Design and characterization of sgRNAs aimed at the control of the phytopathogen *Pseudocercospora fijiensis* that causes Black Sigatoka

Diseño y caracterización de sgRNAs dirigidos al control del fitopatógeno *Pseudocercospora fijiensis* causante de la Sigatoka Negra

Projeto e caracterização de sgRNAs visando o controle do fitopatógeno *Pseudocercospora fijiensis* causador da Sigatoka Negra

Luis Moncayo[1]
Paulo Centanaro[2]
Diego Arcos-Jácome[2,8]
Alex Castro[2]
Cristina Maldonado[3]
Diego Vaca[4,8]
Gardenia González[8]
Carla Lossada[5]
Aleivi Perez[6]
Lenin González-Paz[7,8*]

Rev. Fac. Agron. (LUZ). 2022, 39(1): e223909
ISSN 2477-9407
DOI: https://doi.org/10.47280/RevFacAgron(LUZ).v39.n1.09

Crop Production
Associate editor: Dr. Jorge Vilchez-Perozo

[1]Universidad Católica de Cuenca, Ecuador.
[2]Universidad Agraria de Ecuador, Facultad de Ciencias Agrarias, Ecuador
[3]Laboratorio de Biotecnología de la Facultad de Ciencias Agropecuarias, Universidad Técnica de Babahoyo, Ecuador.
[4]Universidad Laica Eloy Alfaro de Manabí, Ecuador.
[5]Laboratorio de Caracterización Molecular y Biomolecular – Centro de Investigación y Tecnología de Materiales - Sección Microbiología Molecular y Biofísica. Instituto Venezolano de Investigaciones Científicas - Zulia. Maracaibo, Venezuela
[6]Laboratorio de Microbiología General, FEC-LUZ. Zulia, Maracaibo, Venezuela
[7]Laboratorio de Genética y Biología Molecular, FEC-LUZ. Zulia, Maracaibo, Venezuela. 526
[8]Doctorado en Ciencias Agrarias, Facultad de Agronomía, Universidad del Zulia, Zulia, Venezuela.

Received: 01-10-2021
Accepted: 08-11-2021
Published: 03-01-2022

Keywords:
CRISPR-Cas9
Banana
Crowding
Thermodynamics

Abstract

Black Sigatoka, caused by the fungus *Pseudocercospora fijiensis* (*Mycosphaerella fijiensis*) is an important disease of bananas and plantain. The design of sgRNAs molecules for gene silencing offers the possible control of this phytopathogen. The sgRNAs, are molecules that bind to enzymes to specifically edit genes of interest. The use of these molecules requires the use of bioinformatics tools for their study. Therefore, the objective of this research was to design and characterize sgRNAs to silence the *Fus*3 virulence gene and *CYP*51 gene growth in *P. fijiensis*, through the analysis of structural, thermodynamic and functional characteristics that allow to discriminate the sgRNAs candidates for control of the phytopathogen. Several thermodynamically stable sgRNAs with high specificity for the target genes were achieved, as well as with sequences easily recognizable by the SpCas9 nuclease, and with sizes that allow efficient diffusion in eukaryotic cytoplasms. The results suggest that all the designed and characterized sgRNAs can promote the correct silencing of the genes selected for the control of *P. fijiensis*. Additionally, the most optimal designs were identified, based on the characteristics considered in this study. These results, although they require additional studies to improve the technology, are promising as they show the possibility of using non-toxic and highly specific molecular tools in plant biotechnology for genetic improvement, directed mutagenesis, plant sanitation and control of phytopathogens.

*Corresponding author: lgonzalezpaz@gmail.com

Resumen

La Sigatoka Negra, causada por el hongo *Pseudocercospora fijiensis* (*Mycosphaerella fijiensis*) es una enfermedad importante del banano y plátano. El diseño de moléculas sgRNAs para el silenciamiento de genes ofrece un posible control de este fitopatógeno. Los sgRNAs son moléculas que se unen a enzimas para cortar de forma específica genes de interés. El aprovechamiento de estas moléculas requiere usar herramientas bioinformáticas para su estudio. Por lo que el objetivo de esta investigación fue diseñar y caracterizar sgRNAs para silenciar el gen de virulencia *Fus*3 y el gen de crecimiento *CYP51* en *P. fijiensis*, mediante el análisis de características estructurales, termodinámicas y funcionales que permiten discriminar los sgRNAs candidatos a control del fitopatógeno. Se obtuvieron diversos sgRNAs termodinámicamente estables y con alta especificidad para los genes diana, así como con secuencias fácilmente reconocibles por la nucleasa SpCas9, y con tamaños que permiten la difusión eficiente en citoplasmas eucariotas. Los resultados sugieren que todos los sgRNAs diseñados y caracterizados, pueden promover el correcto silenciamiento de los genes seleccionados para el control de *P. fijiensis*. Adicionalmente, se identificaron los diseños más óptimos, en función de las características consideradas en este estudio. Estos resultados, aunque requieren de estudios adicionales para perfeccionar la tecnología, son prometedores pues muestran la posibilidad de usar herramientas moleculares no tóxicas y de alta especificidad en biotecnología vegetal para el mejoramiento genético, mutagénesis dirigida, saneamiento vegetal y control de fitopatógenos.

Palabras clave: CRISPR-Cas9, banano, *crowding*, termodinámica.

Resumo

A Sigatoka Negra, causada pelo fungo *Pseudocercospora fijiensis* (*Mycosphaerella fijiensis*), é uma doença importante da banana e da banana-da-terra. O desenho de moléculas de sgRNAs para silenciamento de genes oferece um possível controle deste fitopatógeno. sgRNAs são moléculas que se ligam a enzimas para cortar especificamente genes de interesse. O uso dessas moléculas requer o uso de ferramentas de bioinformática para seu estudo. Portanto, o objetivo desta pesquisa foi projetar e caracterizar sgRNAs para silenciar o gene de virulência *Fus*3 e o gene de crescimento *CYP51* em P. fijiensis, por meio da análise de características estruturais, termodinâmicas e funcionais que permitem discriminar sgRNAs candidatos para controlar fitopatógenos. Vários sgRNAs termodinamicamente estáveis foram obtidos com alta especificidade para os genes alvo, bem como sequências facilmente reconhecíveis pela nuclease SpCas9, e com tamanhos que permitem difusão eficiente em citoplasmas eucarióticos. Os resultados sugerem que todos os sgRNAs projetados e caracterizados podem promover o correto silenciamento dos genes selecionados para o controle de *P. fijiensis*. Além disso, os designs mais ideais foram identificados, com base nas características consideradas neste estudo. Esses resultados, embora necessitem de estudos adicionais para o aprimoramento da tecnologia, são promissores, pois mostram a possibilidade do uso de ferramentas moleculares atóxicas e altamente específicas em biotecnologia vegetal para melhoramento genético, mutagênese dirigida, saneamento vegetal e controle de fitopatógenos.

Palavras chave: CRISPR-Cas9, banana, aglomeração, termodinâmica.

Introduction

Black Sigatoka, caused by the fungus *Pseudocercospora fijiensis* (*Mycosphaerella fijiensis*) is one of the most important diseases of banana and plantain from the economic point of view, causing a decrease of more than 40% of the yield (Díaz-Trujillo *et al.,* 2018). The control of the disease is carried out mainly through the extensive application of chemical fungicides, which causes a negative environmental impact (Escobar-Tovar *et al.,* 2015).

Interestingly, a genetic system applicable to control pathogens has been described in bacteria and is based on the use of non-coding RNA molecules or simple RNA guides (sgRNAs, single guide RNAs) that direct nucleases of the CAS family, such as Cas9, encoded by genes associated with CRISPR (clustered regularly interspaced short palindromic repeats) or *cas* genes, to form a ribonucleoprotein complex (RNP) made up of Cas9-sgRNA that specifically cuts genetic material thanks to the recognition of sequences called PAM (protospacer adjacent motif) (Kocak *et al.,* 2019). This system has been proposed as a tool for programmable gene and genome editing (Campenhout *et al.,* 2019). This has created experimental opportunities and ethical challenges since the technique can produce off-target cuts (off-target events) (Bartkowski *et al*., 2018). However, the study of CRISPR/Cas9 systems is important because they are precursors of next generation tools for genetic manipulation (Belhaj *et al.,* 2015).

The sgRNAs can be designed to inactivate genes of importance in pathogens. For example, the *CYP51* gene encodes for cytochrome P450 14α-demethylase, a key enzyme in the biosynthesis of ergosterol in *M. fijiensis* Morelet, so its inhibition can prevent the formation of cell membranes and the growth of fungi (Ma and Tredway, 2013). El gen *Pfcyp51* de *P. fijiensis* ha sido asociado a la resistencia a los inhibidores de 14α-desmetilasa (DMI), de modo que, su silenciamiento contribuye a la recuperación de la sensibilidad a DMI (Díaz-Trujillo *et al*., 2018; Chong *et al.,* 2019). The *Fus3* gene regulates invasion pathways such as the formation of infection, sporulation, invasive and filamentous growth structures of phytopathogenic fungi (Onyilo *et al*., 2018). The *Fus3* gene of *P. fijiensis* has been experimentally silenced, achieving reduced virulence, as well as decreased invasive growth (Onyilo *et al*., 2018).

CRISPR-Cas9 technology has already been applied to silence genes of banana pathogens (Tripathi *et al*., 2019). Likewise, it has been used in plants for plant improvement purposes (Zaynab *et al*., 2020). However, little progress has been made in *P. fijiensis* (Escobar-Tovar *et al*., 2015). Filamentous fungi such as *P. fijiensis* are more complex to genetically manipulate due to their morphology, cell differentiation, membranes, and thick chitinous cell walls (Jiang *et al*., 2013). Although some species have been manipulated, the technology is still under development (Estrela and Cate, 2016). On the other hand, RNAi has been proposed for the silencing of virulence and growth genes in *P. fijiensis* (Mumbanza *et al*., 2013) and in related pathogens (Koch *et al*., 2013), and additionally, efforts have been made to introduce CRISPR-Cas9 systems in various filamentous fungi with promising results (Estrela and Cate, 2016)

To use these systems it is necessary to consider factors such as possible cuts in off-target sites (Tripathi *et al*., 2019), thermodynamic stability (Kocak *et al*., 2019) and even the size of said molecules by the cytoplasmic congestion phenomenon (Dupuis *et al*., 2014). The latter is a novel aspect little considered (Kocak *et al*., 2019). Although all these parameters must be estimated for the validation and optimization of sgRNAs designed for the study and control of *P.*

fijiensis (Escobar-Tovar *et al.,* 2015; Díaz-Trujillo *et al*., 2018). In this sense, a comparative analysis of designed sgRNAs targeting genes of interest in *P. fijiensis* was carried out, studying functional, structural and biophysical aspects in order to characterize and discriminate candidate sgRNAs for CRISPR-Cas9 tools.

Materials and methods

Data sources and Design of sgRNAs for the control of *P. fijiensis*

The genomic sequence used in this study for the design of sgRNAs was obtained from DOE JGI (http://www.jgi.doe.gov) and NCBI (http://www.ncbi.nlm.nih.gov/). Two target genes were selected, *Fus3* (Xu, 2000) and *CYP51* (Podust *et al.,* 2001), associated with virulence and cell division in *P. fijiensis*, respectively. For the design, the CRISPOR program (http://crispor.tefor.net/) was used, which includes the genome of *P. fijiensis* CIRAD86-NCBI GCF_000340215.1. *Streptococcus pyogenes* Cas9 (SpCas9) with PAM recognition sequence: "NGG" in the 3 'sense was used as nuclease (Campenhout *et al.,* 2019).

Characterization of the designed sgRNAs

Each sgRNA, including the PAM motif, was compared to the *P. fijiensis* genome using DOE JGI's BLASTN program (http://www.jgi.doe.gov) to identify potential off-target sites. The results of the DOE JGI will be compared with the possible non-destination sites offered by the CRISPOR algorithm. The secondary structures of sgRNA were predicted with RNA fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/barriers.cgi) as well as the number of possible suboptimal structures by calculating the folding energy (*K*) of RNAs using the barriers web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/barriers.cgi). The Gibbs minimum free energy thermodynamic parameters for formation (ΔG), entropy (ΔS), enthalpy (ΔH) and melting temperature (Tm) were obtained with mfold (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). In all cases, the default parameters were considered. % GC, molecular mass (MW) and regions of maximum flexibility were determined using the Unipro UGENE-v.1.32.0 software.

The sgRNA size was determined by calculating the solvent accessible surface area with the Shrake-Rupley algorithm (SASA method, solvent accessible surface area) (Dupuis *et al*., 2014). To determine the diffusion coefficient (*D*) of sgRNAs in a model of high and normal degree of crowding (Regan *et al*., 2018). The Stokes-Sutherland-Einstein (SSE) equation was used, which establishes the diffusion relationship between the viscosity of the cytoplasm, the thermal energy and the size of the molecule. The coefficient *D* was calculated using accepted cell models such as HeLa (cytoplasmic viscosity of ≈ $4.4x10^{-2}$ Pa.s^{-1} a 37º C) and the Swiss 3T3 normal cell model (viscosity of $2.4x10^{-2}$ Pa.s^{-1} a 37º C).

Complementary analysis

Simple and multiple correlation analyzes were applied (as the case may be) to look for co-linearities in the variables considered, and to be able to reduce them and / or propose predictive models. The t test (Student's) was also used to corroborate the difference in the means of the sgRNA groups designed and treated together with descriptive statistics. Discriminant multivariate analysis was also used, after treating primary variables in search of orthogonality, in order to have only predictor variables to classify the sgRNAs and to be able to explain the membership of each designed molecule to one or another pre-established design group. Multivariate normality and homoscedasticity tests were applied. The Microsoft Excel 2010 application and the SPSS statistical package (IBM SPSS Statistics 23) (George and Mallery, 2016) were used.

Results and discussion

Design of sgRNAs for the control of *P. fijiensis*

From the possible guide sequences calculated for the *Fus3* and *CYP51* genes, three of the best predicted sgRNAs were chosen for each gene, taking into account those that presented the highest CRISPOR score and that did not exhibit off-target. The sgRNAs were designated as sgRNA-F1, F2 and F3; and sgRNA-C1, C2 and C3; for sgRNAs designed for the *Fus3* gene (designs F) and *CYP51* (designs C), respectively (figure 1). The specificity mediated by the lack of off-target was corroborated after the use of the DOE JGI alignment tools, finding that the sequences chosen as potential sgRNA do not present homology with other sequences outside the regions of interest, promoting only the cleavage of the sequences homologous to sgRNAs designed from the *P. fijiensis* genome.

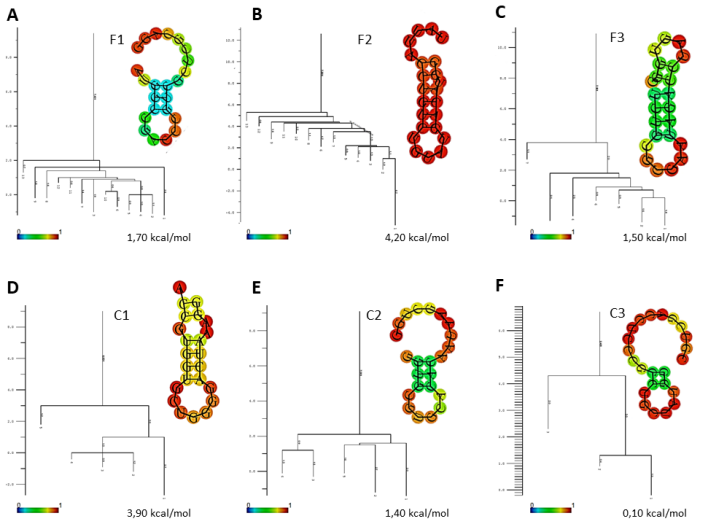

Figure 1. Prediction of the secondary structure of the designed sgRNAs and their energy folding kinetics. The A) sgRNA-F1, B) F2 and C) F3, and D) sgRNA-C1, E) C2 and F) C3 are shown, for the sgRNAs designed for the silencing of the Fus3 gene (designs F) and CYP51 (designs C), respectively. As well as the Gibbs minimum free energy for its formation (ΔG) in kcal.mol^{-1} and the energy folding kinetics (plotted by tree diagrams indicative of the number of possible sub-optimal minimum energy structures) next to each one of the sgRNA molecules designed in this study. The bar with the colorimetric gradient from 0 (blue) to 1 (red) at the bottom of each figure represents the probability of the correct base pairing and position.

The predominant PAM sequences in the designs were "TGG" (3/6) for the sgRNA-F2, C2 and C3, and "AGG" (2/6) in the sgRNA-F1 and C1, and only the sgRNA-F3 presented the PAM sequence "GGG" (1/6) (table 1). The importance of designing sgRNA to inactivate (knockout) the *Fus3* and *CYP51* genes for the control of *P. fijiensis* lies in the fact that this phytopathogenic fungus is the cause of Black Sigatoka, one of the most significant diseases of banana and plantain. These genes are important for the virulence and growth of this pathogen (Xu, 2000; Podust *et al.,* 2001), at the same time that the strategy of blocking genetic determinants has already been tested in related organisms (Mumbanza et al., 2013; Escobar-Tovar *et al.*, 2015; Díaz-Trujillo *et al*., 2018). However, many sgRNAs can be inefficient in achieving knockout as a result of genetic variability, which plays an important role in the complementarity and/or recognition of the target by sgRNAs (Scott and Zhang, 2017).

Table 1. Thermodynamic characteristics of sgRNAs directed at *P. fijiensis*.

Design/Target	PAM	Off-target	ΔG (kcal.mol^{-1})	ΔH (kcal.mol^{-1})	ΔS (cal.K^{-1}*mol^{-1})
sgRNA-F15'-ATGGCCGACCCGTCGCTCGC-3'	AGG	0	- 1.7	- 51.5	- 160.5
sgRNA-F2 5'-TATTACCTGTCTGTATGACA-3'	TGG	0	- 4.2	- 64.8	- 195.3
sgRNA-F3 5'-TCTGCGCGAATAGACCTAGT-3'	GGG	0	- 1.5	- 52.4	- 164.1
sgRNA-C1 5'- CCGTGGTGCTGGGGACTAA-3'	AGG	0	- 3.9	- 65.8	- 199.5
sgRNA-C2 5'- GTGCGTGCACACAATAAGC-3'	TGG	0	- 1.4	- 36.2	- 112.2
sgRNA-C3 5'-CGTCGACCTCCCGCCTGCTA-3'	TGG	0	- 0.1	- 16.6	- 53.2

The thermodynamic characteristics of the sgRNAs designed for the control of *P. fijiensis* are presented; sgRNA-F1, F2 and F3 (molecules designed for the silencing of the *Fus3* gene); sgRNA-C1, C2 and C3 (molecules designed for the silencing of the *CYP51* gene); PAM: nucleotide sequence or motif recognized by nuclease to generate cuts; Off-target: number of cuts outside the sequence of interest; ΔG: minimum Gibbs free energy for the formation of the structure; ΔH: enthalpy contribution to the formation energy; ΔS: entropic contribution to the formation energy.

In this study, the three best designs offered by the CRISPOR software were selected, to guarantee a greater probability of excision of the gene of interest (Liang *et al.,* 2017). Because one of the main problems behind the design of sgRNA is represented by off-target, a phenomenon product of genetic polymorphisms (Scott and Zhang, 2017), which causes various unwanted events (Zhang *et al*., 2015; Tripathi *et al.,* 2019). Therefore, as off-target sites are not found in this study from the selected sgRNAs, we are in the presence of an important result indicative that the CRISPOR algorithm is capable of offering specific guides, a promising fact in the constant search to reduce the off-target (Zhang *et al*., 2015). Much of the efficiency of the guides is given by the PAM sequences, as they are the specific nuclease recognition regions. These sequences are diverse, however, in this study, the pool of selected sequences corresponds to PAM typical of the Cas9 enzyme, specifically, sequences recognizable by SpCas9 (Campenhout *et al*., 2019).

Characterization of the designed sgRNAs

sgRNA with secondary structures typical of CRISPR-associated primary transcripts with "stem-loop" conformations were predicted. These structures presented a ΔG with a mean in terms of absolute value of -2.13 kcal.mol^{-1}. The mean ΔG for the Fus3 gene was -2.46 kcal.mol^{-1} and for *CYP51* it was -1.80 kcal.mol^{-1}. In the case of sgRNAs for the *Fus3* gene, the minimum ΔG was -1.50 kcal.mol^{-1} (sgRNA-F3) and a maximum of -4.20 kcal.mol^{-1} (sgRNA-F2). While for the *CYP51* gene, the minimum ΔG was -0.10 kcal.mol^{-1} (sgRNA-C3) and maximum -3.90 kcal.mol^{-1} (sgRNA-C1). Therefore, all the sgRNAs presented a ΔG <0 (figure 1, table 1). No significant difference (p> 0.05) was found between the ΔG presented by the sgRNA groups studied.

The sgRNAs present very stable secondary structures, with ΔG for their formation thermodynamically favored, as was reported for similar native CRISPR-type structures (Li *et al*., 2017). The thermodynamic stability of sgRNAs is a critical aspect to guarantee the success of CRISPR systems (Kuan *et al*., 2017). It has been shown that the thermodynamic stability of the "stem-loops" can increase the specificity of the designs in several orders of magnitude of the SpCas9, especially if the ΔG is between 0 and -10 kcal.mol^{-1}, range in which were distributed all the sgRNAs designed in this study (Kocak *et al*., 2019). This stability depends on the length of the stems, the longer the stem, the greater the stability of the structure (Li *et al*., 2017). A characteristic shared by the sgRNAs analyzed, and that explains why there was a high correlation between the size expressed in r and ΔG of the sgRNAs, with a coefficient of 0.68.

The folding energy of each design determined that there is a statistically significant difference (p <0.05) between the means of the possible sub-optimal structures predicted for the guides. The sgRNAs presented a mean value of ≈128 possible minimum energy structures. With a minimum of 41 possible conformations for sgRNA-C3 and a maximum of 266 for sgRNA-F2. All the guides directed to the *Fus3* gene present between 140-266 possible structures of minimum energy, with the sgRNA-F3 being the one with the lowest probability of change (≈140), while for the *CYP51* gene the range of the folding kinetics of its guides was between 41 to 80 possible structures (figure 1, table 1).

In relation to the thermodynamic parameters that contribute to the ΔG, it was found that there is no statistically significant difference (p> 0.05) between the groups of sgRNA studied and that the mean in terms of the absolute value of the entropic contribution (ΔS) in the designs it was -147.47 cal.K^{-1}.mol^{-1}, an enthalpic contribution (ΔH) of -47.88 kcal.mol^{-1} and a melting temperature (Tm) of 49.45º C. Specifically, the mean ΔS for the *Fus3* gene was -173.3 cal.K^{-1}.mol^{-1}, with a ΔH of -56.23 kcal.mol^{-1} and a Tm of 50.66º C. While the thermodynamic contributions for the designs directed to the *CYP51* gene were a ΔS = -121.63 cal.K^{-1}.mol^{-1}, ΔH = -39.53 kcal.mol^{-1} and a Tm = 48.23º C. The design that presented the greatest entropic contribution for the *Fus3* gene was the sgRNA-F2 with a ΔS = -195.3 cal.K^{-1}.mol^{-1}, while for *CYP51* it was the sgRNA-C1 with an ΔS = -199.5 cal.K^{-1}.mol^{-1}, however, all designs targeting these genes presented ΔS> ΔH, with ΔS <0 and ΔH <0.

The mean Tm calculated for all designs was 49.45º C. The Tm of the guides for the *Fus3* gene was 50.66º C and for *CYP51* it was 48.23º C. The sgRNA-F2 and sgRNA-C1 were the designs that presented the highest Tm, this being 58.4º C and 56.5º C, respectively. It should be noted that all sgRNAs presented Tm> 45º C, except for sgRNA-C3 (table 2). The thermodynamic stability determined is a reflection of enthalpic and entropic contributions

and melting temperatures, parameters that are strongly correlated with coefficients between 0.85 and 1.00, and show that sgRNAs present unimolecular folding with a diverse number of possible variations. conformational at 37º C, depending on the nucleotide sequences and according to the calculations of the energy folding kinetics. As reported, the sgRNA melting temperature (Tm) seems to have little impact on the activity of the guide, except in the guides with very high Tm, which are slightly less active (Brendan *et al.,* 2020). Although designs such as sgRNA-C3 can achieve a stable conformation faster compared to the rest of the guides, by presenting only ≈41 conformations, it was determined that all sgRNAs are structures assembled by exothermic processes, with structural tension, rigidity, little rotation, vibration and movement of electronic charge, aspects that favor stability and coupling with Cas9 (Kocak *et al*., 2019). This explains the correlation between all the thermodynamic parameters and the parameters associated with the size (*r*) and dynamics (*D*) of the sgRNA, with correlation coefficients of up to 0.90.

The mean GC content for the sgRNAs was 59.42%, with a molecular weight of 7209.59Da. The GC content of the designs directed to Fus3 presented a mean value of 56.52%, and a mean molecular weight of 7199.59 Da. On the other hand, the designs for CYP51 presented a %GC of 62.32 and a molecular weight of 7219.59 Da. Being the sgRNA-F1 and sgRNA-C3, the designs with the highest %GC, while the highest molecular weight was calculated from the sgRNA-F3 and sgRNA-C1 (table 2). A high GC content is important because it stabilizes the RNA-DNA duplex and destabilizes the off-targets (Ren *et al.,* 2014).

The difference between the molecular weight of the designs was ≈20 Da. No statistically significant differences ($p > 0.05$) were found in these determined parameters in the sgRNA groups studied, and regions of maximum flexibility were not predicted. These results show that the use of bioinformatics tools makes possible the selection of specific sgRNAs, thermodynamically stable and with suitable biophysical and molecular parameters. Aspects associated with editing specificity and efficacy (Kuan *et al.,* 2017).

The sgRNAs presented an r with a mean value of 3.172 Å, equivalent to 6.344 Å in diameter, without significant differences ($p > 0.05$). The smallest size was calculated for the sgRNAs directed to *CYP51* with a mean of r = 2.451 Å, while for *Fus3* it was r = 3.892 Å. The smallest sgRNAs for each gene were sgRNA-F2 (*r* = 3.796 Å) and sgRNA-C3 (*r* = 0.821 Å) (table 2). The difference in size between the groups (sgRNA targeting *Fus3* versus *CYP51*) had a mean value of just *r* = 1.441 Å (approximate radius of the water atom). From the calculated sizes it was possible to determine that the *D* coefficient of the sgRNAs in the congested HeLa cytoplasm (high crowding) is ≈ 2.255×10^{-18} $\mu m^2.s^{-1}$ and in the normal cytoplasm 3T3 (normal crowding) of *D* = ≈ 4.131×10^{-18} $\mu m^2.s^{-1}$. With a mean for the guides directed to the *Fus3* gene of *D* = 1.328×10^{-18} $\mu m^2.s^{-1}$ in HeLa and a *D* = 2.433×10^{-18} $\mu m^2.s^{-1}$ in 3T3. On the other hand, the values for the sgRNAs designed for *CYP51* were *D* = 2.433×10^{-18} $\mu m^2.s^{-1}$ for HeLa and *D* = 5.828×10^{-18} $\mu m^2.s^{-1}$ for 3T3. The guide with the best *D* coefficient in HeLa for the *Fus3* gene was specifically the sgRNA-F2 with a *D* = 1.361×10^{-18} $\mu m^2.s^{-1}$, and for *CYP51* the sgRNA-C3 (D = 6.300x10-18 $\mu m^2.s^{-1}$). While the guides with the best *D* coefficients under 3T3 were sgRNA-F2 (*D* = 2.494×10^{-18} $\mu m^2.s^{-1}$) and sgRNA-C3 (*D* = 1.153×10^{-17} $\mu m^2.s^{-1}$) (table 2). Although no statistically significant differences were found ($p > 0.05$) between the calculated *D* coefficients, the sgRNA-C3 presented the best coefficients in both HeLa and 3T3. Knowing the size of the guide molecules is important because it has been described that cytoplasms can cause changes of up to $\Delta G \approx -2.5$ $kcal.mol^{-1}$ (Dupuis *et al*., 2014). A high correlation between *r* and *D* was predicted in the sgRNAs, with a coefficient of 0.96. Therefore, when obtaining sgRNAs with *D* coefficients in the order of ≈10-18 $\mu m^2.s^{-1}$ in HeLa, it can be inferred that the sgRNAs considered exhibit a three-dimensional diffusion similar to Cas9 (Knight et al., 2015).

The number of possible energetically feasible sub-optimal conformations or structures (*k*) of each sgRNA, turned out to be the independent variable that best allows to differentiate the groups of designs in a significant way ($p < 0.05$). An increase in *k* (above the mean) will make it more likely that a sgRNA will obtain a positive score and, thus, that it will conform to the sgRNA pattern typical of designs targeting the *Fus3* gene in a significant way. Additionally, the structure matrix generated by the multivalent analysis excluded

Table 2. Structural and functional characteristics of guide RNAs aimed at controlling *P. fijiensis*.

Design/Target	T_m (°C)	%GC	MM	*r* (Å)	D_{HeLa} ($\mu m^2.s^{-1}$)	D_{3T3} ($\mu m^2.s^{-1}$)	*K*
sgRNA-F1 5'-ATGGCCGACCCGTCGCTCGC-3'	47.5	73.91	7167.53	3.94	1.31×10^{-18}	2.40×10^{-18}	164
sgRNA-F2 5'-TATTACCTGTCTGTATGACA-3'	58.4	39.13	7185.62	3.80	1.36×10^{-18}	2.49×10^{-18}	266
sgRNA-F3 5'-TCTGCGCGAATAGACCTAGT-3'	46.1	56.52	7245.63	3.94	1.31×10^{-18}	2.40×10^{-18}	140
sgRNA-C1 5'- CCGTGGTGCTGGGGACTAA-3'	56.5	60.87	7310.67	3.80	1.36×10^{-18}	2.49×10^{-18}	79
sgRNA-C2 5'- GTGCGTGCACACAATAAGC-3'	49.4	56.52	7254.64	2.74	1.89×10^{-18}	3.46×10^{-18}	80
sgRNA-C3 5'-CGTCGACCTCCCGCCTGCTA-3'	38.8	69.57	7093.48	0.82	6.30×10^{-18}	1.15×10^{-17}	41

The structural and functional characteristics of the sgRNAs designed for the control of P. fijiensis are shown; sgRNA-F1, F2 and F3 (molecules designed for the silencing of the Fus3 gene); sgRNA-C1, C2 and C3 (molecules designed for the silencing of the CYP51 gene); Tm: melting temperature of guide RNAs; % GC: guanine and cytosine contents; MM: molecular mass; r: radius of the RNA molecule; DHeLa: diffusion coefficient of guide RNA in a cytoplasm considered congested (high crowding) for this study from the HeLa cell line; D3T3: diffusion coefficient of guide RNA in a cytoplasm considered normal (low crowding) for this study from the 3T3 cell line; K: number of possible sub-optimal structures of minimum energy.

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the rest of the variables for not providing relevant classificatory information. It is recommended to apply this multivariate statistical model with a greater diversity of sgRNAs, to obtain other discriminant functions or to corroborate the weight of the function predicted here.

Conclusions

Optimal sgRNAs could be designed and identified using bioinformatic tools based on structural, thermodynamic and functional characteristics. The methods used to improve the efficiency of sgRNAs point to sgRNA-F3 and sgRNA-C3 as the molecules with the most optimal characteristics for the knockout of *Fus3* and *CYP51* in *P. fijiensis*. Likewise, the number of possible conformations has an important predictive weight to differentiate between suitable sgRNAs for *P. fijiensis*. These results, although preliminary and require more studies, are promising because they show the possibility of using non-toxic alternatives for genetic improvement, and specific control of plant diseases, as more research is carried out.

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