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PhD THESIS

Development of molecular and genomic tools for functional analysis in the cucurbit powdery mildew fungus Podosphaera fusca

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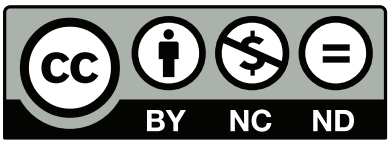

The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*) is the main causal agent of cucurbit powdery mildew and one of the most important limiting factors for cucurbit production worldwide. Despite the fungus' economic importance, very little is known about the physiological and molecular processes involved in *P. fusca* biology and pathogenesis. In this study, we isolated and characterised the β-tubulin-encoding gene of *P. fusca* (*PfTUB*2) to develop molecular tools with different applications in powdery mildew research. *PfTUB*2 is predicted to encode a protein of 447 amino acid residues. The coding region is interrupted by six introns that occur at approximately the same positions as the introns present in other fungal *TUB*2-like genes. Once cloned, the *PfTUB*2 sequence information was used in different applications. Our results showed that the *TUB*2 gene is a good marker for molecular phylogenetics in powdery mildew fungi but it is unsuitable for the analysis of intraspecific diversity in *P. fusca*. The expression of *PfTUB*2 was proven to be stable in different temperature conditions, supporting its use as a reference gene in quantitative gene expression studies. Furthermore, an allele-specific PCR assay for the detection of resistance to methyl-2-benzimidazole carbamate (MBC) fungicides in *P. fusca* was developed based on the correlation between the single amino acid change E198A in β-tubulin and the MBC resistance phenotype. Lastly, *PfTUB2* was used as a target gene in the development of a high-throughput method to quantify fungal growth in plant tissues.

The cucurbit powdery mildew fungus *Podosphaera fusca*, is a major limiting factor for cucurbit production worldwide. Despite its agronomic and economic importance, very little is known about fundamental aspects of *P. fusca* biology such as obligate biotrophy and pathogenesis. In order to design novel and more durable control strategies, genomic information of *P. fusca* is needed. In order to reduce genome complexity, in this work we aimed to obtain and analyse the epiphytic transcriptome of *P. fusca* as starting point. Total RNA was isolated from epiphytic fungal material composed by mycelia and conidia, and the corresponding cDNA library was sequenced using a 454 GS FLX platform. Over 676,562 reads were obtained and assembled into 39,346 contigs. Annotation data was acquired for 62.6% of the assembled sequences, identifying 9,713 putative genes with different orthologues. In the transcript data set, the most represented protein functions were those with role in gene expression, protein metabolism, regulation of biological process and organelle organization. Our analysis also confirmed the existence of "missing ascomycete core genes" (MACGs) found in previous studies. To get insight into the plant-pathogen relationships, special attention was focused on the analysis of the pool of fungal secreted proteins. After analysis, 119 putative secreted proteins were identified, including 35 "candidate secreted effector proteins" (CSEPs) specific for *P. fusca*. In order to validate the *in silico* assembly, a time-course transcriptional profiling of some selected CSEP genes was performed during the course of infection. The expression profile observed for these CSEPs was consequent with a canonical effector expression pattern, with a maximum of expression at the beginning of the infection process 24-48 h after inoculation. Our data open the genomics era of this very important cucurbit pathogen.

Methyl benzimidazole carbamates (MBC) such as carbendazim and benomyl are fungicidal compounds that exert their biological activity preventing cell division, due to inhibition of the polymerization of tubulin, the major component of microtubules. MBC fungicides have been widely used against powdery mildews (*Erysiphales*), a constant threat to many crops worldwide. Despite the effectiveness of MBC fungicides in disease control, their misuse in the past has led to the problem of fungicide resistance and thus the loss of efficacy. Although diverse mutations seem to contribute to MBC resistance in powdery mildews and other fungal pathogens, the most common mechanism of resistance against MBCs is provided by the substitution of glutamic acid to alanine at position 198 (E198A). However, the precise mechanism by which this amino acid change affects fungicide binding is still unknown. The aim of this work was to elucidate the mode of action and the molecular basis of resistance to MBC fungicides in *Podosphaera fusca*, the main causal agent of cucurbits powdery mildew in Spain. By a combination of techniques, we show that carbendazim, a paradigm of MBC fungicides, induces conformational changes to β-tubulin, leading to the formation of aberrant tubulin structures. Furthermore, by computational analysis we have evaluated topologically the interaction of the fungicide molecule and the target protein. Our results allowed us to propose a novel binding site for MBCs to β-tubulin in a protein region very close to the GTP binding site. In absence of more conclusive crystallographic data, our results provided new insights about the mode of action and resistance to MBC fungicides, information that could help on new fungicide design.

CONCLUSIONS

1. Cloning of the *P. fusca TUB2* gene has enabled the development of a number of molecular tools with different applications in cucurbit powdery mildew research, such as a high-throughput method for quantification of fungal growth or a selection marker for genetic transformation.

2. Although transient, electroporation of conidia has been an effective method for genetic transformation of *P. fusca*. This is the first report on transformation of *P. fusca* and on transformation of powdery mildew fungi by electroporation.

3. NGS technologies have made it possible for the first time to obtain massive genomic information of *P. fusca* through the sequencing of its epiphytic transcriptome.

4. The identification of the *P. fusca* predicted epiphytic secretome has led to a major understanding of the interaction between the fungus and its host plant, for example, through the identification of a number of candidate effector proteins.

5. Binding of carbendazim to sensitive β-tubulin induces a conformational change with lethal consequences. In the resistant protein, however, subtle conformational modifications provoked by the E198A substitution abolish fungicide binding.

6. By a computational approach a new binding site for carbendazim and benomyl has been proposed, which is located in the proximity of the GTP binding domain. This computational model along with the reported competition between carbendazim and GTP suggest a possible mode of action of those fungicides as GTP competitive inhibitors, thus blocking microtubule polymerization.