In silico **annotation of five candidate genes associated with pathogenicity in** ***Fusarium circinatum*****.**

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Running Head

Annotation of candidate genes in *Fusarium circinatum*

Summary

The Pine Pitch Canker disease caused by the pathogenic fungus *Fusarium circinatum* is one of the most devastating diseases in pine forests, afforestation and nurseries around the world. Despite the importance of this phytopathogen, only a little is known about the genes that drive the infection traits and the virulence factors. In this work, five candidate genes (i.e. *Fcfga1, Fcfgb1, Fcac, Fcrho1* and *FcpacC*) were *in silico* annotated using the whole genome of *F. circinatum* as reference. The similarity of these proposed genes at nucleotide and protein levels with genes previously described in other *Fusarium* species was > 90 % of identity and > 90 % query coverage in all cases. In addition, the gene ontology of each candidate gene was also investigated.

Keywords: cAMP-PKA, damping-off, gene annotation, MAPK, Pine Pitch Canker disease.

1 INTRODUCTION

Fusarium circinatum Nirenberg & O´Donell is the causal agent of one of the most devastating forest diseases around the world: the Pine Pitch Canker disease. This pathogen infects coniferous species (mainly *Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco), causing wilting and bleeding cankers in mature trees, drastically reducing the value of timber and predisposing the trees to break during windstorms (Wingfield et al., 2008). In addition, it causes severe damping-off in seeds and seedlings in nurseries. Despite the high threat that this fungus embodies for native forests and pine plantations, there is limited knowledge about the genes involved in the infection process. On the other hand, the complete genome of *F. circinatum* has been sequenced and made accessible through public databases (Wingfield et al., 2012; De Vos et al., 2014).

Several virulence-related genes have been annotated in other species of *Fusarium*. Regarding the cell signaling pathways, the guanine nucleotide-binding protein subunits α and β have been reported as related with pathogenesis in fungi. These two subunits play a key role as essential elements upstream of cellular signaling process affecting either the mitogen-activated protein kinase (MAPK) cascade or the cAMP-dependent protein kinase pathway (cAMP-PKA) by triggering virulence factors as in the case of *Fusarium oxysporum* Schltdl. (Guo et al., 2016). Likewise, adenylate cyclase has been described as a major element in cAMP-PKA signaling. This pathway is involved with pathogenesis development in several fungi (e. g. *Colletotrichum orbiculare* (Berk.) Arx and *Ustilago maydis* (DC.) Corda), and it is linked to the MAPK pathway in virulence regulation (Kohut, Oláh, Ádám, García-Martínez, & Hornok, 2010)

A number of genes known to be involved in metabolic process are also implicated in virulence. More specifically, Martínez-Rocha et al. (2008) highlighted the importance of GTP-binding protein as an element implied in the maintenance of a correct cell wall structure in *F. oxysporum*. According to these authors, the loss of the structure could facilitate the recognition of fungal membrane proteins by potential hosts. On the other hand, Caracuel et al. (2003) studied the pH signaling transcription factor in the same species and reported that the expression of this gene was reduced when pH becomes acidic (closer to host conditions) favoring the synthesis of virulence factors (e. g. cell-wall degrading enzymes).

In this study, we hypothesized that the genome of *F. circinatum* could include pathogenicity genes with high similarity with other species of *Fusarium*. In consequence, the goal of this research was to annotate candidate genes of *F. circinatum* that could be related with virulence.

2 MATERIAL AND METHODS

The prediction of five candidate genes was performed using an empiric approach based on homology between annotated genes available in GenBank (https://www.ncbi.nlm.nih.gov/) and the genome of *F. circinatum* (ASM49732v2; genomes of fungal strains FSP34 and GL1327). The following criteria were fulfilled by the genes selected as queries: a) the reference genes were identified in other pathogenic *Fusarium* spp. (e.g. genes of *F. oxysporum* reviewed by Michielse, & Rep, 2009), b) the complete nucleotide and protein sequences were available in GenBank and c) the biological function of the gene as a pathogenicity trait was previously reported in scientific literature. The accession numbers of genes selected as queries were summarized in Table 1.

The encoding regions of each reference sequence was accessed from GenBank, without removing intron sequences. These reference sequences were compared with the complete genome of *F. circinatum* using MegaBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (database: whole-genome shotgun contigs (wgs); taxid:48490), to obtain the candidate sequences. The nucleotide sequences for *F. circinatum* were compared with those deposited in GenBank using BLAST and MegaBLAST algorithms in order to ensure the putative homology with target genes (Table 1). The annotation was assessed if the identity between candidate genes and queries was ≥ 90 %. In parallel, the similarity at protein level between the candidate sequences and those available in GenBank was also checked by BLASTx using the non-redundant protein sequences (nr) database. The specific databases of Fungi (taxid: 4751) and *Fusarium* sp. (taxid 5506) were selected for more accurate searches (Table 1). Only proteins with a similarity higher than 90 % in at least one search were selected for annotation. The software Geneious 6.0.6 (http://www.geneious.com) was used for sequence trimming and alignment (methods: CluscalW for nucleotide sequences; BLOSUM matrix for proteins), while the software AUGUSTUS 2.5.5 (http://augustus.gobics.de/) was required to predict amino acid sequences available in S2. Regarding gene ontology, the 100 best matches of the BLASTx search (i.e. BLASTx, nr database, *Fusarium* spp. (taxid 5506) as organism and word size of 3) were used as input data in

TABLE 1. GenBank best matches for five candidate genes related with the pathogenicity of *F. circinatum*. QC: Query cover; E: E-value; ID: Similarity between sequences; Coord.: Number of first nucleotide position in the 5´/3´ extreme. nt: nucleotides.

Gene	Accession number (Reference gene)	Type of sequence	Algorithms	Database	Limit	Best match ID	Accession number (Best match)	QC (%)	E	ID (%)	Coord.	Length (nt)
Fcfga1	GU168785.1	Protein	BLASTx	nr	Fungi (taxid: 4751)	Guanine nucleotide-binding protein subunit alpha [*Hirsutella minnesotensis* 3608]	KJZ72861.1	90%	0	91%	2594/3849	1256
					Fusarium (taxid:5506)	guanine nucleotide-binding protein [*Fusarium oxysporum* f. *cubense*]	ACF20294.1	93%	0	91%		
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)	*Fusarium oxysporum* fga1 gene for guanine nucleotide-binding protein alpha subunit, partial cds	AB072451.1	100%	0	96%		
			MegaBLAST		*Fusarium* (taxid:5506)	*Fusarium oxysporum* fga1 gene for guanine nucleotide-binding protein alpha subunit, partial cds	AB072451.1	100%	0	96%		
Fcfgb1	DQ457053	Protein	BLASTx	nr	Fungi (taxid: 4751)	Guanine nucleotide-binding protein subunit beta [*Fusarium oxysporum* f. sp. *cubense* race 1]	ENH75085.1	76%	0	98%	494810/496220	1411
					Fusarium (taxid:5506)	Guanine nucleotide-binding protein subunit beta [*Fusarium oxysporum* f. sp. *cubense* race 1]	ENH75085.1	76%	0	98%		
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			MegaBLAST		*Fusarium* (taxid:5506)	*Fusarium oxysporum* fgb1 gene for guanine nucleotide-binding protein beta subunit, partial cds	AB072452.1	100%	0	95%		
Fcac	HF563555.1	Protein	BLASTx	nr	Fungi (taxid: 4751)	putative adenylate cyclase [*Fusarium fujikuroi*]	KLO87628.1	89%	0	99%	330884/338072	7189
					Fusarium (taxid:5506)	putative adenylate cyclase [*Fusarium fujikuroi*]	KLO87628.1	89%	0	99%		
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)	*Gibberella fujikuroi* ac gene for adenylate cyclase, strain IMI58289	HF563555.1	100%	0	95%		
			MegaBLAST		*Fusarium* (taxid:5506)	*Gibberella fujikuroi* ac gene for adenylate cyclase, strain IMI58289	HF563555.1	100%	0	95%		
Fcrho1	XM_018889260.1	Protein	BLASTx	nr	Fungi (taxid: 4751)	mitochondrial *Rho* GTPase 1 [*Fusarium oxysporum* f. sp. *cubense* tropical race 4 54006]	EXM09411.1	99%	0	99%	89545/91402	1858
					Fusarium (taxid:5506)	mitochondrial *Rho* GTPase 1 [*Fusarium oxysporum* f. sp. *cubense* tropical race 4 54006]	EXM09411.1	99%	0	99%		
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)	*Fusarium oxysporum* f. sp. *lycopersici* 4287 mitochondrial *Rho* GTPase 1 partial mRNA	XM_018380855.1	99%	0	96%		
			MegaBLAST		*Fusarium* (taxid:5506)	*Fusarium oxysporum* f. sp. *lycopersici* 4287 mitochondrial *Rho* GTPase 1 partial mRNA	XM_018380855.1	99%	0	96%		
FcpacC	XM_018893598.1	Protein	BLASTx	nr	Fungi (taxid: 4751)	pH-response transcription factor *pacC* [*Fusarium oxysporum* f. sp. *vasinfectum* 25433]	EXM37240.1	99%	0	98%	144218/145606	1389
					Fusarium (taxid:5506)	pH-response transcription factor *pacC* [*Fusarium oxysporum* f. sp. *vasinfectum* 25433]	EXM37240.1	99%	0	98%		
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)	*Gibberella fujikuroi pacC* gene for transcription factor *PACC*, exons 1-4	AJ514259.1	100%	0	96%		
			MegaBLAST		*Fusarium* (taxid:5506)	*Fusarium verticillioides* 7600 pH-response transcription factor *pacC*/RIM101 mRNA	XM_018893598.1	100%	0	96%		
All	-	Nucleotide	MegaBLAST	wgs	*Fusarium circinatum* (taxid:48490)	-	see text	100%	0	100%	-	-

BLAST2GO 4.1 software (https://www.blast2go.com/; required e-value: 10^{-10}). This software assigned the ontology of each gene using GO database (http://www.geneontology.org/page/go-database).

3 RESULTS AND DISCUSSION

In this study, five candidate pathogenicity genes were detected in the *F. circinatum* genome using *in silico* methods (i.e. *Fcfga1*, *Fcfgb1*, *Fcac*, *Fcrho1* and *FcpacC*; Table 1, Supplementary files S1, S2 and S3). The role that these gene products plays in cellular signaling pathways has already been addressed in other species of the genus *Fusarium*, but these candidate genes have not been previously described in *F. circinatum*. The proposed annotation was supported by gene ontology and results provided by BLAST2GO agreed with the suggested function of each putative gene (Table 2).

Minor differences in amino-acid sequence can affect the functionality of the protein. In consequence, high similarities between queries and translated proteins do not guarantee the same biological function. In this study, the similarities between proteins did not reach 100 %, nevertheless even in that case the annotation could not be directly assigned (Punta, & Ofran, 2008). The results reported here (i.e. high homology of sequences and ontology) supported high probability of the same functions between queries and translated proteins. Hence, definitive annotation will require either structural characterization of proteins or biological assays focused on gene expression.

The candidate genes *F. circinatum* putative guanine nucleotide-binding protein subunit alpha (*Fcfga1*) and beta (*Fcfgb1*) were identified in the reference genomic shotguns AYJV02000016.1 (genome of fungal strain FSP34) and JRVE01000002.1 (fungal strain GL1327), respectively (Table 1). Guo et al. (2016) studied the disruption effect of *fga2* and *fgb1* genes disruption in *F. oxysporum* f. sp. *cubense* and they found that when the encoding gene of Gα subunit (*fga2*) was silenced, the *in vivo* virulence resulted strongly reduced.

A putative adenylate cyclase (*Fcac*) was found in the genomic region of *F. circinatum* identified as JRVE01000018 (fungal strain GL1327. Table 1). In *Fusarium proliferatum* (Matsush.) Nirenberg either virulence (female fertility and *in planta* pathogenicity) or resistance factors (i.e.

TABLE 2. Best matches of gene ontology (based on GO database) for five candidate genes of *F. circinatum*.

Candidate gene	Domain	GO term description	GO term ID
Fcfga1	Molecular function	G-protein coupled receptor binding	GO:0001664
	Biological process	regulation of MAPK export from nucleus	GO:0071701
Fcfgb1	Molecular function	signal transducer activity	GO:0004871
	Biological process	heterotrimeric G-protein complex cycle	GO:0031684
Fcac	Molecular function	adenylate cyclase activity	GO:0004016
	Biological process	cAMP biosynthetic process	GO:0006171
Fcrho1	Molecular function	nucleic acid binding	GO:0003676
	Biological process	fungal-type cell wall biogenesis	GO:0009272
FcpacC	Molecular function	nucleic acid binding	GO:0003676
	Biological process	cellular response to alkaline pH	GO:0071469

thermo-tolerance and resistance against oxidative stress) were regulated by a homolog gene of *Fcac* called *Fpacy1* (Kohut et al., 2010).

The candidate gene in *F. circinatum* a putative GTP-binding protein (*Fcrho1*) was located in JRVE01000119.1 (fungal strain GL1327. Table 1). The loss of function of the possible orthologue *Rho1* in *F. oxysporum* reduced fungal virulence according to Martínez-Rocha et al. (2008). The last candidate gene analyzed was a putative pH signaling transcription factor (*FcpacC*), whose sequence was found in the genomic shotgun JRVE01000056.1 of *F. circinatum* (fungal strain GL1327. Table 1). Caracuel et al. (2003) reported that a plausible orthologue of *FcpacC* could repress the expression of some pathogenicity genes in alkaline conditions triggering the virulence factors in acidic environments (plant-fungus interface).

The metabolic pathways that drive the response of *F. circinatum* against external stimuli (e.g. host metabolites, nutrients, etc.) have not been deeply investigated. Consequently, the results proposed here could improve the knowledge about the basis of pathogenicity in this phylamentous fungus, contributing to better understand the etiology of Pine Pitch Canker disease.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

S1. Nucleotide sequences of five candidate genes in *Fusarium circinatum*.

S2. Amino acid sequences of five candidate genes in *Fusarium circinatum*.

S3. Alignments of candidate genes and reference ones as nucleotide sequences and protein.

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			MegaBLAST		*Fusarium* (taxid:5506)	*Fusarium verticillioides* 7600 pH-response transcription factor *pacC*/RIM101 mRNA	XM_018893598.1	100%	0	96%		
All	-	Nucleotide	MegaBLAST	wgs	*Fusarium circinatum* (taxid:48490)	-	see text	100%	0	100%	-	-

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	Biological process	fungal-type cell wall biogenesis	GO:0009272
FcpacC	Molecular function	nucleic acid binding	GO:0003676
	Biological process	cellular response to alkaline pH	GO:0071469