orthologue to the effector protein GoEC2 of *Golovinomyces orontii* were also found. The secretome prediction based on the presence of a peptide signal, absence of transmembrane domain, and absence of mitochondria-targeting motifs resulted in 217 transcripts, of which 14 exhibited homology to proteins described in the PHI database, and were considered as candidate secreted effectors proteins (CSEPs). In addition, 242 non-redundant primer pairs were identified in the transcriptome sequenced with potential to amplify *P. pannosa* SSRs. These results provide a basis for continued studies to better understand the *P. pannosa*-eucalypt pathosystem, and could parallel studies of the eucalypt transcriptome to help determine host resistance mechanisms.

Key words: RNA-Seq, powdery mildew, Erysiphales, eucalypt, effectors.

1 INTRODUCTION

Powdery mildew is a disease caused by a group of biotrophic plant pathogens belonging to Erysiphales. This disease is easily recognizable by the signs, such as, superficial white powdery patches or film produced by anamorphic mycelia, conidiophores, and conidia affecting leaves, stems, flowers, and fruits of almost 10,000 host species (Braun and Cook 2012). On eucalypt (*Eucalyptus* spp.), powdery mildew is an important nursery disease causing leaf and shoot distortion, shoot discoloration, and reduction in growth, which consequently causes losses in mini-cuttings production (Keane et al. 2000; Alfenas et al. 2009). The eucalypt powdery mildew pathogen in Brazil was traditionally identified as *Oidium eucalypti* Rostr. (1902) or generically as *Oidium* sp. (Alfenas et al. 2009) based on its anamorph morphology. Recently, studies based on ITS and 28S rDNA sequences concluded that *Podosphaera pannosa* (Wallr.:Fr.) de Bary (1870) is the cause of powdery mildew on eucalypt in Brazil (N. R. Fonseca, unpublished data). However, studies about the powdery mildew pathosystem are limited, and definitive information is critical to develop technologies for the managing of this disease.

Podosphaera pannosa is a pathogen with a broad host range, including *Rosa* spp. (Rosaceae), *Prunus* spp. (Rosaceae), *Cotinus coggygria* (Anacardiaceae),

Forsythia spp. (Oleaceae), *Eucalyptus* spp. (Myrtaceae) (Braun and Cook 2012), and *Corymbia citriodora* (Myrtaceae) (Cho et al. 2016). On eucalypt, *P. pannosa* has been reported in several countries as Argentina, Brazil, Australia, Denmark, Italy, New Zealand, Poland, Portugal, United Kingdom, South Africa, and recently in Korea (Old et al. 2003; Cho et al. 2016).

Podosphaera pannosa like other obligate/biotrophic fungal pathogens cannot be cultured on artificial media. These pathogens live in intimate cellular contact (i.e., haustorium) with their hosts and depend on their ability to secrete molecules into host cells that manipulate host physiology and defense responses, which allow the pathogen to avoid host recognition (Godfrey et al. 2010). While necrotrophs and hemibiotrophs mainly secrete secondary metabolites and cell-wall degrading enzymes during their necrotrophic phase (Kemen et al. 2015), obligate biotrophs possess large repertoires of effector proteins, as has been demonstrated for the barley powdery mildew pathogen, *Blumeria graminis* (Godfrey et al. 2010; Spanu et al. 2010) and rust fungi (Bruce et al. 2014; Lorrain et al. 2015). Identification of these effector proteins is a fundamental step toward developing management disease strategies.

Whole transcriptome sequencing using next-generation sequencing technologies or RNA Sequencing (RNA-Seq) has increased the quality and utility of transcriptome analysis through the sequencing of entire transcriptome of an organism under a given condition. RNA-Seq is a powerful and relatively cost-effective, high-throughput sequencing method that uses deep sequencing to produce millions of short-sequence reads. These reads can be aligned to a reference genome, when available, or assembled *de novo* without the genomic reference to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene (Wang et al. 2009). For non-model organisms, such as *P. pannosa*, the use of RNA-Seq is a practical approach, because a reference genome is not required. In addition, the large numbers of repetitive elements throughout powdery mildew pathogen genomes present a serious challenge for whole-genome sequencing and assembly (Spanu et al. 2010). Transcriptome sequencing by RNA-Seq provides fundamental information for gene discovery and quantification of gene expression (Kim et al. 2014; Huynh et al. 2015), comparative genomic studies

(Hurtado Páez et al. 2015; Wang et al. 2015), secretome analysis, and prediction of fungal candidate effectors (Bruce et al. 2014; Meinhardt et al. 2014; Liu et al. 2015).

Transcriptome sequencing also provides a valuable tool to identify and develop genetic markers, such as Simple Sequence Repeats (SSRs) and Single Nucleotide Polymorphisms (SNPs), which are useful for examining population structure within a species (Salgado et al. 2014). In previous work, low genetic variability was identified in the ITS rDNA region among isolates of *P. pannosa* infecting eucalypt cuttings in disparate regions of Brazil (N. R. Fonseca, unpublished data). The development of genetic markers for *P. pannosa* is essential to advance population genetic studies of this important pathogen, and develop management strategies. Thus, the aim of our study was to analyze the transcriptional profile of *P. pannosa* infecting *Eucalyptus urophylla.*

2 MATERIAL AND METHODS

2.1 Fungal and plant material

To obtain powdery mildew materials for RNA extraction, ten *E. urophylla* plants of clone 1183 were inoculated. Ninety-day-old cuttings were transplanted to 2 L pots that contained the commercial substrate MecPlant supplemented with 3 kg m^{-3} of superphosphate and 1.5 kg m^{-3} of Osmocote® (19-6-12). The plants were kept in greenhouse conditions and inoculated 30 days after transplanting. Eucalypt powdery mildew pathogen isolate LPF 615, collected in Viçosa, MG, Brazil and maintained on eucalypt cuttings in a growth chamber free of other inoculum sources, was used for inoculation. Inoculations were performed with a small soft brush by dusting conidia from an infected eucalypt leaf onto new leaves of eucalypt plants of clone 1183. Inoculated plants were placed in growth chamber at 19 ± 2 ^{0}C with a 12-h photoperiod and light density of 40 µmol/s/m². After 4 weeks, *P. pannosa*-infected *E. urophylla* leaves were collected and mycelia and conidia were scraped off, and immediately placed into 2.0-mL microcentrifuge tubes containing 1.0 mL of RNA*later*® Stabilization Solution (ThermoFisher Scientific, NY, USA). The tubes were sealed and maintained in -80^{0}C until the RNA extraction.

2.2 RNA extraction

Total RNA was extracted from the mycelia and conidia scraped from *P. pannosa*-infected eucalypt leaves using the ZR Fungal/Bacterial RNA Mini Prep Kit (Zymo Research, Orange, CA, USA) with following modifications, 1 mL sterilized MiliQ water was added to the RNA*later*®-containing tubes, followed by vortexing and centrifugation at 20,800 x g. The supernatant was discarded and the pellet was resuspended in 800 µL of RNA Lysis Buffer; the mixture was split into two 2.0-mL FastPrep tubes (MP Biomedicals, Solon, Ohio, USA) containing 0.18 g of garnet matrix (MP Biomedicals) and two 6.25-mm ceramic spheres (MP Biomedicals). The tubes were processed in a FastPrep FP120 cell disrupter (Thermo Savant, Holbrook, NY, USA) at 5.5 speed for 30 sec; tubes were placed on ice for 5 min and processed again using the same conditions. All centrifugation steps was performed at 15,300 x g (centrifuge model S417R, Eppendorf, Hauppauge, NY). The RNA was eluted in 15 µL DNase/RNase-free water. RNA concentration was measured using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific) and RNA integrity number (RIN) was checked using Agilent RNA Screen Tape System (Agilent Technologies, Germany) in a 2200 TapeStation (Agilent Technologies).

2.3 Illumina sequencing and *de novo* assembly

Illumina next-generation sequencing was performed at Macrogen Korea (Geumcheon-gu, Seoul, Republic of Korea). First, the rRNA in total RNA was depleted by Ribo-Zero kit (Illumina, San Diego, CA, USA). The enriched mRNA samples were subjected to Illumina cDNA library construction using TruSeq stranded mRNA (Microbe) kit (Illumina Inc., San Diego, CA, USA). The RNA was purified, fragmented, and primed for cDNA synthesis. The RNA fragments were transcribed into first strand cDNA using reverse transcriptase and random hexamers, followed by second strand cDNA synthesis. These fragments were prepared for sequencing with an end-repair process and addition of a single 'A' base at the 3' end. Paired-end adapters were ligated to the ends of these 3' adenylated cDNA fragments. Products were then purified and enriched with PCR to create the final cDNA library. The cDNA library was sequenced using Illumina HiSeq™ 2000 (Illumina Inc.) with a read length of 101 bp.

The resulting sequence reads were subjected to a quality control check using FastQC. Sequences were trimmed and the adapters were removed using Trimmomatic V0.32 with settings at ILLUMINACLIP: TruSeq3-PE-2.fa:2:151:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. *De novo* transcriptome assembly of the sequences reads was performed by Trinity (version r20140717), a de Brujin graph-based assembler (Grabherr et al. 2011). Assembled sequences with a minimum length of 101 bp were recorded as a contig.

2.4 Identification of *P. pannosa* transcripts and functional annotation

The assembled contigs were utilized for homology searches against the NCBI non-redundant (nr) protein database (http://www.ncbi.nlm.nih.gov/) with BLASTX algorithm with an E-value $\leq 1e^{-5}$. Because RNA was extracted from mycelia and conidia scraped off from infected eucalypt leaves, it was expected that eucalypt, insect, and/or other microbe transcripts would be present in the samples. To remove transcripts that were not produced by *P. pannosa*, the results were filtered based on BLASTX searches and only those transcripts with matches to previously described proteins from Erysiphales were used to further analysis of the *P. pannosa* transcriptome. Gene annotation on Gene Ontology Functional Classification System was performed using Blast2GO program (Conesa et al. 2005), which retrieved Gene Ontology (GO) terms according to the categories, molecular function, biological process, and cellular component assigned to the powdery mildew pathogen genes based on their homologies to the NCBI nr database.

Transcript abundance was estimated using RSEM 1.2.15 (RNA-Seq by Expectation-Maximization), an accurate software tool for quantifying transcript abundance from RNASeq data (Li and Dewey 2011), the results were based on FPKM (Fragments Per Kilobase of transcripts per Million mapped reads) values that quantify the expression (relative abundance) of each contig.

2.5 Prediction of secretory proteins

Transcripts homologous to powdery mildew pathogen proteins in the NCBI nr database were scanned for putative signal peptides by SignalP v4.1 at default D-cut-off value (Petersen et al. 2011). The resulting peptides then were scanned for transmembrane helices sequences using TMHMM program v2.0 (Krogh et al. 2001)

and for mitochondria-targeted sequences using TargetP v1.1 (Emanuelsson and Nielsen 2000). All transcripts with a secretion signal and absent of a transmembrane domain and mitochondria-targeted sequence were considered as secretome candidates. In addition, the proteome was used as query for BLASTP search against pathogen-host interaction database (PHI-base v4.0) that catalogues experimentally verified pathogenicity, virulence, and effector genes from fungal, oomycete, and bacterial pathogens (Winnenburg et al. 2008) to identify genes involved in pathogenicity. Searches for transcripts described as putative effectors or AVR proteins on BLAST results (BLASTX, E-value $\leq 1e^{-5}$) were also performed. Searches for Candidate Effectors Secreted Proteins (CSEPs) in the secretome of *P. pannosa* were made as reported by Spanu et al. (2010), which defined CSEPs as proteins encoded by bioinformatically annotated genes whose products are predicted to be secreted and that do not have orthologs in non-powdery mildew fungi by BLAST searches (Bindschedler et al. 2016).

2.6 Screening for Simple Sequence Repeat (SSR) loci

Searches for polymorphic tandem repeat (i.e., microsatellites/SSR) motif regions from the contig data set of *P. pannosa* were performed using the Batch Primer3 web software [http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi]. The same webserver was used to design primer pairs. The pattern types screened were perfect tri-, tetra-, penta-, and hexanucleotide motifs. For trinucleotide motifs was set the minimum number of repeats for four. For tetra-, penta-, and hexanucleotide motifs the minimum number of repeats was set for three. Primer design parameters were set as follows: PCR product size range = 100 to 300 bp with 150 bp as optimum; primer size range = 18 to 23 nucleotides, with 21 as optimum; annealing temperature (Tm) optimum = 55 °C; and GC content ranging from 40 to 60%, with 50% as optimum, accordingly with default parameters.

3 RESULTS

3.1 Illumina sequencing and *de novo* assembly

In total, we obtained 185,128,706 reads containing 18,697,999,306 bases from the cDNA library. After adaptor sequences and low-quality reads were removed,

over 178 x 10^6 RNA-Seq 101-bp paired-end reads remained with a Q30 of 93.6% and 49.3% of GC. The *de novo* assembly produced 200,473 contigs, which were assembled from 98,973,062 bases with a median contig length of 493.7 bp and an N50 metric of 586 bp. Considering only the longest isoform per gene, 180,413 contigs were identified with a median contig length of 300 bp and an N50 metric of 497 bp (Table 1).

Table 1. Basic assembly statistics of *de novo* transcriptome assembly.

	All transcript contigs	**Only longest isoform per 'gene'**
Total Trinity transcripts	200,473	180,413
N50 (bp)	586	497
Maximum contig length (bp)	13,361	13,361
Minimum contig length (bp)	201	201
Median contig length (bp)	310	300
Average contig length (bp)	493.7	453.48
Total assembled bases	98,973,062	81,813,563

3.2 Identification of *P. pannosa* transcripts and functional annotation

Among all assembled transcripts, 146,546 (73.1%) were successfully annotated using BLASTX algorithm against NCBI nr protein database with an e-value threshold of $1e^{-5}$. The 53,927 (26.9%) transcripts with no hits to nr database were saved as a separated library for future investigations. After annotation, the transcripts were subjected to a manual filtering, to retrieve only the most reliable pathogen transcripts. Of the 146,546 annotated transcripts, 12,107 (8.2%) transcripts were retrieved with significant similarity to Erysiphales fungal proteins (i.e., *B. graminis*, *Erysiphe necator*, *G. orontii*, *P. macularis*, and *P. fusca*). The resulting transcripts dataset was considered as *P. pannosa* transcriptome for further analysis. Reference transcriptome of *P. pannosa* (12,107 contigs > 200 nt) was submitted to the NCBI Transcriptome Shotgun Assembly (TSA) database under the accession number xxxx.

Gene annotation on Gene Ontology Functional Classification System (GO terms) resulted in 42.8% of total transcripts (5,181 out of 12,107) successfully annotated, and 23.5% (2,851 of 12,107) of these were assigned to at least one GO term. Of the total 12,107 transcripts, 5,101 (42.1%) had similarity with proteins defined only as hypothetical. Within contigs that had homology hits to Erysiphales, a BLASTX top-hit species distribution of gene annotations showed highest homology to *B. graminis* (51.3%), followed by *E. necator* (48.1%). In addition, 42 (0.34%)

transcripts had homology with *G. orontii* proteins sequences, and 16 (0.13%) assembled transcripts aligned with *P. macularis* and *P. fusca* proteins sequences.

Three GO annotation categories are provided by GO database, biological process (BP), molecular function (MF), and cellular component (CC). In this study, the distribution among these three GO domains was sorted based on level 2 classification. Biological process domain comprised the majority of assignments (9,755; 80.5%); metabolic process (3,187; 26.3%), cellular process (2,889; 23.8%), and single-organism process (1,554; 12.8%) were the most abundant categories. GO terms with most assigned sequences under the domains of cellular component (7,255; 60%) were cell (1,783; 14.7%), and cell part (1,770; 14.6%). Binding (2,801; 23.1%) and catalytic activity (2,432; 20%) were prominently represented in the molecular function domain (Figure 1).

To quantify the expression of each transcript, the reads were assembled back to the contigs and adopted FPKM values. Of all 12,107 transcripts, the most abundant expressed gene for the *P. pannosa* transcriptome was homologous to dihydrofolate reductase (DHFR) (c169657_g1_i1), an enzyme common in all organisms. Other expressed enzyme-encoding genes found on the most expressed transcripts of the *P. pannosa* transcriptome were genes encoding transferase, hydrolase, and enzymes belonging to oxidoreductase group, such as desaturase and dehydrogenase (Table 2). A highly expressed transcript (c11058_g1_i1), homologous to effector protein EC2 of *Golovinomyces orontii*, was also identified in this study.

Table 2. Summary of the 10 most abundant expressed genes in the transcriptome of *Podosphaera pannosa* accordingly to FPKM values.

Contig ID	FPKM*	Accession	Annotation	Source
c169657_g1_i1	532,61	CCU82453.1	Dihydrofolate reductase	*B. graminis*
c74378_g1_i1	495,70	KHJ35908.1	Putative fungal protein	*E. necator*
c130120_g1_i1	294,08	KHJ33619.1	Putative methyltransferase	*E. necator*
c92601_g1_i1	288,67	CAD66431.1	Aquaporin 1	*B. graminis*
c149315_g1_i1	288,18	KHJ34064.1	Putative acyl-desaturase	*E. necator*
c149758_g1_i1	276,43	KHJ30843.1	Putative glycoside hydrolase	*E. necator*
c152156_g1_i1	273,43	KHJ31592.1	Hypothetical protein	*E. necator*
c11058_g1_i1	269,02	AEQ16464.1	Effector protein EC2	*G. orontii*
c130884_g1_i1	248,64	KHJ35817.1	Putative dehydrogenase	*E. necator*
c91266_g1_i1	236,90	CCU78156.1	Allergen F4-like/hypothetical protein	*B. graminis*

*FPKM – Fragments per kilobase of exon per million fragments mapped. The information of accession, annotation and source was from BLASTX against NCBI non-redundant protein database.

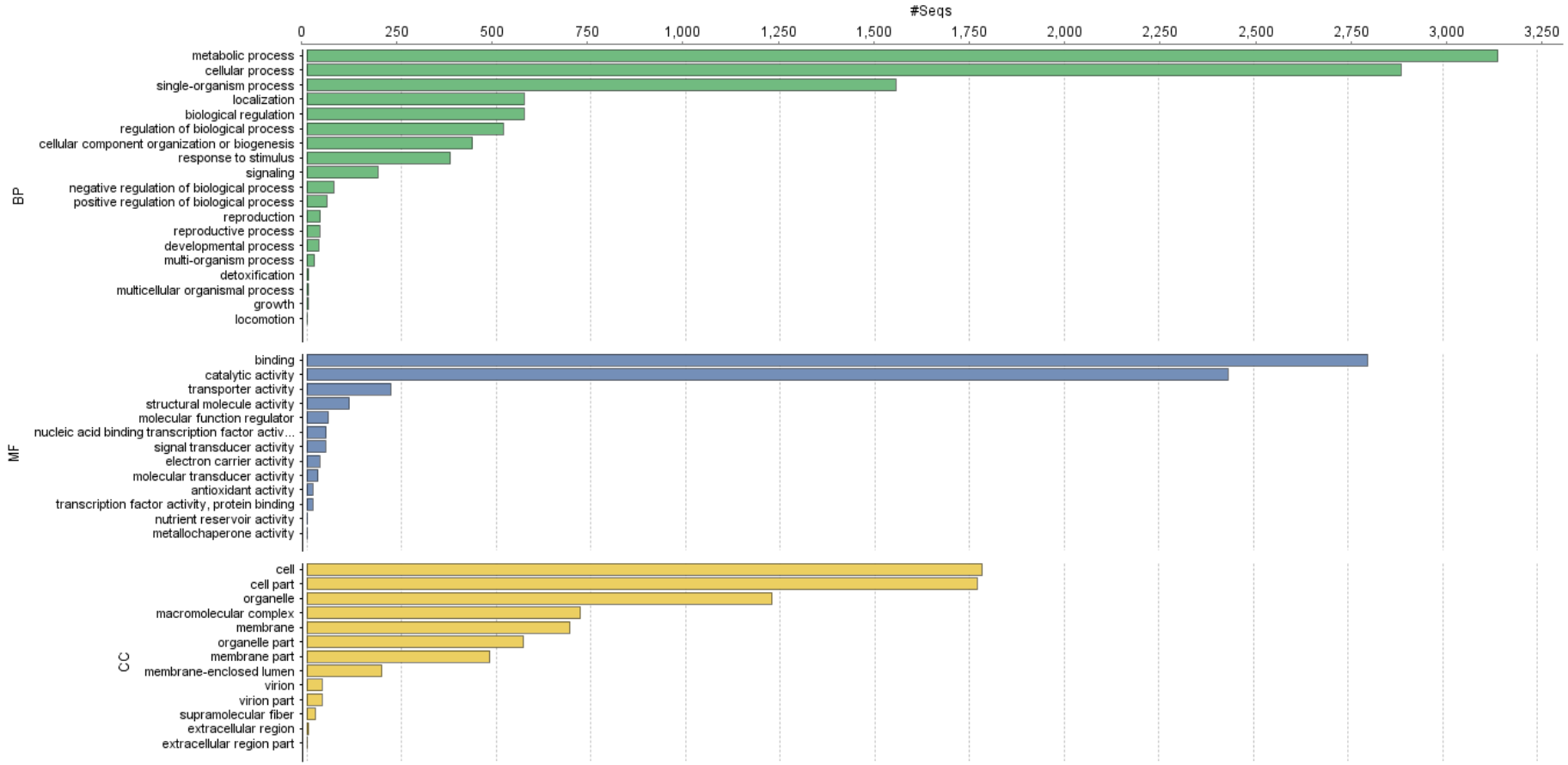

Figure 1. Summary of Top 20 functional annotations of *Podosphaera pannosa* transcriptome by Gene Ontology (GO) terms divided into three main domains, Biological Process (BF), Molecular Function (MF), and Cellular Component (CC).

3.3 Secretome prediction

Secreted proteins play important roles in communication between the fungal pathogen and the host plant, some of which may be effectors that enhance disease development. Analyses of the *P. pannosa* transcriptome were performed to identify potential secretory proteins and candidate secreted effector proteins (CSEPs).

The search for peptide signals followed by scanning for transmembrane domains and mitochondria-targeted sequences resulted in 217 transcripts coding for putative secreted proteins, with no similarities to other proteins in the NCBI nr database except for matches with powdery mildew pathogens proteins. These results, accordingly to CSEP definition used by Spanu et al. (2010), could be considered CSEPs of *P. pannosa*; however, only genes encoding products that had homology to PHI-base proteins were considered as CSEPs in this study. To recover additional secreted proteins, the *P. pannosa* proteome was used as a query for BLAST search (BLASTP, E-value $\leq 1e^{-5}$), against the PHI-base. A total of 736 proteins were identified as homologous to proteins with a potential role in pathogenicity, virulence, or as an effector. A third search was made on the results of BLASTX against NCBI nr database for orthologs to other powdery mildew pathogens proteins described as effectors or AVR proteins found 190 proteins (Figure 2).

Comparisons among the three different methods used to identify putative secreted proteins resulted in very few shared contigs among the three methods (Figure 3). Three contigs were found in both PHI-base and nr database, two of them are contigs from the same gene, c68091_g1_i3 and c68091_g1_i1, described as putative glucose-repressible alcohol dehydrogenase transcriptional effector, and c69581_g4_i1 homologue to a putative effector with unknown function. Three contigs c37233_g1_i1, c116000_g1_i1, and c88397_g1_i1 homologous to putative effectors with unknown functions was observed comparing results from nr database and resulting contigs found by the secretome identification workflow. Among the 217 transcripts, 14 contigs with signal peptide and absence of transmembrane domain and mitochondria-targeted sequences showed homology to PHI database proteins. No contigs were shared among all three methods used.

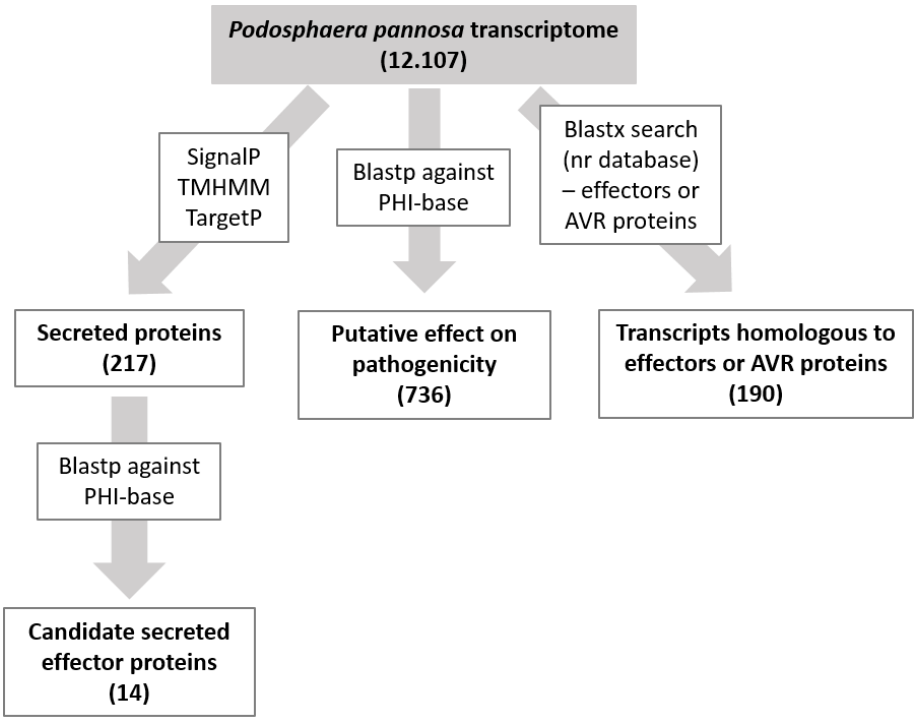

Figure 2. Workflow of secretome prediction for *Podosphaera pannosa* during infection of eucalypt (*Eucalyptus urophylla*) using bioinformatics tools. Number of transcripts after each methodology applied is in parenthesis.

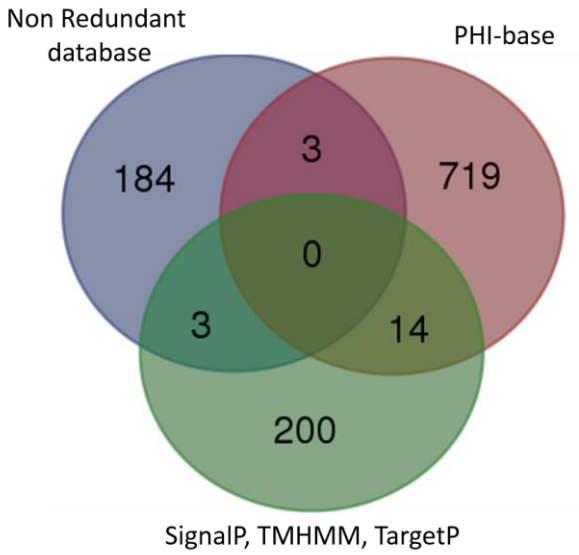

Figure 3. Venn diagram comparing the three different methods used to identify putative secreted proteins of *Podosphaera pannosa*. Number of contigs are indicated for each workflow used for secretome prediction.

The 14 transcripts homologous to PHI-base proteins are described in Table 3. The transcripts c65010_g1_i1 and c65010_g1_i2, isoforms of the same gene, and c62496_g2_i1 were similar to proteins encoded by genes of *Magnaporthe oryzae*, for which transgenic strains without functioning orthologous genes lost their pathogenicity when inoculated in host plants. Reduction of virulence was observed in transgenic *M. oryzae* strains missing genes that had homologies to three transcripts of *P. pannosa* (c68391_g1_i1, c54939_g1_i4, c47594_g1_i1), generated in this study. Five transcripts (c64682_g1_i1, c63200_g1_i1, c103991_g1_i1, c63853_g1_i1, c53011_g1_i1) were similar to gene products of *Fusarium graminearum* and *M. oryzae*, which were determined to have no effect on pathogenicity through gene deletion experiments. One transcript (c111832_g1_i1) was also identified that was homologous to the effector BEC1005, a virulence protein of *B. graminis*.

Table 3. Secreted transcripts with putative functions in pathogenicity, accordingly to PHI database.

Contig	PHI name	Gene name	Species	Mutant phenotype	E-value	Reference
c65010_g1_i1	PHI:1061	MgRho3	*Magnaporthe oryzae*	Loss of pathogenicity	1.39e-100	Zheng et al., 2007
c65010_g1_i2	PHI:1061	MgRho3	*Magnaporthe oryzae*	Loss of pathogenicity	2.10e-103	Zheng et al., 2007
c68391_g1_i1	PHI:1202	Sc Cla4	*Fusarium graminearum*	Reduced virulence	0	Wang et al., 2011
c64682_g1_i1	PHI:1398	GzC2H061	*Fusarium graminearum*	Unaffected pathogenicity	1.29e-99	Son et al., 2011
c63200_g1_i1	PHI:1543	GzMyb007	*Fusarium graminearum*	Unaffected pathogenicity	1.50e-64	Son et al., 2011
c103991_g1_i1	PHI:1655	GzWing028	*Fusarium graminearum*	Unaffected pathogenicity	6.61e-25	Son et al., 2011
c63853_g1_i1	PHI:1658	GzCCCH002	*Fusarium graminearum*	Unaffected pathogenicity	7.22e-68	Son et al., 2011
c53011_g1_i1	PHI:2038	Mir1	*Magnaporthe oryzae*	Unaffected pathogenicity	1.33e-08	Li et al., 2007
c62496_g2_i1	PHI:2075	Moatg7	*Magnaporthe oryzae*	Loss of pathogenicity	0	Kershaw and Talbot, 2009
c54939_g1_i4	PHI:2638	Ilv3A	*Aspergillus fumigatus*	Reduced virulence	0	Oliver et al., 2012
c111832_g1_i1	PHI:2896	BEC1005	*Blumeria graminis*	Effector (reduced virulence)	6.09e-82	Pliego et al., 2013
c47594_g1_i1	PHI:2981	Ctf1	*Fusarium oxysporum*	Reduced virulence	0	Rocha et al., 2008
c64726_g1_i2	PHI:3310	Mocod1	*Magnaporthe oryzae*	Mixed outcome	1.11e-133	Lu et al., 2014
c68308_g1_i5	PHI:3630	Rv2467	*Mycobacterium tuberculosis*	Increased virulence	5.49e-36	McAdam et al., 2002

3.4 Microsatellites marker identification

All 12,107 contigs were used to mine potential SSRs defined as tri- to hexa-nucleotide motifs with a minimum of four repeats for tri-nucleotide motifs and three repeats for tetra- to hexa-nucleotide motifs. A total of 1,571 putative SSRs were identified and, of these, 1,152 (73.3%) had sufficient flanking sequences to permit the primer design. Tri-nucleotide SSR motifs were the most abundant, 711 were detected (45.2%), followed by tetra-nucleotide motifs (570; 36.2%) and hexa-nucleotide motifs (183; 11.5%). Penta-nucleotide motifs were found less frequently, with only 107 (6.8%) (Table 4). The length of SSR motifs ranged from 12 to 48 nucleotides. If the thresholds were raised from four to five repeats for tri-nucleotide motifs and from three to four repeats for tetra-, penta-, and hexa-nucleotide motifs, the number of detectable SSRs decreased to 392. Of these, 289 SSR sequences permitted suitable primer pair design. In total, 309 primer pairs were identified from the transcripts capable to amplify the SSRs. However, if we remove all redundant primers pairs that were detected amplifying the same SSR region of different isoforms of the same contig, will result in 242 primer pairs identified with potential to amplify these SSRs (Supplementary material).

Table 4. Report statistics of microsatellites (simple sequence repeats; SSRs) detection and primer design. Minimum number of repeats described as unit size/minimum number of repeats.

	Tri/4x, Tetra/3x, Penta/3x, Hexa/3x	**Tri/5x, Tetra/4x, Penta/4x, Hexa/4x**
Total of SSRs detected	1,571	392
Number of trinucleotide SSRs detected	711	237
Number of tetranucleotide SSRs detected	570	121
Number of pentanucleotide SSRs detected	107	15
Number of hexanucleotide SSRs detected	183	19
Number of sequences with primers pairs	1,152	289
Total primer pairs picked	1,389	309 (242)

4 DISCUSSION

A transcriptome of *P. pannosa* infecting *E. urophylla* was generated in the present study. Although powdery mildew is an important disease on the economically important eucalypt crop, little is known about the pathogen, which was only recently identified as *P. pannosa* (N. R. Fonseca, unpublished data). The transcriptome sequencing of *P. pannosa* produced more than 178 million reads, *de novo* assembled into ~200,000 transcripts. *De novo* transcriptome assembly method was used because a whole genome sequence is not currently available for *P. pannosa*, and that method has yielded good results for several non-model organisms, such as fungi (Ross-Davis et al. 2013, Thakur et al. 2013; Yazawa et al. 2013; Liu et al. 2015) and plants (Li et al. 2012; Castro et al. 2015).

As revealed by BLASTX search against NCBI nr database, the majority of annotated transcripts derived from scraped *P. pannosa*-infected leaves of *E. urophylla* originated from non-*P. pannosa* organisms. After manual filtering, the resulting 12,107 (8.2%) transcripts were identified as the *P. pannosa* transcriptome dataset. Yazawa et al. (2013) applied a similar strategy to recover transcripts of *Bipolaris sorghicola* from infected sorghum leaves, which was also based on a *de novo* transcriptome assembly. The mixed transcriptome of *B. sorghicola* in infected sorghum leaves resulted in 160 transcripts of the pathogen. If we compare previous transcriptome-sequencing strategies of other powdery mildew pathogens, Tollenaere et al. (2012) using a 454 GS-FLX sequencing platform and *de novo* assembly acquired 29,505 transcripts from conidia scraped off of *P. plantaginis*-infected *Plantago lanceolata* leaves. However, that study had a low depth of sequencing (1.76 reads per nucleotide) compared to the high, deep sequencing used in this study that provided an average 148-fold coverage. Previous transcriptome sequencing of *G. orontii* haustoria using deep 454 pyrosequencing resulted in 7,077 contigs assembled by mapping back to *G. orontii* draft genome with 3,725 annotated contigs (Weßling et al. 2012). In this study, the sequencing depth and the number of transcripts of *P. pannosa* obtained demonstrates that the merit of the methodology chosen to identify pathogen transcripts resulted in good representation of total transcriptome of *P. pannosa*. With this method, however, putative *P. pannosa* transcripts that had no homology to previously identified genes from Erysiphales fungi were filtered out of

the transcriptome data. Thus, it is likely that some highly conserved genes and other uncharacterized genes are underrepresented in our *P. pannosa* transcriptome.

The 10 most abundant transcripts included genes encoding enzymes likely involved in fungal establishment and growth, such as dihydrofolate reductase (DHFR), putative methyltransferases, acyl-desaturase, glycoside hydrolase, and dehydrogenase. Additional transcribed genes putatively encoded an aquaporin and an effector protein (see: 3.3 Secretome prediction).

DHFR had the highest FPKM value, representing the *P. pannosa* gene that was most transcribed during eucalypt infection. DHFR is important for cell proliferation and cell growth and it also plays a critical role in regulating the amount of tetrahydrofolate in the cell, which is essential for purine and thymidylate synthesis (Schnell et al. 2004). The transcripts of a putative methyltransferase gene were also found in high number. In *M. oryzae,* this enzyme has been reported as important for DNA methylation contributing to fungal development and genome defense (Jeon et al. 2015), and in the entomopathogenic fungus *Beauveria bassiana* as contributing to spore viability, fungal development, protein secretion, and virulence (Qin et al. 2014).

Glycoside hydrolase (or glycosyl hydrolase) family proteins are known to be involved in host cell wall degradation. Despite *P. pannosa* being an obligate biotrophic pathogen, which requires living host cells to develop, a plant-cell-wall-degrading enzyme was identified among the most abundant transcripts. Although high levels of cell-wall-degrading enzyme gene transcription might be expected from necrotrophic or facultative plant pathogens, this enzyme could augment appressorium formation and cellular penetration. Glycoside hydrolase family proteins were previously reported as occurring during initial infection stages of *M. oryzae* on rice, where it was found involved in host cell wall degradation and appressorium formation (Kawahara et al. 2012). In addition, the same authors observed the upregulation of glycoside hydrolase-encoding transcripts in both host and pathogen, suggesting that these cell-wall-degrading enzymes play important roles in both host defense and pathogen attack. Similarly, glycoside hydrolases were also characterized on the biotrophic rust fungus, *Uromyces fabae* (Murphy et al. 2011).

Aquaporins are special pore proteins that help channel water, and enhance the permeability of cell membranes. Genes encoding aquaporins are found in

mammals, plants, and some microorganisms including the powdery mildew pathogen, *B. graminis* (Tanghe et al. 2006). Although the function of these aquaporins is not well established in fungi, a high level of aquaporin transcription was observed during mycelial growth of the ectomycorrhizal fungus *Laccaria bicolor*. Such findings suggest a demand for elevated water permeability in the plasma membrane when hyphae are growing (Nehls and Dietz 2014). As expected, genes encoding products that contribute to establishment, development, and growth of *P. pannosa* were highly expressed during eucalypt infection.

Secreted proteins play an important role in communication between the fungal pathogen and the host plant, some of which may be effectors, which are defined as proteins and other compounds that enhance disease development by targeting host processes, but are redundant to basal growth processes in the pathogen (Godfrey et al. 2010). Our methodology to search for secreted proteins in the *P. pannosa* transcriptome were based on previous studies to predict the secretome of other plant pathogenic fungi. *Blumeria graminis*, *Venturia inaequalis*, *Sclerotinia sclerotiorum*, *Melampsora lini* and *Cronartium ribicola* are some examples for which secretomes have been predicted based on the presence of a peptide signal, absence of transmembrane domain, and absence of mitochondria-targeting motifs (Pedersen et al. 2012; Thakur et al. 2013; Guyon et al. 2014; Nemri et al. 2014; Liu et al. 2015).

In this study, the search for candidate effectors secreted proteins (CSEPs) was based on the methodology of Spanu et al. (2010). Among the 14 transcripts with homology to PHI-base proteins (Table 3), the transcript c111832_g1_i1 showed homology to the previously described effector BEC1005 of *B. graminis*, which had high sequence similarity to fungal glucosyltransferases. Previous gene-silencing studies with BEC1005 demonstrated its role in virulence and suggested that the protein could play a role in cell wall remodeling during haustorial formation (Pliego et al. 2013).

The transcript c11058_g1_i1, an orthologue of the effector protein GoEC2 of *G. orontii*, was one of the most abundant transcripts during *P. pannosa* infection of eucalypt (Table 2); however, this transcript did not appear in the search results against PHI-base. GoEC2 functionality during *G. orontii* infection of *Arabidopsis thaliana* was explored by Schmidt et al. (2014), who reported the potential of GoEC2 to

enhance host susceptibility by promoting fungal entry. In *B. graminis*, high transcript levels of the orthologue gene, BEC2, were observed at the time of appressorium formation, after which transcript levels decreased (Schmidt et al. 2014). In addition to being found among *P. pannosa* transcripts in this study, and the orthologous genes in *G. orontii* and *B. graminis*, orthologous genes have also been described in the genome of *Erysiphe pisi*, causal agent of pea powdery mildew (Spanu et al. 2010), indicating gene conservation among powdery mildew pathogen species.

Nineteen transcripts were homologous to genes encoding putative AVR_{A10}-like proteins by BLASTX search against nr database, 11 to *B. graminis*, and eight to *E. necator* proteins. AVR_{A10} are avirulence (AVR) genes that encode proteins with potential dual roles as an AVR protein and as an effector, and are also known to increase the pathogenicity of *B. graminis* f. sp. *hordei* in barley plants (Amselem et al. 2015). Resistant barley encodes Mla10 resistance proteins that recognize the presence of AVR_{A10} eliciting the hypersensitive response (localized cell death) typical of gene-for-gene interactions (Ridout et al. 2006). These transcripts were not selected as putative effectors by the workflow used in this study because unlike other known effectors, the AVR_{A10} genes encode proteins that do not contain a secretory signal peptide (Ridout et al. 2006). AVR_{A10} genes belong to a large gene family along with their homologous AVRk1 genes and the EKA (Effectors homologous to AVRk1 and AVR_{A10}) family, with more than 1,350 homologues in *B. graminis* genome (Spanu et al. 2010). It has recently been hypothesized that this enormous number of homologues in the genome could act as an reservoirs from which new effector genes may quickly evolve to overcome the host resistance (Amselem et al. 2015).

Studies of population biology rely on the use of molecular markers that can detect polymorphisms among the populations being studied. SSR markers have the advantage over other DNA fingerprinting because they are codominant, highly polymorphic, and species specific. The search for SSRs within the 12,107 transcripts of *P. pannosa* yielded 392 SSR motifs, of which 289 sequences allowed for putative primer design. Searches for repeat motifs within nucleotide sequences from EST libraries, whole genomes, and transcriptomes have been successfully used for several fungi and plants (Parchman et al. 2010; Frenkel et al. 2012; Salgado et al. 2014; Tucker et al. 2015). Although SSR markers derived from expressed sequences or transcriptome data can be considered less informative due DNA sequence

conservation compared to genomic sequences (Bouck and Vision, 2007), such markers are cost and time effective, while providing useful polymorphic markers from protein-coding sequences (Frenkel et al. 2012; Tucker et al. 2015). The SSR sequences identified here provide substantial resources to design of SSRs markers for examining the population structure of *P. pannosa*, which can be used not only for the pathosystem *P. pannosa*-eucalypt but also can be expanded to other important hosts, as roses and *Prunus* spp.

5 CONCLUSIONS

This study describes the generation of transcriptome sequences of *P. pannosa* using next-generation sequencing technology and *de novo* assembly for protein identification, secretome prediction, and SSR motifs discovery to design molecular markers that allow further study of *P. pannosa*. To our knowledge, this is the first study of *P. pannosa* transcriptome during infection of *E. urophylla* using RNA-Seq. The results generated in this work increases the knowledge about powdery mildew pathogens, providing useful information for new advances. Furthermore, this study provides a basis for better understanding the *P. pannosa*-eucalypt pathosystem, which could parallel studies of the eucalypt transcriptome to help determine host resistance mechanisms.

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SUPPLEMENTARY MATERIAL

Table S1. Characterization of SSR makers.

	Contig ID	Orientation	Tm	GC%	Primer Sequence	Orientation	Tm	GC%	Primer Sequence	Prod size	SSR
1	c2443_g1_i2	FORWARD	55.36	33.33	TCTCAAAAACATGTCGATTTC	REVERSE	54.62	38.10	CGAATGGGTACTACTTTTTCA	157	AGAAGAAGAAGAAGA
2	c7608_g1_i1	FORWARD	55.10	42.11	CGATGAATTGAAACAGACG	REVERSE	55.34	42.86	AAAAGTCCAGAAGAGATCGAG	153	CTGGCTGGCTGGCTGG
3	c8385_g1_i1	FORWARD	54.66	38.10	ATACTCTAACCCAGCCAAAAT	REVERSE	54.73	42.86	TGGATGGTCTAAGGTATCAGA	155	GAAGAAGAAGAAGAA
4	c11240_g1_i1	FORWARD	54.82	42.86	GGGTCATTCTCCAGTCTTATT	REVERSE	55.36	42.86	TCCAGGAACGTATAAACAGTG	147	ATCATCATCATCATC
5	c14056_g1_i1	FORWARD	55.21	33.33	AAGGTCAAAGAAAAATGAAGG	REVERSE	54.89	38.10	TGATAAAAGAGTGTTCCGTGT	147	GAAGAAGAAGAAGAA
6	c14056_g1_i1	FORWARD	55.21	33.33	AAGGTCAAAGAAAAATGAAGG	REVERSE	54.89	38.10	TGATAAAAGAGTGTTCCGTGT	147	GAGCGAGCGAGCGAGC
7	c21029_g1_i1	FORWARD	54.55	38.10	TGAGTGTATTTGGATGGAGAT	REVERSE	55.95	47.37	GCCCACAACAGACGATAAT	151	GTGTGTGTGTGTGTGT
8	c21881_g1_i2	FORWARD	55.49	42.86	AAAGAGGATGTGCCTATGACT	REVERSE	54.95	42.86	GCCTAGGGTATGATCTTCATT	168	AGCAGCAGCAGCAGC
9	c21993_g1_i1	FORWARD	54.64	38.10	CCTTGCAACGTATGACTTAAT	REVERSE	55.05	33.33	TGGATCTCGTAGATTTTTCAA	174	AGCTAGCTAGCTAGCT
10	c24623_g1_i1	FORWARD	54.90	42.86	AGAATACCCAAGGTTTCAGAC	REVERSE	55.18	38.10	ACAGGACACAATAAGGGAAAT	151	GAGGAGGAGGAGGAGGAG
11	c27929_g1_i1	FORWARD	55.05	38.10	AACTAGCCACAATTTGTCAGA	REVERSE	54.98	42.86	GGTTCATATCTCGAGGAAAGT	147	GGCGGCGGCGGCGGC
12	c27929_g1_i1	FORWARD	54.63	38.10	AATCCCCTACGGATAAAACTA	REVERSE	54.66	42.86	GCATGTGAGTGTTTGTAGTCA	160	CACCACCACCACCACCACCAC
13	c28282_g1_i1	FORWARD	55.27	38.10	GCGAAATTCTCCGTATATTCT	REVERSE	55.06	50.00	TACGTGGGCTTAGTGAGTCT	163	CCGCCGCCGCCGCCGCCGCCG
14	c28290_g1_i1	FORWARD	55.23	47.62	CATCTAGCTTACGTACCATCG	REVERSE	54.57	33.33	TGAATGAACAGCATATAGCAA	146	CCTCCTCCTCCTCCT
15	c33156_g1_i1	FORWARD	54.90	33.33	ACGCAAACAACGTAAAAATAG	REVERSE	54.99	42.86	CCTTTAACGCCTTACTAATCC	147	AAGAAGAAGAAGAAG
16	c35294_g1_i1	FORWARD	55.73	52.63	GGAAACAACGCTGACCTAC	REVERSE	54.89	47.62	CTGTGATGCGTGTGAGTAGTA	130	CCGCCGCCGCCGCCG
17	c35294_g1_i2	FORWARD	54.92	52.38	GAGGCCTCTCAACTTACTCTC	REVERSE	55.62	52.38	GCGTGTGAGTAGTAGTCCGTA	182	CCGCCGCCGCCGCCG
18	c38296_g1_i1	FORWARD	55.48	38.10	CGTCCAACTCAAAATGAAGTA	REVERSE	54.82	38.10	AAGTAAGGGAAAGGAAGACAA	150	CATTCATTCATTCATT
19	c38516_g1_i1	FORWARD	54.65	42.86	TATGAGGCTATCGACTGTTTC	REVERSE	55.02	38.10	TCTGTACGCTTCCATATTTGT	157	TCATCATCATCATCATCA
20	c38909_g1_i1	FORWARD	54.93	42.86	AATGGTGGTGTACAGCTTCTA	REVERSE	55.39	50.00	GGATCGGGATTATTGCTC	180	CACCACCACCACCAC

21	c38909_g1_i2	FORWARD	54.93	42.86	AATGGTGGTGTACAGCTTCTA	REVERSE	55.39	50.00	GGATCGGGATTATTGCTC	180	CACCACCACCACCAC
22	c39420_g1_i1	FORWARD	55.79	33.33	GACCTTTCGATTGATCATTTT	REVERSE	55.13	42.86	GAGAAGTGTCCAAATGGTGTA	154	TCATCATCATCATCA
23	c40364_g1_i1	FORWARD	55.04	47.62	ACGTGGAATCCTCTCTTCTAC	REVERSE	55.43	38.10	AAACAGGAGTTTTCTTTCGAG	147	CTCCCTCCCTCCCTCC
24	c43241_g1_i2	FORWARD	54.27	33.33	ATCGATAAATGGATATCAACG	REVERSE	55.24	45.00	CAGCTGGAAGACCATTACAT	144	GCTGGCTGGCTGGCTGGCTG
25	c43245_g1_i1	FORWARD	54.90	42.86	TCTGGAACTTTAGGTACACCA	REVERSE	55.68	38.10	TGCAGAGTTGTGATGGATTAT	165	CAGCAGCAGCAGCAG
26	c43440_g1_i1	FORWARD	54.70	42.86	GGCATCATTTATCTCACTCAC	REVERSE	54.97	42.86	GCTCATCACTATCAAACAAGG	160	ACTCACTCACTCACTCACTC
27	c44315_g1_i1	FORWARD	54.36	42.86	ATCAGATAGGATGAGCATGAG	REVERSE	56.46	38.10	TAATTTCCATCTCGTCGACTT	148	CTCACTCACTCACTCACTCA
28	c45784_g1_i1	FORWARD	53.69	33.33	GCAAGGTAATATTGGGAATAA	REVERSE	54.61	38.10	TTAAGGCACTTCCATTAACAC	152	TAATAATAATAATAA
29	c46548_g1_i1	FORWARD	54.70	38.10	TCGATTTTCTCGTGAAGTTAC	REVERSE	54.46	42.86	CAAGACCATTCTTAGTGTCGT	155	CAAGCAAGCAAGCAAG
30	c49921_g1_i2	FORWARD	55.53	40.00	ATCGACCTCGAATTTTCTCT	REVERSE	60.32	55.56	AGCGACGGATCAGGATGA	139	CCCACCCCACCCCACCCCAC
31	c51169_g1_i1	FORWARD	55.13	50.00	ACCACTCATAGACCAGTTGC	REVERSE	55.68	47.62	TGGTGACGTAGAATAGAGCAG	173	CTGGTCTGGTCTGGTCTGGTCTGGT
32	c51638_g1_i1	FORWARD	54.54	38.10	TTTAGTGACGACTTTTGCTCT	REVERSE	55.13	38.10	TGATAGCGTTGTCTCAGATTT	151	ACCACCACCACCACCACC
33	c51800_g1_i1	FORWARD	54.48	33.33	TCGCTTTGTTCATAGAAAACT	REVERSE	54.93	40.00	AAGATGGATGACGACAAATC	158	TTCGTTCGTTCGTTCG
34	c51800_g1_i2	FORWARD	55.28	42.86	CGGTACTTCCTTCATCATACA	REVERSE	54.93	40.00	AAGATGGATGACGACAAATC	174	TTCGTTCGTTCGTTCG
35	c52172_g1_i1	FORWARD	55.21	33.33	TCATTGGAAGAAATAGCTGAA	REVERSE	54.85	47.62	GGTATGTGGATGTGTACGAGT	141	GAAGAAGAAGAAGAA
36	c52216_g1_i1	FORWARD	55.06	42.86	TCTAGCTGGATGACCTATCAA	REVERSE	54.86	47.62	GGATACGTATGGTCTTCTCCT	151	ACCACCACCACCACC
37	c52520_g1_i1	FORWARD	54.72	42.86	GGTGGCTTACATAAGATGCTA	REVERSE	55.37	42.86	ATTTATACAGGCTCCCACAGT	158	CTCTCTCTCTCTCTCT
38	c53004_g1_i1	FORWARD	54.92	47.37	TCTCTGCTGATGAAGTTGC	REVERSE	55.63	42.86	GTCATCTGGATCTTCCATCTT	152	CGACGACGACGACGACGA
39	c53119_g1_i1	FORWARD	55.03	42.86	GGAAGTAATCTTGGGCTACAT	REVERSE	54.93	33.33	CCGAAAAATCCCAGATATAAT	178	ATGATGATGATGATG
40	c53165_g1_i1	FORWARD	54.76	38.10	CAATTAAGTGGGATTCAGATG	REVERSE	54.77	45.00	GGCTACAACTTCAGTGCTTT	155	GAAGAAGAAGAAGAA
41	c53334_g1_i1	FORWARD	55.22	47.62	AAGACGATGAGGAGTTAGAGG	REVERSE	54.87	47.62	CCTTCTCCTTCTTCGACTATC	127	ATGATGATGATGATG
42	c53470_g1_i1	FORWARD	54.40	38.10	CTGCAATATAACCACCAGAAT	REVERSE	55.32	42.86	TAGACGTACCGGTAAATGATG	123	CGCATCGCATCGCATCGCAT
43	c53620_g1_i1	FORWARD	55.05	33.33	TCCTGTTAAACCTCAAACAAA	REVERSE	54.85	42.86	TTCTGGATTACTGAAGAGCTG	166	ACCACCACCACCACC

44	c53894_g1_i2	FORWARD	54.70	38.10	GGATTTGCAAGACTTTGTAGA	REVERSE	55.09	42.86	AGAAGTCCCTTTGAACAGAAC	130	TATTATTATTATTAT
45	c54244_g1_i2	FORWARD	54.65	38.10	CTGAGAATAGCATCGTTGAAT	REVERSE	54.97	38.10	AGCAGTGAATGGTCTGAAATA	148	AGAAGAAGAAGAAGA
46	c54804_g1_i1	FORWARD	54.88	36.36	TGTGCACCATCTAGATTTATGT	REVERSE	54.98	42.86	TCTCACTCGTCTGTCTTTCAT	145	GACGACGACGACGAC GACGAC
47	c55594_g1_i1	FORWARD	54.84	33.33	ATGATTTGCTGCTTATCTTTG	REVERSE	55.18	38.10	ACAAATCAACACCCTTCCTAT	157	TGGCTGGCTGGCTGG CTGGC
48	c55666_g1_i1	FORWARD	54.86	47.62	AATCAGTAGAGGGACGGATAC	REVERSE	55.01	42.86	TCAACTTAGAACCTGGTCAGA	156	ATGATGATGATGATG
49	c56053_g1_i1	FORWARD	55.17	33.33	CAATAACCATCATGGAAAGAA	REVERSE	55.18	38.10	ATGTTGTTGTTGGTCGAATAG	145	ATCATCATCATCATCA TCATC
50	c57287_g1_i2	FORWARD	56.16	42.86	AAAAGAGGTCTTGAAGGAGGT	REVERSE	55.16	38.10	CTCCGGATAAGGGTAAAATAA	139	GACGACGACGACGAC
51	c57386_g1_i1	FORWARD	54.90	42.86	ACCAGAGGTGCTTTTAAGACT	REVERSE	55.45	47.62	CGGTGTACAAGCACTGAGTAT	140	TGACTGACTGACTGA C
52	c57676_g1_i1	FORWARD	55.14	50.00	AAGATACAGACCACCGACTG	REVERSE	54.80	42.86	CCTTCGATTTCTAGCTCTTCT	150	GATGATGATGATGAT
53	c58399_g1_i1	FORWARD	54.58	38.10	ATAAATCAACACCTTGCTGAG	REVERSE	54.90	47.62	TCGTCTTGTAGAGTGAGCTTC	162	ATGATGATGATGATG ATGATG
54	c58399_g1_i2	FORWARD	54.87	31.82	TGAATAATGATAACGAGATGGA	REVERSE	54.90	47.62	TCGTCTTGTAGAGTGAGCTTC	164	ATGATGATGATGATG ATGATG
55	c58666_g1_i1	FORWARD	54.68	38.10	CGGCATATCTCCTATTGTTTA	REVERSE	56.01	33.33	GAAAAACAATTTCAGGCAGAT	153	TGGTTGGTTGGTTGGT
56	c59091_g1_i2	FORWARD	55.45	52.38	CTGACGACTGACTGACTGACT	REVERSE	55.05	42.86	GCTCCAATGACTGTAACTTTG	132	GACCGACCGACCGAC C
57	c60422_g1_i1	FORWARD	55.12	38.10	CAGAAAGAAAACGCAGTAGAA	REVERSE	55.21	45.00	CGACGTGTTGTATACCCTTT	150	GCTGCTGCTGCTGCTG CT
58	c60911_g1_i1	FORWARD	55.39	45.00	GACAAGAAAAGGGATTAGGG	REVERSE	54.55	38.10	TAATCTCTGACATGGTGGATT	158	GCTGCTGCTGCTGCTG CT
59	c61016_g1_i1	FORWARD	57.71	55.56	CAACAGCAACAGCAGCAG	REVERSE	55.83	47.62	CAGGAAGATGTTGTGACTGAG	151	CAACAACAACAACAA
60	c61430_g1_i2	FORWARD	54.96	38.10	CGACTTACGAAGTTCTTTTCA	REVERSE	54.98	33.33	TTTTGAAGATTGAGCTTATCG	154	TGATGATGATGATGA TGATGATGA
61	c61501_g1_i1	FORWARD	54.93	38.10	CTTCCAATGGGAGATTCTTAT	REVERSE	54.32	33.33	AGACTTTGCCTTGTAATTTGA	140	AAGAAGAAGAAGAAG
62	c61550_g1_i2	FORWARD	55.42	42.86	CATCAATACTGTCGATGCTCT	REVERSE	55.12	42.86	CAGCGGATGATTCAATAGTAG	167	GACGACGACGACGAC
63	c61880_g1_i1	FORWARD	54.84	38.10	TTGATATTCCCTAGATGACGA	REVERSE	55.03	38.10	TCCATGTTTCTATCTCTCGAA	171	GATGATGATGATGAT GATGATGAT
64	c62366_g1_i1	FORWARD	55.21	38.10	AAAATATTCCGAGACCTAACG	REVERSE	54.73	42.86	TATCCCATGAGTCTGGTTCTA	168	CATCATCATCATCAT
65	c62406_g1_i1	FORWARD	54.95	38.10	TCCTTGAATACACTCCTTTCA	REVERSE	54.61	38.10	GTGACAACATAAAGCCCTAAA	138	TGATTGATTGATTGAT TGAT
66	c62496_g2_i1	FORWARD	54.41	38.10	CGGACAAGAAATACGTTTTAG	REVERSE	55.04	42.86	AACTTGTCTGATGCTACCTGA	159	TGATGATGATGATGA

67	c62766_g1_i1	FORWARD	55.63	42.86	GCCGTTAGCTCTGTTAACTTT	REVERSE	55.06	38.10	GTGCGTAATCAAACAAACTTC	181	CTTTCTTTCTTTCTTTC TTT
68	c63148_g1_i2	FORWARD	54.85	47.62	CCTCGTACTACCATTGATCTG	REVERSE	54.76	38.10	GAGGTTTCTGGGAATTTTAAG	172	ATCATCATCATCATC
69	c63167_g1_i1	FORWARD	54.54	38.10	TGGAAGTCGAAGTTATTATGG	REVERSE	54.92	38.10	TCCTCTATTATTTCCACACGA	139	ATGATGATGATGATG
70	c63167_g1_i2	FORWARD	54.54	38.10	TGGAAGTCGAAGTTATTATGG	REVERSE	54.92	38.10	TCCTCTATTATTTCCACACGA	139	ATGATGATGATGATG
71	c63824_g1_i2	FORWARD	54.82	33.33	TTCATTGAATGGCTTAAAGAG	REVERSE	55.18	38.10	ATACTCGTAATCGTTCTGCAA	145	GATGATGATGATGAT GAT
72	c63824_g1_i3	FORWARD	56.24	45.00	CCCGATATTTTCCTAACGAC	REVERSE	54.96	52.63	CTCCTCCTCGATCTTTCTG	178	GCAGCAGCAGCAGCA
73	c63824_g1_i4	FORWARD	56.24	45.00	CCCGATATTTTCCTAACGAC	REVERSE	54.96	52.63	CTCCTCCTCGATCTTTCTG	178	GCAGCAGCAGCAGCA
74	c63824_g1_i4	FORWARD	54.82	33.33	TTCATTGAATGGCTTAAAGAG	REVERSE	55.18	38.10	ATACTCGTAATCGTTCTGCAA	145	GATGATGATGATGAT GAT
75	c63836_g2_i1	FORWARD	55.00	38.10	TATTCGTGCTCTTGCTATTTC	REVERSE	56.46	33.33	ATACATTGGCAAATCTTGCTT	152	ATCATCATCATCATC
76	c64048_g1_i1	FORWARD	55.73	35.00	CAACAGCAACAACAAAATCA	REVERSE	55.19	42.86	GGAAAGCTGATCCGTAGTTAT	159	ACTACTACTACTACT
77	c64206_g1_i1	FORWARD	55.59	47.62	CAGCTGAACCTGTACCTTGTA	REVERSE	55.64	47.62	CCTGCAGTCTCTTCATCACTA	145	GATGATGATGATGAT GAT
78	c11043_g1_i1	FORWARD	55.17	47.62	GACAGATACGACAACCTACCA	REVERSE	55.30	33.33	GTTGCTGAAATTGTTGTTGAT	156	CAGCAGCAGCAGCAG
79	c19977_g1_i1	FORWARD	54.93	38.10	TGTTTTAGAGCTACGGAAATG	REVERSE	54.96	47.62	CTAGAGGGAGAAGGTGAAAAG	151	CGGTCGGTCGGTCGG T
80	c22949_g1_i1	FORWARD	55.25	55.00	AGTACCACACCAGCTACGTC	REVERSE	55.26	42.86	GCCATGAGTTATGTAGGACAA	152	ACCACCACCACCACC
81	c31618_g1_i1	FORWARD	55.17	42.86	ATGTCGTTATCCAACACTGAG	REVERSE	55.16	45.00	CTCACGACTTGGTTATGGTT	155	CGCACGCACGCACGC A
82	c34163_g1_i1	FORWARD	55.39	42.86	CATCATGACCTTCATCATCTC	REVERSE	55.28	33.33	CAAAAACAAAGGAGGAAACTT	149	ATTTTATTTTATTTTAT TTT
83	c36513_g1_i1	FORWARD	55.71	38.10	AATTCAATTCAGGGAACTCAC	REVERSE	54.97	38.10	TGAGTGTGCAATCTTCCTTAT	150	TATATATATATATATA
84	c36980_g1_i1	FORWARD	54.93	42.86	TCCCCTTACTCTAATTCCATC	REVERSE	57.46	55.56	GTGTGACATGTGCCGAGA	157	ACTGACTGACTGACT GACTGACTG
85	c38131_g1_i1	FORWARD	55.09	47.62	CTGCTGGTCAACTCGTTATAC	REVERSE	54.97	42.86	TTTACTGCTATCGTTCTACGG	139	AGAAGAAGAAGAAGA
86	c39562_g1_i2	FORWARD	55.42	38.10	GCTTGCATTGACAAGTATGAT	REVERSE	55.62	38.10	AAGCACCTCATCTGAAAGAAT	144	CGACGACGACGACGA
87	c39562_g1_i2	FORWARD	55.42	38.10	GCTTGCATTGACAAGTATGAT	REVERSE	55.62	38.10	AAGCACCTCATCTGAAAGAAT	144	GATGATGATGATGAT
88	c39615_g1_i1	FORWARD	54.97	47.62	CAGGTCTTACTTGCTCGACTA	REVERSE	55.19	45.00	TCTCCTCGTTTGTAGTTGCT	159	GCTCGCTCGCTCGCTC
89	c40172_g1_i2	FORWARD	54.34	38.10	CTTCACATGATACCAATGACA	REVERSE	55.23	38.10	ATTTTTCCTTGACTTGTAGGC	145	TGGTTGGTTGGTTGGT TGGT

90	c40397_g1_i2	FORWARD	54.58	42.86	GTTGCTGTCTAAAGGACCATA	REVERSE	55.51	42.86	TACTTTCTGCGGTGACATTAC	161	TAATAATAATAATAATAA
91	c41425_g1_i1	FORWARD	54.96	33.33	TATGAGCCGAACATGTTTATT	REVERSE	55.15	33.33	TTTTAACCTTGTGTGTTGCTT	150	GCCGCCGCCGCCGCC
92	c49687_g1_i1	FORWARD	55.99	47.62	CTGTGTACCAGTCGTGATGAT	REVERSE	55.09	31.82	CCATTATTTACGACCTGAATTT	137	CAGCAGCAGCAGCAGCAG
93	c52172_g1_i2	FORWARD	55.21	33.33	TCATTGGAAGAAATAGCTGAA	REVERSE	54.85	47.62	GGTATGTGGATGTGTACGAGT	141	GAAGAAGAAGAAGAA
94	c52368_g1_i1	FORWARD	54.97	42.86	CTCAGAACAATAATGCACTCC	REVERSE	54.86	38.10	TGAGACTGCGAATAAAAGTTC	148	CCTCCTCCTCCTCCT
95	c52776_g1_i1	FORWARD	55.16	38.10	CCTTCCATCAATACAAGATGA	REVERSE	55.67	47.62	CAGGAGTCTCTTGAACTTTCC	165	TAATAATAATAATAA
96	c54189_g1_i2	FORWARD	54.93	38.10	GAACAGAGAACAATCGAAATG	REVERSE	54.82	42.86	CTCATCATGATCTTCTTCGTC	135	GATACCGATACCGATACCGATACC
97	c57368_g1_i2	FORWARD	55.22	42.86	TCGATCAGGATTTACTGTCAC	REVERSE	55.01	47.62	GGTCTGGCAGGAATATCTAGT	145	TGTAATGTAATGTAATGTAA
98	c58728_g1_i1	FORWARD	55.02	30.00	AGAAAATGAGGCATCAAAAA	REVERSE	54.97	42.86	GAGAAAATATCTGCTGTGGTG	153	CAGCAGCAGCAGCAGCAG
99	c61119_g1_i1	FORWARD	54.92	33.33	GATTCAAAATTCGCAATACAC	REVERSE	54.83	33.33	GCTGTTGCTGTAATTTGTTTT	149	CAGCAGCAGCAGCAG
100	c61343_g2_i3	FORWARD	54.20	38.10	AACAGTTTCCTAACCTGCATA	REVERSE	54.92	47.62	CTAGAAGGGGAGCAGTTATTC	163	TCTCATTCTCATTCTCATTCTCAT
101	c62060_g1_i1	FORWARD	54.71	47.62	AGACAGAAGTTACTCCCTCGT	REVERSE	54.46	42.86	GTCGTCTTTTTCACAGTGTCT	194	TGATGATGATGATGA
102	c62797_g1_i1	FORWARD	54.97	42.11	ATTAGTTGGCTGGTTGCTT	REVERSE	55.02	47.62	CCTTTTGTACCTAGTGACGTG	125	GCCGGCCGGCCGGCCG
103	c62797_g1_i1	FORWARD	55.33	52.38	CAGTAAGCTCACGTCACTAGG	REVERSE	55.13	42.86	TCAGAAAGTACCACAATGGAC	145	CGTGCGTGCGTGCGTG
104	c62797_g1_i3	FORWARD	54.97	42.11	ATTAGTTGGCTGGTTGCTT	REVERSE	55.02	47.62	CCTTTTGTACCTAGTGACGTG	125	GCCGGCCGGCCGGCCG
105	c63063_g2_i1	FORWARD	54.95	38.10	TCTTCTTTTACCTCATCACCA	REVERSE	55.01	42.86	TTGACCAGTTAGAACCTCTGA	152	TATATATATATATATA
106	c63218_g1_i1	FORWARD	55.82	61.11	CCTGGAGTAGACCGAAGC	REVERSE	55.12	45.00	GTTCGATAGATCCCAGAACA	166	TCGTCGTCGTCGTCG
107	c63945_g2_i1	FORWARD	55.57	33.33	TTACAAGATCAAATTGGCAAG	REVERSE	55.04	47.62	GTAGTCATAGAAGCAGCGAGA	149	CCACCACCACCACCACCA
108	c64515_g1_i2	FORWARD	55.53	42.86	ATTGAGCCGTAACTACCAGAT	REVERSE	55.53	42.86	GTATGTACAGAGCGCAAATGT	150	ATATATATATATATATATAT
109	c64618_g2_i1	FORWARD	55.15	38.10	TGTGTAAATTTGACCAAGTCC	REVERSE	55.06	38.10	CGCCTATTGCTATGTTTTCTA	156	TCATCATCATCATCA
110	c66541_g1_i1	FORWARD	54.54	38.10	CGGAACAATCCAGAACTATAA	REVERSE	54.73	42.86	CCATCCGAGCTACAATTACTA	153	CAACAACAACAACAA
111	c66610_g1_i1	FORWARD	54.83	38.10	CGCGAATATCCTAATTCTGTA	REVERSE	55.76	33.33	CGTGCAATTTTCATTATCATC	136	GATGATGATGATGAT
112	c66870_g1_i1	FORWARD	55.97	42.86	CCTCCCTCATCTGAGATAAAA	REVERSE	55.26	42.86	TATCAACTTCAGCTCTCCTCA	173	TGATTGATTGATTGAT

113	c66963_g1_i4	FORWARD	54.56	38.10	AGTGTCATTGCGATTTACTTC	REVERSE	54.85	47.62	CTGTAAGGTAGGGGTGGTATT	181	TTCTTCTTCTTCTTCTTC
114	c67089_g3_i1	FORWARD	55.57	47.62	CTTTAGAGCAGCTGGGATAGT	REVERSE	55.48	42.86	TGAGGAGTCGTTCTTAATGTG	151	ATGGATGGATGGATGG
115	c67358_g1_i1	FORWARD	55.65	33.33	AAATTAAATTGTCAGCGTCCT	REVERSE	54.58	38.10	CAATGAATATGACTTGGGTTC	131	CTGCCTGCCTGCCTGCCTGC
116	c67425_g1_i1	FORWARD	55.92	33.33	TTCGAGGAAAGCAAAAATACT	REVERSE	55.16	38.10	TTTAGACGAACTTACCGATGA	155	CCAACCAACCAACCAACCAACCAACCAACCAACCAA
117	c67425_g1_i3	FORWARD	55.05	33.33	TATGCCCAATATTCATTCATC	REVERSE	53.84	33.33	GATGAGCCCTTATTAAACAAA	154	CCAACCAACCAACCAACCAACCAACCAA
118	c67615_g1_i3	FORWARD	55.28	42.86	TATCTTCATACACGGTTCAGG	REVERSE	54.99	47.62	ATCACAGAGGAGAAGAACCTC	143	AAGAAGAAGAAGAAGAAG
119	c68918_g1_i1	FORWARD	54.69	42.86	TAGATCGAAGTTCCTGACTTG	REVERSE	55.61	36.36	CGCTCAAAATACATGTCTATCA	164	ATGATGATGATGATGATG
120	c68918_g1_i1	FORWARD	55.43	33.33	ACATGTATTTTGAGCGATTTG	REVERSE	57.82	55.56	GCATGGCGAGAAAGTCAC	171	CTCCCTCCCTCCCTCC
121	c68918_g1_i3	FORWARD	54.69	42.86	TAGATCGAAGTTCCTGACTTG	REVERSE	55.61	36.36	CGCTCAAAATACATGTCTATCA	164	ATGATGATGATGATGATG
122	c68918_g1_i3	FORWARD	55.43	33.33	ACATGTATTTTGAGCGATTTG	REVERSE	57.82	55.56	GCATGGCGAGAAAGTCAC	171	CTCCCTCCCTCCCTCC
123	c70036_g1_i1	FORWARD	54.91	33.33	TTGTGATCATTTCTGTCATCA	REVERSE	54.88	40.00	GAAACACTGCGATTTTATCC	144	CAGCAGCAGCAGCAG
124	c70344_g6_i1	FORWARD	54.69	33.33	AGATAAAAAGAAACGGAAAGC	REVERSE	54.92	42.86	TCTGAGGAGGAGTGTTATGAA	159	TACCTACCTACCTACC
125	c70344_g6_i2	FORWARD	55.14	42.86	TAGGTACGTCGCTGTTACATT	REVERSE	54.92	42.86	TCTGAGGAGGAGTGTTATGAA	164	TACCTACCTACCTACC
126	c70602_g3_i2	FORWARD	56.02	38.10	AACATCAGTTCGGGTCATATT	REVERSE	55.08	38.10	TTGGCTTGGTTAGATCTGATA	140	GAAGAAGAAGAAGAAGAAGAAGAA
127	c73291_g1_i8	FORWARD	55.18	42.86	TGGTATCCCTTCTTTTCTAGG	REVERSE	54.84	42.86	GAGCATGCTATCTGTTGCTAT	177	GTCGTCGTCGTCGTC
128	c73291_g1_i8	FORWARD	54.64	47.62	TAGAAGAGAGGATGCTGTCAC	REVERSE	55.10	38.10	TTTATACCCACATACCATTCG	131	GGCACGGCACGGCACGGCAC
129	c170312_g1_i1	FORWARD	55.09	44.44	CCGAACCTCAAAGGATTT	REVERSE	55.04	42.86	AGTAGAAGGACAATGTGCTGA	153	CTTCTTCTTCTTCTT
130	c169087_g1_i1	FORWARD	55.42	47.62	GGTTGAGTCCATACATCAGTG	REVERSE	55.32	38.10	CGCTCTCAATTTGTCAATAAG	154	AGCTGAGCTGAGCTGAGCTGAGCTG
131	c135199_g1_i1	FORWARD	54.57	38.10	CAGAAAATAGCTGAAAGATCG	REVERSE	55.16	38.10	CCTGAAGATATGCAGTTTTTG	163	CCATCCATCCATCCAT
132	c132765_g1_i1	FORWARD	51.54	30.00	TGCATACACAAGAATGATTT	REVERSE	54.89	45.00	CTGAATCGTAAATGGAGGAG	133	AATAATAATAATAAT
133	c73291_g1_i1	FORWARD	54.64	47.62	TAGAAGAGAGGATGCTGTCAC	REVERSE	55.10	38.10	TTTATACCCACATACCATTCG	131	GGCACGGCACGGCACGGCAC
134	c73028_g3_i3	FORWARD	55.07	42.86	CTATCAACTCCAGGGACTTTT	REVERSE	55.01	40.00	TTGGCCTGTTACGACTTAAT	111	ATTGATTGATTGATTG

135	c72577_g5_i1	FORWARD	55.22	47.37	GTAGGCCGTTGAATTGAGT	REVERSE	54.96	45.00	CATAGCAACAACCAACACAC	144	CGGCACGGCACGGCACGGCA
136	c71999_g1_i1	FORWARD	55.45	33.33	TGGATTTTAATTACACGAACG	REVERSE	55.12	33.33	GTCTGAAATTCCGAAAAGATT	139	GAAGAAGAAGAAGAA
137	c70602_g3_i3	FORWARD	56.02	38.10	AACATCAGTTCGGGTCATATT	REVERSE	55.08	38.10	TTGGCTTGGTTAGATCTGATA	140	GAAGAAGAAGAAGAAGAAGAAGAA
138	c69150_g1_i1	FORWARD	55.24	38.10	GAAAAGCAGAAGGAGAAAAAG	REVERSE	54.79	50.00	GTCATCAGAGATGGTGTCCT	132	GAAGAAGAAGAAGAAGAA
139	c68918_g1_i2	FORWARD	54.69	42.86	TAGATCGAAGTTCCTGACTTG	REVERSE	55.61	36.36	CGCTCAAAATACATGTCTATCA	164	ATGATGATGATGATGATG
140	c68918_g1_i2	FORWARD	55.43	33.33	ACATGTATTTTGAGCGATTTG	REVERSE	57.82	55.56	GCATGGCGAGAAAGTCAC	171	CTCCCTCCCTCCCTCC
141	c67714_g3_i1	FORWARD	54.36	33.33	CGATTCCTTGACATGTAATTT	REVERSE	55.09	42.86	ACACTATTGGGATCCATCTCT	152	CTCTCTCTCTCTCTCT
142	c67615_g1_i4	FORWARD	55.28	42.86	TATCTTCATACACGGTTCAGG	REVERSE	54.99	47.62	ATCACAGAGGAGAAGAACCTC	143	AAGAAGAAGAAGAAGAAG
143	c67425_g1_i2	FORWARD	55.05	33.33	TATGCCCAATATTCATTCATC	REVERSE	54.11	38.10	TAGAATGAGCATAGGTTGGTT	132	CCAACCAACCAACCAACCAACCAACCAACCAACCAACCAA
144	c67358_g1_i2	FORWARD	55.65	33.33	AAATTAAATTGTCAGCGTCCT	REVERSE	54.58	38.10	CAATGAATATGACTTGGGTTC	131	CTGCCTGCCTGCCTGCCTGC
145	c67089_g3_i2	FORWARD	55.57	47.62	CTTTAGAGCAGCTGGGATAGT	REVERSE	55.48	42.86	TGAGGAGTCGTTCTTAATGTG	151	ATGGATGGATGGATGG
146	c66963_g1_i5	FORWARD	54.56	38.10	AGTGTCATTGCGATTTACTTC	REVERSE	54.85	47.62	CTGTAAGGTAGGGGTGGTATT	181	TTCTTCTTCTTCTTCTTC
147	c66870_g1_i2	FORWARD	55.05	47.62	GTCACGGTAGCTATACAATGC	REVERSE	54.20	33.33	CTCAGGATGATTGATGATTTT	176	TGATTGATTGATTGAT
148	c66685_g1_i1	FORWARD	54.85	33.33	TTCAGGTTTTGATGTCTTTGT	REVERSE	54.85	40.00	GGGAAAACACTGATCACAAT	148	TGATGATGATGATGATGATGA
149	c66610_g1_i3	FORWARD	54.83	38.10	CGCGAATATCCTAATTCTGTA	REVERSE	55.76	33.33	CGTGCAATTTTCATTATCATC	136	GATGATGATGATGAT
150	c66541_g1_i2	FORWARD	54.54	38.10	CGGAACAATCCAGAACTATAA	REVERSE	54.73	42.86	CCATCCGAGCTACAATTACTA	153	CAACAACAACAACAA
151	c66183_g1_i3	FORWARD	55.07	38.10	CGTTCGTGTAAAAGAAACATC	REVERSE	55.20	38.10	CTTTCCCACAAAGAGAGAAAT	158	ATCATCATCATCATCATC
152	c64714_g1_i2	FORWARD	55.53	38.10	TCTTCATCGACTTTGAATGAG	REVERSE	56.06	42.86	ATATCGGTGACCACGTATTCT	154	TACCTACCTACCTACCTACC
153	c64618_g2_i2	FORWARD	55.15	38.10	TGTGTAAATTTGACCAAGTCC	REVERSE	55.06	38.10	CGCCTATTGCTATGTTTTCTA	156	TCATCATCATCATCA
154	c64515_g1_i1	FORWARD	55.53	42.86	ATTGAGCCGTAACTACCAGAT	REVERSE	55.53	42.86	GTATGTACAGAGCGCAAATGT	150	ATATATATATATATATATAT
155	c63945_g2_i2	FORWARD	55.57	33.33	TTACAAGATCAAATTGGCAAG	REVERSE	55.04	47.62	GTAGTCATAGAAGCAGCGAGA	149	CCACCACCACCACCACCA
156	c62797_g1_i2	FORWARD	54.97	42.11	ATTAGTTGGCTGGTTGCTT	REVERSE	55.02	47.62	CCTTTTGTACCTAGTGACGTG	125	GCCGGCCGGCCGGCC

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157	c62797_g1_i2	FORWARD	55.33	52.38	CAGTAAGCTCACGTCACTAGG	REVERSE	55.13	42.86	TCAGAAAGTACCACAATGGAC	145	CGTGCGTGCGTGCGTG
158	c62409_g2_i2	FORWARD	54.05	38.10	CTATCTTGCCCGTCTATAAAA	REVERSE	54.90	42.86	CTGATTTGTAGGAGGTGTTTG	150	GCTGCTGCTGCTGCT
159	c62060_g1_i2	FORWARD	54.71	47.62	AGACAGAAGTTACTCCCTCGT	REVERSE	54.46	42.86	GTCGTCTTTTTCACAGTGTCT	194	TGATGATGATGATGA
160	c61343_g2_i2	FORWARD	54.20	38.10	AACAGTTTCCTAACCTGCATA	REVERSE	54.92	47.62	CTAGAAGGGGAGCAGTTATTC	163	TCTCATTCTCATTCTCATTCTCAT
161	c61119_g1_i2	FORWARD	54.92	33.33	GATTCAAAATTCGCAATACAC	REVERSE	54.83	33.33	GCTGTTGCTGTAATTTGTTTT	149	CAGCAGCAGCAGCAG
162	c58728_g1_i2	FORWARD	55.02	30.00	AGAAAATGAGGCATCAAAAA	REVERSE	54.97	42.86	GAGAAAATATCTGCTGTGGTG	153	CAGCAGCAGCAGCAGCAG
163	c54244_g1_i1	FORWARD	54.65	38.10	CTGAGAATAGCATCGTTGAAT	REVERSE	54.97	38.10	AGCAGTGAATGGTCTGAAATA	148	AGAAGAAGAAGAAGA
164	c54189_g1_i3	FORWARD	54.93	38.10	GAACAGAGAACAATCGAAATG	REVERSE	54.82	42.86	CTCATCATGATCTTCTTCGTC	135	GATACCGATACCGATACCGATACC
165	c52368_g1_i2	FORWARD	54.97	42.86	CTCAGAACAATAATGCACTCC	REVERSE	54.86	38.10	TGAGACTGCGAATAAAAGTTC	148	CCTCCTCCTCCTCCT
166	c52210_g1_i1	FORWARD	55.13	42.86	CATCCCTCATATTCCCTAATC	REVERSE	54.00	38.10	CGGTTTCTAATTTACTGTGCT	157	ATATATATATATATATATAT
167	c52210_g1_i1	FORWARD	55.08	38.10	GCGAGCACAGTAAATTAGAAA	REVERSE	55.96	42.86	TCTCGGATCTTCACTAATTCC	124	GGTGCCGGTGCCGGTGCCGGTGCC
168	c51616_g1_i1	FORWARD	55.64	42.86	AGCTCTTCGCGATAGTTAATC	REVERSE	56.56	33.33	CGCGGAAAACAAAATATAAAC	142	CGTCCGTCCGTCCGTC
169	c51616_g1_i1	FORWARD	55.64	42.86	AGCTCTTCGCGATAGTTAATC	REVERSE	56.56	33.33	CGCGGAAAACAAAATATAAAC	142	AGAAAGAAAGAAAGAA
170	c49687_g1_i2	FORWARD	55.99	47.62	CTGTGTACCAGTCGTGATGAT	REVERSE	55.09	31.82	CCATTATTTACGACCTGAATTT	137	CAGCAGCAGCAGCAGCAG
171	c44245_g1_i1	FORWARD	55.11	38.10	CGAGAACTGATCAAGAAATTG	REVERSE	54.73	38.10	TTCTAGCCAGAGAAAATGCTA	134	ATATATATATATATAT
172	c40397_g1_i1	FORWARD	54.58	42.86	GTTGCTGTCTAAAGGACCATA	REVERSE	55.51	42.86	TACTTTCTGCGGTGACATTAC	161	TAATAATAATAATAATAA
173	c40172_g1_i1	FORWARD	54.34	38.10	CTTCACATGATACCAATGACA	REVERSE	55.23	38.10	ATTTTTCCTTGACTTGTAGGC	145	TGGTTGGTTGGTTGGTTGGT
174	c39775_g1_i1	FORWARD	55.05	42.86	GCATTGACGATAGAATGAGAG	REVERSE	55.20	33.33	ATTTAGGCAACATGCTTTACA	123	AATAATAATAATAAT
175	c39615_g1_i2	FORWARD	54.68	42.86	CTAATTGCGTCTTACTTGCTC	REVERSE	54.16	47.62	GTCTCTCCTCGTTTGTAGTTG	167	GCTCGCTCGCTCGCTC
176	c39562_g1_i1	FORWARD	55.42	38.10	GCTTGCATTGACAAGTATGAT	REVERSE	55.62	38.10	AAGCACCTCATCTGAAAGAAT	144	CGACGACGACGACGA
177	c39562_g1_i1	FORWARD	55.42	38.10	GCTTGCATTGACAAGTATGAT	REVERSE	55.62	38.10	AAGCACCTCATCTGAAAGAAT	144	GATGATGATGATGAT
178	c17751_g1_i1	FORWARD	55.09	31.82	CCATTATTTACGACCTGAATTT	REVERSE	55.99	47.62	CTGTGTACCAGTCGTGATGAT	137	GCTGCTGCTGCTGCTGCT

179	c9613_g1_i1	FORWARD	55.57	55.56	CCTCACGACAATGACCAC	REVERSE	55.22	42.86	GTGTTCACGCTCAAGAAATAG	192	CCGCCGCCGCCGCCGCCG
180	c2851_g2_i1	FORWARD	54.95	38.10	TGTCTCCATTTCCAACTCTAA	REVERSE	54.26	42.86	GTATTTGCTGGTCTATTGCTC	146	AGACAGACAGACAGAC
181	c64714_g1_i1	FORWARD	55.53	38.10	TCTTCATCGACTTTGAATGAG	REVERSE	56.06	42.86	ATATCGGTGACCACGTATTCT	154	TACCTACCTACCTACCTACC
182	c64957_g2_i1	FORWARD	54.87	45.00	CAATTGAATCCCAGCTCTAC	REVERSE	54.60	38.10	AGGTCGAGATTATTTCTTGGT	161	TGATGATGATGATGA
183	c65447_g1_i1	FORWARD	54.01	42.86	GGTGGTGATCTACTGACAAAT	REVERSE	56.73	55.56	ACAGGTTGACGGGTTGAC	192	GACGACGACGACGAC
184	c65447_g1_i2	FORWARD	54.01	42.86	GGTGGTGATCTACTGACAAAT	REVERSE	56.73	55.56	ACAGGTTGACGGGTTGAC	192	GACGACGACGACGAC
185	c65501_g1_i1	FORWARD	55.05	42.86	CTGCGAATTCATCATCTCTAC	REVERSE	55.54	38.10	ATCCAACGGGTATCATTTAAC	164	GGCCGGCCGGCCGGCC
186	c65680_g2_i1	FORWARD	54.48	33.33	GACGTTTTACCCACAAAAATA	REVERSE	55.92	38.10	ATATGTTGCAAGTCGTCTGAA	152	AACAACAACAACAACAAC
187	c65680_g2_i3	FORWARD	55.51	33.33	AAAAACAACCCGGATAAAGTA	REVERSE	55.13	38.10	TGATTAACCGGTAGAAGCATA	154	AACAACAACAACAACAAC
188	c66009_g1_i2	FORWARD	55.23	42.86	CTGAAGAGCAGTGGTTAAATG	REVERSE	54.04	42.86	TACCTGAGGGTGAATTCTCTA	153	GATGATGATGATGAT
189	c66136_g1_i1	FORWARD	55.05	42.86	TTACCGCTACTAGAAAACACG	REVERSE	55.58	42.86	GTTCCTGAATTCTTTCGTCTC	169	CGATGACGATGACGATGACGATGA
190	c66161_g1_i1	FORWARD	54.53	38.10	AATTTCCACCTACAACCTTCT	REVERSE	55.50	38.10	ATGGTTAAATGACCTCAGCTT	140	CTGTCTGTCTGTCTGTCTGT
191	c66344_g1_i1	FORWARD	54.60	47.62	GAGAGTTAGCGAGAGAGGATT	REVERSE	54.46	42.86	TTAGGAGGTAGTGAAAGCTGA	152	AACGAACGAACGAACG
192	c66393_g1_i1	FORWARD	55.02	33.33	AACTTTTCCCGTAATTGATTC	REVERSE	55.32	38.10	TGGCGTATCTGAGTTAGAAAA	139	ACGACGACGACGACG
193	c66610_g1_i4	FORWARD	54.83	38.10	CGCGAATATCCTAATTCTGTA	REVERSE	55.76	33.33	CGTGCAATTTTCATTATCATC	136	GATGATGATGATGAT
194	c66721_g1_i1	FORWARD	54.82	55.56	GACGGTTTGGTGTAGTCG	REVERSE	54.37	42.11	GATCGGGGTAATTGAAATC	149	AACGAACGAACGAACG
195	c66875_g1_i1	FORWARD	54.75	33.33	TTTGTTGTTTTCCCTGTGTAT	REVERSE	55.63	38.10	GATTTCAATTCCTGTGAGGAT	152	CCAGCCAGCCAGCCAG
196	c66875_g1_i2	FORWARD	55.21	47.62	AGACGAGAGATCGTAAGTTCC	REVERSE	55.21	50.00	GATAGGGAAAGAGGACCAAC	147	CCAGCCAGCCAGCCAG
197	c66963_g1_i1	FORWARD	54.56	38.10	AGTGTCATTGCGATTTACTTC	REVERSE	54.85	47.62	CTGTAAGGTAGGGGTGGTATT	181	TTCTTCTTCTTCTTCTTC
198	c67366_g1_i3	FORWARD	55.92	38.10	CCATTCAAGCAACAAATACAG	REVERSE	55.46	42.86	AGGTTTGCTAAGGTCTTTCTG	152	CAACAACAACAACAA
199	c67366_g1_i3	FORWARD	54.88	33.33	CTTGATTATCGCAAACCTTTA	REVERSE	54.77	42.86	GAAGCTAAGGAGAAGGAGAAA	144	TGATGATGATGATGATGA
200	c67366_g1_i4	FORWARD	54.88	33.33	CTTGATTATCGCAAACCTTTA	REVERSE	54.77	42.86	GAAGCTAAGGAGAAGGAGAAA	144	TGATGATGATGATGATGA

201	c67423_g1_i3	FORWARD	54.59	33.33	TTGGTTAAACAGGATTCTTTG	REVERSE	55.32	33.33	GCGTTATTTCTTGAAATCCTT	150	GCCGCCGCCGCCGCC
202	c67474_g1_i3	FORWARD	54.85	38.10	AGTTGTATGATCATGGGATTG	REVERSE	54.45	42.86	AAGTCATCATCTTCAGCTGTC	148	CTAGCTAGCTAGCTAG
203	c67917_g1_i2	FORWARD	54.54	38.10	CTGGATAAAGTGGTCGATAAA	REVERSE	55.18	38.10	ATTGGTTACGACAAGAATGTG	151	GCAGCAGCAGCAGCA
204	c67924_g1_i1	FORWARD	56.72	47.62	CGTATGACTCTGGACCTTCAT	REVERSE	55.01	42.86	AAGCGTCTGATGTGGATACTA	149	GGTGGTGGTGGTGGT
205	c68034_g1_i2	FORWARD	54.94	33.33	CCGTAAATCAAATCAAATCAG	REVERSE	54.95	42.86	ATCAGAGTCCCCATTTATAGC	163	GATGATGATGATGAT
206	c68113_g1_i1	FORWARD	55.67	33.33	TGAACACTTTCAAAGCAAAAC	REVERSE	54.81	42.86	TTGCGGTAGTATTAGAAGTGG	153	GCCGCCGCCGCCGCC
207	c68189_g1_i1	FORWARD	55.98	42.86	AGAGATGAATAATCGGCTGAC	REVERSE	54.88	42.86	GGGTCGTTTATCTATGGACTT	143	GAAAGAAAGAAAGAAAGAAA
208	c68288_g1_i1	FORWARD	55.65	33.33	CGCTTAGATGATGATGAAAAA	REVERSE	54.01	33.33	CCATCAATAGGCTTTCTTTTA	155	GATGATGATGATGAT
209	c68516_g1_i1	FORWARD	54.86	47.62	GAGACTAGCCTCCAATTCTTC	REVERSE	55.42	42.86	CACAGGTCACTTGCTTTACAT	147	TGATGATGATGATGA
210	c68516_g2_i1	FORWARD	54.89	42.86	CAACAACAGTATCGGAGTTTC	REVERSE	54.40	38.10	GTGGCAAGAATGAATAAGTGT	157	TATTATTATTATTAT
211	c68516_g2_i2	FORWARD	54.89	38.10	TCAACAACAGTATCGGAGTTT	REVERSE	54.95	38.10	TAAGGAACCTCACAAGAATGA	166	TATTATTATTATTAT
212	c68516_g2_i4	FORWARD	54.89	42.86	CAACAACAGTATCGGAGTTTC	REVERSE	54.88	38.10	CCACAGAAGCTTTAAGAATGA	165	TATTATTATTATTAT
213	c68620_g1_i1	FORWARD	54.72	45.00	AGTCAATGATGACTGCCTCT	REVERSE	55.11	38.10	GGTCTTTTGACTGGTTTCTTT	148	CAACAACAACAACAACAACAA
214	c68822_g2_i1	FORWARD	54.86	42.86	CTTACGTTCAAGCACTCGTAT	REVERSE	54.26	50.00	GGATTAGGCGTAGCAATG	171	CCTCCTCCTCCTCCTCCTCCT
215	c68822_g2_i3	FORWARD	55.77	47.62	TGGGAGAGAGAGAAGGAACTA	REVERSE	55.31	42.86	AGAATAGATTCCTCAGCCAAG	133	AAGCAAAGCAAAGCAAAGCAAAGCAAAGCA
216	c68822_g2_i4	FORWARD	54.86	42.86	CTTACGTTCAAGCACTCGTAT	REVERSE	54.26	50.00	GGATTAGGCGTAGCAATG	171	CCTCCTCCTCCTCCTCCTCCT
217	c68822_g2_i11	FORWARD	55.77	47.62	TGGGAGAGAGAGAAGGAACTA	REVERSE	55.31	42.86	AGAATAGATTCCTCAGCCAAG	128	AAGCAAAGCAAAGCAAAGCAAAGCA
218	c68822_g2_i14	FORWARD	54.86	42.86	CTTACGTTCAAGCACTCGTAT	REVERSE	54.26	50.00	GGATTAGGCGTAGCAATG	171	CCTCCTCCTCCTCCTCCTCCT
219	c68954_g1_i1	FORWARD	55.02	50.00	CTGCTTTTACACCAGTCTCC	REVERSE	55.08	35.00	ATTGACGCTAGCAAAATGAT	178	ACCACCACCACCACC
220	c68954_g2_i1	FORWARD	55.08	35.00	ATTGACGCTAGCAAAATGAT	REVERSE	55.02	50.00	CTGCTTTTACACCAGTCTCC	178	GGTGGTGGTGGTGGT
221	c68954_g2_i6	FORWARD	54.94	38.10	AAGCTCTTTCAGGTTTCATCT	REVERSE	55.00	38.10	GTTCATCATCATCGTCGTTAT	155	TGATGCTGATGCTGATGCTGATGCTGATGCTGATGCTGATGC
222	c68954_g2_i7	FORWARD	55.08	35.00	ATTGACGCTAGCAAAATGAT	REVERSE	55.02	50.00	CTGCTTTTACACCAGTCTCC	178	GGTGGTGGTGGTGGT
223	c69374_g1_i1	FORWARD	55.37	45.00	GTCAGTTGCTCAGGGAATAA	REVERSE	55.07	33.33	TGTTGTAAAGCTTGACCATTT	150	CAACAACAACAACAACAA

224	c69450_g1_i4	FORWARD	54.83	38.10	CGGAAAAGAGGATAAAGATTC	REVERSE	55.70	38.10	GATGGAGAAATCGTTAAAAGC	151	AGCAGCAGCAGCAGC
225	c69658_g1_i1	FORWARD	55.29	33.33	TCCGTATTTTTACGCTGATTA	REVERSE	55.24	38.10	CTTATTTCTCCCAAAGTTCGT	163	ATATATATATATATATATAT
226	c70602_g3_i1	FORWARD	56.02	38.10	AACATCAGTTCGGGTCATATT	REVERSE	55.08	38.10	TTGGCTTGGTTAGATCTGATA	140	GAAGAAGAAGAAGAAGAAGAAGAA
227	c70805_g2_i2	FORWARD	53.95	33.33	GATATGAAAAGGAATCGAATG	REVERSE	55.01	42.86	CTGTGATTTTGGAGAGTTCTG	147	GAAGAAGAAGAAGAA
228	c72037_g7_i1	FORWARD	55.42	42.11	TTGAAGGGAATTCTTCTCG	REVERSE	55.01	42.86	TAGACATGATCTTAGCCGTGT	124	TTGTTTGTTTGTTTGT
229	c72252_g2_i2	FORWARD	55.05	42.86	CAGTTGAACCTGTCTAAATGC	REVERSE	55.11	50.00	GATAGCTCTAGCGTCCTATGTC	157	TGATGATGATGATGATGA
230	c72835_g1_i2	FORWARD	54.64	38.10	TGGTAACATGAGTCCATTCTT	REVERSE	54.90	42.86	CCAGTTCGAGAGATGATAATG	153	GACGACGACGACGACGAC
231	c72899_g2_i1	FORWARD	54.98	47.62	AGCCGTAGGTTACGTAGAAGT	REVERSE	54.66	45.00	GACAAGCTCGGTTATAGCAT	154	ACAAACAAACAAACAA
232	c73198_g1_i3	FORWARD	58.39	66.67	GAGTGGAGCTGGGGAGAG	REVERSE	57.24	42.86	TATTCTAAAGAGGTGCGAACG	149	TGTTTGTTTGTTTGTT
233	c73276_g1_i1	FORWARD	56.05	47.62	AAGCTGCCCAGACTCTACTTA	REVERSE	55.29	38.10	CCCACAACAGACGATAAATAA	133	TGTGTGTGTGTGTGTG
234	c73358_g2_i1	FORWARD	54.82	38.10	TATCACCCCAGACGTTAAATA	REVERSE	54.28	42.86	GACGTGACCTGTCAAGTAAAT	148	ATCATCATCATCATCATC
235	c74748_g1_i1	FORWARD	55.31	33.33	TGCTCAAATATCTGCAACTTT	REVERSE	54.80	38.10	CAAGTACATTGGCTTATGCTT	154	AGAAGAAGAAGAAGA
236	c91956_g1_i1	FORWARD	55.36	38.10	GGCTTAAGGTTTTAACGGATA	REVERSE	55.13	42.86	CACTGAGCGATAATGTTCTTC	150	ATGATGATGATGATG
237	c93711_g1_i1	FORWARD	55.64	38.10	AAAGTATATTCGCTTCGCTTC	REVERSE	54.93	45.00	CTATCCGCCGCTATCTATAA	168	GCGGTGCGGTGCGGTGCGGT
238	c100224_g1_i1	FORWARD	55.96	45.00	TAACTGTGGGTCCATAATGC	REVERSE	54.91	47.62	GCGATACACCAGTAGTTGTTC	160	GAAGAAGAAGAAGAA
239	c103749_g1_i1	FORWARD	54.98	42.86	GGTTCATATCTCGAGGAAAGT	REVERSE	55.05	38.10	AACTAGCCACAATTTGTCAGA	147	GCCGCCGCCGCCGCC
240	c115725_g1_i1	FORWARD	55.54	42.86	CAACTGTGAGCAAGAATGACT	REVERSE	54.98	40.00	CTTTGGGCTGAAACAAATAC	138	ACTACTACTACTACT
241	c134253_g1_i1	FORWARD	55.34	38.10	ATTAGGTGTTGTGAAATGCAG	REVERSE	54.75	42.86	CTTGATCTGGGCTAGTTTGTA	148	AAGAAGAAGAAGAAG
242	c152481_g1_i1	FORWARD	54.67	47.62	GATAGATGGTCACTGGTTACG	REVERSE	54.56	38.10	TCAAGAAGTGGACTGAAAAAC	170	CACGCCACGCCACGCCACGC

GENERAL CONCLUSIONS

The results generated in this study allows the following conclusions:

1. The ITS and 28S rDNA sequencing and morphological characterization allowed us identified all powdery mildew pathogen isolates collected on eucalypt from different localities in Brazil as *Podosphaera pannosa*;
2. *Podosphaera pannosa* is able to infect both roses and eucalypt through cross inoculations;
3. The transcriptome sequencing of *P. pannosa* during *E. urophylla* infection using RNA sequencing (RNA-Seq) was successful and after filtering steps resulted in 12,107 transcripts of *P. pannosa*;
4. The 10 most abundant transcripts included genes encoding enzymes involved in fungal establishment and growth;
5. The secretome prediction used in this study resulted in 217 transcripts, where 14 of them were considered as candidate secreted effector proteins (CSEPs);
6. A total of 242 primer pairs were identified from the transcripts with potential to amplify *P. pannosa* microsatellites (SSRs) regions;
7. *Erysiphe australiana* is the causal agent of powdery mildew on *Lagerstroemia speciosa* seedlings in Brazil.