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"Genome editing for crop improvement: Challenges and opportunities"

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Los estudios presentados en esta tesis fueron realizados en el Centro de Investigação de Montanha da Escola Superior Agrária del Instituto Politécnico de Bragança.

Desde os primórdios da Humanidade, o Homem tem-se deslumbrado com a beleza da vida e com os mistérios do mundo que o rodeia. Nós, seres humanos, preocupámo-nos em compreender o passado e ficamos intrigados com o futuro, de algum modo, isso torna-nos únicos. "De onde viemos?", "Para onde vamos?" será que "Estamos sozinhos?". Estas, são as três grandes questões fundamentais às quais a Humanidade tem tentado responder ao longo de toda a sua existência. Hoje, graças a todos os avanços que temos conseguido alcançar enquanto espécie e sobretudo à descoberta da ciência, podemos finalmente tentar responder a essas questões utilizando uma abordagem científica. Ainda que não consigamos obter respostas claras ou entender completamente o nosso mundo e os fenómenos que nele ocorrem, estas questões continuarão a fazer parte do que nos torna humanos.

Patrick Ferreira

"O universo é tão extraordinário
Que apenas Deus o poderia ter criado
A minha tarefa é apenas perceber como ELE o fez".

Albert Einstein

A vida pode é feita de momentos memoráveis com pessoas inesquecíveis, pessoas essas que agora chegando ao fim de mais uma etapa muito importante na minha vida, não me podia esquecer de agradecer. Estas pessoas aqui mencionadas contribuíram de forma direta ou indireta para o meu sucesso ao longo deste Doutoramento. Sendo assim, gostaria de agradecer:

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Índice

Resumen

En Castelhano

Esta tesis doctoral, titulada "Edición del genoma para la mejora de cultivos", explora el potencial de la tecnología de edición del genoma para mejorar los cultivos, en particular aquellos de alto interés económico. Se publicaron cuatro artículos científicos que abordan diferentes aspectos de este tema. El primer artículo revisó las herramientas de edición del genoma, centrándose en las ventajas de CRISPR/Cas9. El segundo artículo enfatizó el papel de los ncRNA largos en la regulación genética de las plantas. Tras recopilar toda la información, se utilizó la técnica RNAi para analizar los genes *GIP* y *NPP1* en *Phytophthora cinnamomi*, evitando así los riesgos de la edición del genoma (la destrucción asociada a CRISPR). La enfermedad de la tinta se considera una de las causas más importantes que contribuyen al declive de los huertos de castaños. La reducción del rendimiento de *Castanea sativa Mill* se puede atribuir a dos especies principales: *Phytophthora cinnamomi* y *Phytophthora cambivora*, siendo la primera el principal patógeno responsable de la enfermedad de la tinta en Portugal. *P. cinnamomi* es un patógeno vegetal muy agresivo y ampliamente distribuido, capaz de infectar a casi 1.000 especies hospedadoras. Este oomiceto causa importantes pérdidas económicas y es responsable de la disminución de numerosas especies de plantas en Europa y en todo el mundo. Los dos artículos restantes abordaron la lucha contra *P. cinnamomi* utilizando ARNi para silenciar los genes *NPP1* y *GIP*. Silenciar estos genes imposibilitará o reducirá la producción de las proteínas NPP1 y GIP, responsables de la aparición de la enfermedad de la tinta. Para silenciar parte de la región codificante de los genes *NPP1* y *GIP* se utilizó el vector integrativo pTH210. La integración del casete de silenciamiento se confirmó mediante PCR y secuenciación en transformantes de *P. cinnamomi* resistentes a higromicina. Los transformantes obtenidos con el silenciamiento génico se utilizaron posteriormente para infectar *Castanea sativa*, lo que permitió evaluar los efectos del silenciamiento génico sobre el fenotipo de la planta. Las publicaciones demuestran colectivamente el potencial de la edición de genes para la mejora de cultivos, haciendo hincapié en el ARNi y CRISPR/Cas9, pero también destacando la importancia de los ncRNA en la regulación genética y ofreciendo estrategias para combatir los patógenos. Combatir este tipo de fitopatógeno aumenta el rendimiento de los cultivos y, en última instancia, beneficia la seguridad alimentaria y la sostenibilidad mundial.

In English

This doctoral thesis, entitled "Genome Editing for Crop Improvement", explores the potential of genome editing technology to improve crops, particularly those of high economic interest. Four scientific articles were published addressing different aspects of this topic. The first article reviewed genome editing tools, focusing on the advantages of CRISPR/Cas9. The second article emphasized the role of long ncRNAs in gene regulation in plants. After gathering all the information, the RNAi technique was used to analyze the *GIP* and *NPP1* genes in *Phytophthora cinnamomi*, thus avoiding the risks of genome editing (the destruction associated with CRISPR). Ink disease is one of the most significant causes contributing to the decline of chestnut orchards. The reduction in *Castanea sativa Mill* yield can be attributed to two main species: *Phytophthora cinnamomi* and *Phytophthora cambivora*, the first being the main pathogen responsible for ink disease in Portugal. *P. cinnamomi* is a highly aggressive and widely distributed plant pathogen, capable of infecting nearly 1,000 host species. This oomycete causes substantial economic losses and is responsible for the decline of numerous plant species in Europe and worldwide. The remaining two articles addressed combating *P. cinnamomi* using RNAi to silence the *NPP1* and *GIP* genes. Silencing these genes will either make it impossible or reduce the production of the proteins NPP1 and GIP, which are responsible for the onset of ink disease. To silence part of the coding region of the *NPP1* and *GIP* genes, the integrative vector pTH210 was used. The integration of the cassette was confirmed by PCR and sequencing in hygromycin-resistant *P. cinnamomi* transformants. The transformants obtained with gene silencing were later used to infect *Castanea sativa*, allowing the effects of gene silencing on the plant's phenotype to be evaluated. The publications collectively demonstrate the potential of gene editing for crop improvement, emphasizing RNAi and CRISPR/Cas9, but also highlighting the importance of ncRNAs in gene regulation and offering strategies to combat pathogens. Combating this type of phytopathogen increases crop yields and, ultimately, benefits global food security and sustainability.

1. Introducción

Man's desire to improve plants is not something new; approximately 10,000 years ago, when the domestication of plants began, man was already trying to favor the appearance of certain characteristics in plants to the detriment of others through conventional methods, with the aim, for example, of developing new crop varieties. Much of the development of modern society occurred mainly thanks to this conventional plant breeding, which significantly increased the quantity and quality of food available to humanity [1].

Pre-genomic improvement programs, that is, those essentially guided by mutagenesis through chemical compounds or irradiation, intergeneric crossings, and translocation reproduction to allow the selection of the desired mutations, present some disadvantages, including **the lack of specificity** [2]. What often happens is that large portions of the genome are transferred instead of a single gene; another very common case is the mutation of thousands of nucleotides instead of one. It was to overcome these problems that several transgenic breeding programs emerged at the end of the 20th century [1]. Recently, the **post-genomic** era emerged, which allowed rapid access to very valuable data from a biological point of view, namely, access to the genomic sequences of various plant crops, thus revolutionizing **genetic improvement programs**. Complete genome sequencing, transcriptome sequencing, identification of small nucleotide polymorphisms (SNPs) and other molecular markers have enabled the creation of genetic maps of numerous plant species of agronomic importance [3].

In recent years, the number of tools and techniques that allow adding, removing, or altering genetic material at specific locations in the genome has increased significantly [4, 5]. RNA molecules perform several important tasks in cells, such as transporting genetic information (rRNA - reading the genetic code, and tRNA, protein synthesis). Contrary to what was thought, recently several studies [5–11] have demonstrated the importance that non-coding RNAs have in the performance of numerous genome protection functions against viruses and transposable elements but also in the regulation of gene expression. Some of these newly discovered RNAs will be discussed throughout the introduction of the thesis.

The thesis begins with a literature review article published during the doctorate on the CRISPR/Cas9 technique (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9), known for its simplicity, efficiency, and cost-effectiveness in gene editing. Throughout this review, several topics have been addressed, including the history of the discovery of CRISPR/Cas9 technology, its biological role in the adaptive immune system of bacteria, its mechanism of action, and its ability to edit the genes and genomes of economically important plant species. Furthermore, the advantages of CRISPR/Cas9 were also discussed compared to some previously developed techniques such as RNA interference (RNAi), zinc finger nucleases (ZFNs), and effector domain transcription activating nucleases (TALENs), highlighting their potential applications. In medicine, pharmaceuticals, targeted therapy and improvements in agriculture and the environment [12–15]. Then, a brief introduction to gene expression mediated by non-coding RNAs (ncRNAs) and a literature review publication with a focus on long non-coding RNAs (lncRNAs) are presented. These previously little-known molecules are now recognized as crucial regulators of gene expression and epigenetic processes in eukaryotes, with significant implications for plant biology, especially in the context of crop resistance.

During the doctorate, two bibliographical reviews were published with the aim of analyzing the different genetic manipulation techniques and allowing a more effective fight against the infection process of the phytopathogen *Phytophthora cinnamomi*. The *Phytophthora cinnamomi* species is a threat to several species of plants and trees, particularly chestnut trees (*Castanea sativa*). This plant pathogen is responsible for “ink disease”, a disease that causes serious damage to chestnut trees and has major economic implications, especially in Europe, where chestnuts are a prominent crop. The *P. cinnamomi* infection affects both fruit production and chestnut wood quality, resulting in significant economic losses. The spread of this pathogen occurs fundamentally through water in poorly drained soils, where water-borne sporangia can infect tree roots [16].

Throughout the PhD, two laboratory works were published that highlight the application of the RNAi genomic technique in the interaction between *Phytophthora cinnamomi* and *Castanea sativa*.

In the first article, the investigation focused on the characterization of the *NPP1* gene from *Phytophthora cinnamomi*, responsible for inducing necrosis in chestnut roots. The RNAi was used to silence the *NPP1* (Phytophthora necrosis inducing protein 1) gene, and the objective was to analyze whether there was any significant reduction in disease symptoms in plants infected with *P. cinnamomi* transformants carrying the silenced gene. In the second laboratory article, the investigation focused on the characterization of the Glycanase Inhibitory Protein (GIP) produced by *P. cinnamomi* during chestnut infection. Once again, the RNAi technique was used to suppress the *GIP* gene of *P. cinnamomi*, and the resulting transformants were used to infect *C. sativa*, allowing the evaluation of the effects of gene silencing on the host plant phenotype. This approach intends to contribute some relevant information about the molecular mechanisms underlying the interaction between *Phytophthora* and chestnut trees.

1.1. RNA interference

Eukaryotic cells have an evolutionarily conserved natural gene regulation system called **RNA interference**. Some authors mention that this system was developed to provide protection to cells against foreign DNA invaders while also helping to maintain genomic stability, epigenetic modification and regulation of transposons and control of cellular transcription processes [17, 18].

In 2006, Fire and Mello received the Nobel Prize in Physiology or Medicine. This award was given to these researchers for having discovered that it was the double-stranded RNA that allowed the successful silencing of the target gene. The study aimed to analyze the regulation of muscle protein production in *C. elegans*. The researchers found that it was neither mRNA nor antisense RNA that affected protein production, but double-stranded RNA (dsRNA). Researchers named this phenomenon "RNA interference" (RNAi) [19, 20].

Although investigators have analyzed the RNAi capability using exogenous dsRNA's, the mechanism of RNA interference can also be triggered by the endogenous single-strand hairpin. In the case of gene expression in plants, for example, this can be regulated through small endogenous RNAs (siRNA's), which can be divided into RNAs (siRNA's) and microRNAs (miRNAs) [21]. According to the authors, Lunardon et al., 2021 [22], miRNAs can be used when the objective is to simultaneously silence multiple targets through the production of polycistronic miRNA precursors [22].

In some cases, RNAi can confer resistance to viruses or other pathogen infections. Several transposable elements and viruses produce double-stranded RNA at some point in their cell cycles, so RNAi helps keep these potential invaders under control.

In general, the RNAi mechanism works as follows: double-stranded RNA (dsRNA) can be present in the cell as exogenous RNAs (coming from external origin, for example, viruses or created in the laboratory) or endogenous RNAs (from internal origin, transcribed from nuclear genes). These dsRNAs are recognized and cleaved into small interfering RNAs (21 to 28 nucleotides in length) by the dicer-containing protein complex, resulting in siRNA or miRNA, respectively. The resulting siRNA or miRNA associates with RNA-induced silencing complexes (RISC), which contain a catalytic protein, ARGONAUTE (AGO) [23], and other effector proteins and lead to the degradation of the target messenger RNA [24].

RNAi can be characterized as the ability of endogenous or exogenous dsRNA to inhibit the expression of a gene whose sequence is complementary to it [25]; see Figure 1. If the siRNA pairs 100% with the target mRNA sequence, the AGO cleaves the target mRNA and promotes its degradation. On the other hand, if the pairing is not perfect (<100%) this cleavage does not occur, and the mRNA/RISC complexes associate with P bodies, with consequent inhibition of translation by the ribosomes. However, both pathways result in reduced gene expression [6].

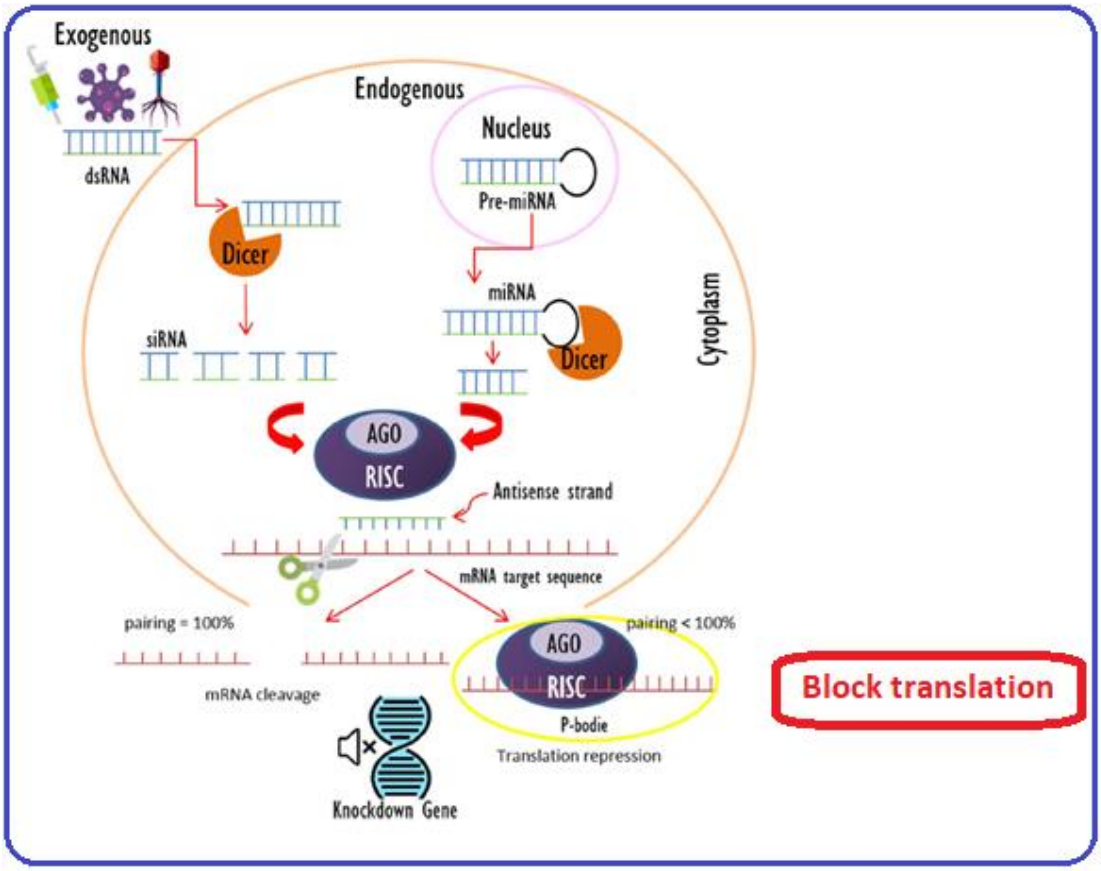

Fig. 1: Illustration of the RNAi mechanism. Adapted from [6].

1.1.1. RNAi comparison with CRISPR

Both RNA interference (RNAi) and CRISPR/Cas9 technology have proven to be powerful tools for manipulating genes and regulating gene expression. Both have advantages and disadvantages, and for this reason, it is important to understand their operating mechanisms before deciding which one to use when developing a research project. Figs. 2 and 3 illustrate the laboratory protocols for these two procedures.

In general, over the last few years, the RNAi technique combined with the insertion of mutants has been widely used to analyze the function of many genes, namely, to try to discover their functions but also to interrupt or reduce their normal expression. The use of knockdowns provided by RNAi can provide several advantages over knockouts, resulting from the emergence of CRISPR/Cas9 technology.

Knockdowns are important when the main objective is the temporary reduction of gene expression and not the modification of the genetic code itself. Furthermore, if complete deletion of gene function is harmful to the cell, the use of knockdowns can also be a great advantage as they only cause a partial loss of function [6, 27, 28].

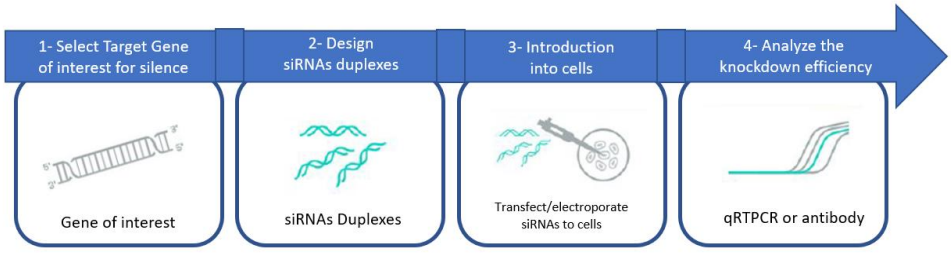

Fig. 2: The laboratory protocol for RNAi-mediated gene silencing involves selecting the gene of interest to silence, designing siRNAs, introducing them into cells, and analyzing knockdown efficiency. Adapted from [26].

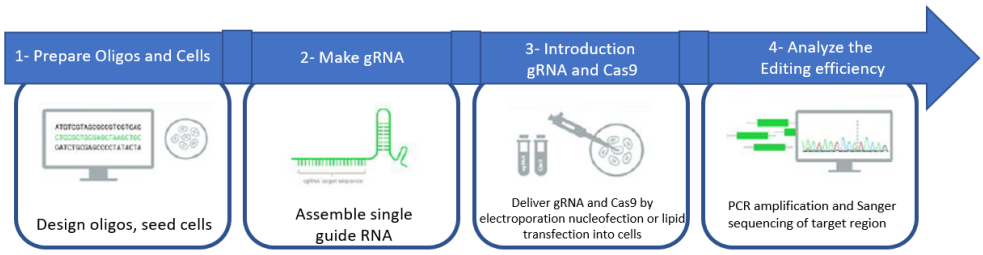

Fig. 3: The laboratory protocol for CRISPR/Cas9: design oligos, assemble single guide RNA, deliver gRNA and Cas9 into cells, PCR amplification, and Sanger sequencing. Adapted from [26].

On the other hand, CRISPR technology also has several advantages, namely allowing the precise editing of DNA (with substitutions, deletions, and additions in genomic sequences). The modifications caused by CRISPR can be transmitted to daughter cells, which constitutes a great therapeutic benefit in the treatment of genetic diseases. The CRISPR technique will be covered in more detail in the review article entitled “CRISPR/Cas9 is a simple, cheap, and effective technique for gene editing” [29] published during the PhD. In general, CRISPR technology allows a series of genomic manipulations from the introduction, inactivation, and correction of new genes; however, this technique also has some disadvantages to be mentioned, such as the respective cost, as it requires certain specific reagents, but also the need to use the sequencing process to assess whether off-target mutations have occurred.

Another disadvantage of CRISPR is the possibility of off-target mutations, i.e., mutations that differ from the intended location [30].

Finally, the use of CRISPR raises ethical questions, particularly regarding its use in human cells (germ cells and embryos) [5, 6, 29, 30].

In general, we can say that both tools enabled great advances in molecular biology (Fig. 4). However, it is important to note that the choice of the desired manipulation tool always depends on the specific objectives of each research project, and as such, a rigorous and considered analysis of the advantages and disadvantages of each technique will always be necessary. If the objective of the research is to use a tool that allows the analysis of the suppression of gene expression for a certain period, RNAi technology is the most recommended. On the other hand, if the objective is to perform genomic manipulation with great precision and cause definitive changes to the genome, the CRISPR editing technique will be the most appropriate. In this PhD, the technology used is RNAi, as this technology allows analyzing the function and expression of the *GIP* and *NPP1* genes in *P. cinnamomi* without the need to resort to genome editing or run the risk of off-target mutations occurring. The DNA sequences will be designed by complementarity from the beginning of the ORFs (open reading frames) of the *GIP* and *NPP1* genes. These artificial sequences were then introduced between an intron in sense and antisense, with this, it was intended that the host (*P. cinnamomi*) produce the miRNA (in this case, double-stranded by hybridization between sense and antisense with the intron circuit). Aiming to silence the respective *GIP* and *NPP1* genes.

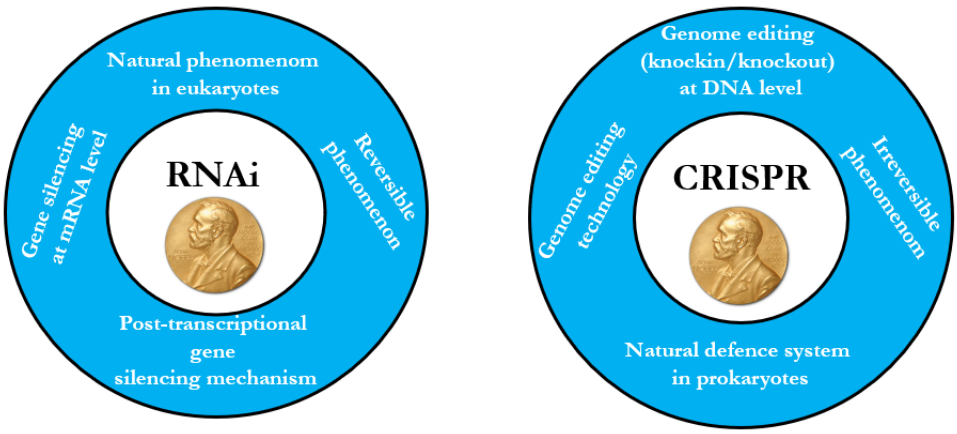

Fig. 4: RNA interference vs. the CRISPR/Cas9 technology. Adapted from [5].

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1.2. Artículo I

CRISPR/Cas9 una técnica sencilla, económica y eficaz para la edición de genes

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CRISPR/Cas9 a simple, inexpensive, and effective technique for gene editing

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Abstract

In recent years, the number of tools and techniques that enable genetic material to be added, removed, or altered at specific locations in the genome has increased significantly. The objective is to know the structure of genomes, the function of genes, and how to improve gene therapy. In this work, we intend to explain the functioning of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) and the advantages that this technique may have compared to previously developed techniques, such as RNA interference (RNAi), zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) in gene and genome editing. We will start with the story of the discovery, then its biological function in the adaptive immune system of bacteria against bacteriophage attack, and end with a description of the mechanism of action and its use in gene editing. We will also discuss other Cas enzymes with great potential for use in genome editing as an alternative to Cas9. CRISPR/Cas9 is a simple, inexpensive, and effective technique for gene editing with multiple applications in the development of functional genomics and epigenetics. This technique will soon have great applications in the development of cell models for use in medical and pharmaceutical processes, targeted therapy, and the improvement of agricultural and environmental species.

1.3. Artículo II

ARN no codificantes regulatorios en la salud y enfermedades de los cultivos.

Regulatory non-coding RNAs in crops health and disease

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Abstract

For many years, it was thought that the function of RNA was limited to the process of producing proteins. In recent years, scientific discoveries have been proving the multiple roles of different RNAs in different regulatory mechanisms. These RNA's are collectively called non-coding RNAs (ncRNA's). This review presents the latest advances on the different classes of non-coding RNAs (ncRNA's), from their function to mechanisms of action. Special emphasis is given to the long non-coding RNAs as new regulatory elements in eukaryote gene expression and in the processes of epigenetic regulation in plants. We believe that increasing studies of regulatory non-coding RNAs in plants will provide a better understanding of the different types of genes related to crop resistance.

2. Justificación y objetivos

Controlling *Phytophthora cinnamomi* is challenging, involving measures such as improvements in soil drainage and the application of fungicides; however, these approaches often have limitations in their effectiveness. The area of molecular biology plays an extremely important role in understanding the natural resistance of chestnut trees to *P. cinnamomi* infection, with the potential to develop more resistant varieties through genetic improvement programs. Furthermore, it is now known that RNA interference (RNAi) constitutes a promising tool for investigating interactions between plant pathogens and host plants. RNAi is a fundamental mechanism used by plants and other organisms to regulate gene expression and defend against pathogens. Through RNAi, small RNA molecules are used to silence specific genes or regulate gene expression. In this sense, the main objective of this doctoral work was to analyze the mechanisms and techniques most commonly used in genome editing, thus allowing a better understanding of its main applications and advantages in plant improvement.

The specific objectives were:

- ✓ Compare the different gene editing techniques and analyze their protection against abiotic and biotic stress on the part of the plant. *Article I*
- ✓ Understand the function of lncRNAs in plants. *Article II*
- ✓ Characterization of the *NPP1* and *GIP* genes from *Phytophthora cinnamomi*, responsible for *Castanea sativa* infection. *Article III and IV*
- ✓ Analysis of the interaction mechanisms between *P. cinnamomi* and *C. sativa*. *Article III and IV*

3. Resultados

The results obtained during the doctorate are represented in two published articles.

3.1. Artículo III

Uso de RNAi en el silenciamiento génico postranscripcional de la proteína 1 de *Phytophthora* (NPP1) inductora de necrosis en *Phytophthora cinnamomi*

Use of RNAi in the post-transcriptional gene silencing of necrosis-inducing *Phytophthora* protein 1 (NPP1) in *Phytophthora cinnamomi*

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Abstract

Background: *Phytophthora cinnamomi* is an oomycete associated with soil. This oomycete is one of the most destructive species of *Phytophthora*, being responsible for the decline of more than 5000 ornamental, forest, or fruit plants. It can secrete a class of protein called NPP1 (Phytophthora necrosis-inducing protein 1), responsible for inducing necrosis in the leaves and roots of plants, leading to their death. **Objective:** This work will report the characterization of the *Phytophthora cinnamomi NPP1* gene responsible for the infection of *Castanea sativa* roots and will characterize the mechanisms of interaction between *Phytophthora cinnamomi* and *Castanea sativa* by gene silencing *NPP1* from *Phytophthora cinnamomi* mediated by RNAi. **Methods and results** for silencing a part of the coding region of the *NPP1* gene were placed in the sense and antisense directions between an intron and ligated to the integrative vector pTH210. Cassette integration was confirmed by PCR and sequencing on the hygromycin-resistant *Phytophthora cinnamomi* transformants. Transformants obtained with the silenced gene were used to infect *Castanea sativa*. **Conclusions:** Plants infected with these transformants showed a great reduction in disease symptoms, confirming RNAi as a potential alternative biological tool in the study of molecular factors and in the control and management of *Phytophthora cinnamomi.*

3.2. Artículo IV

Silenciamiento génico postranscripcional de la proteína inhibidora de la glucanasa en *Phytophthora cinnamomi*

Post-transcriptional gene silencing of glucanase inhibitor protein in *Phytophthora cinnamomi*

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Abstract: Ink disease is considered one of the most significant causes contributing to the decline of chestnut orchards. The reduced yield of *Castanea sativa* Mill can be attributed to two main species: *Phytophthora cinnamomi* and *Phytophthora cambivora*, the latter being the main pathogen responsible for ink disease in Portugal. *P. cinnamomi* is a highly aggressive and widely distributed plant pathogen capable of infecting nearly 1,000 host species. This oomycete causes substantial economic losses and is accountable for the decline of numerous plant species in Europe and worldwide. To date, no effective treatments are available to combat these pathogens. Given chestnut's economic and ecological significance, particularly in Portugal, it is crucial to investigate the molecular mechanisms underlying the interaction between *Phytophthora* species and host plants. This can be achieved through the study of the glucanase inhibitor protein (GIP) produced by *P. cinnamomi* during infection. The technique of RNA interference (RNAi) was employed to suppress the *GIP* gene of *P. cinnamomi*. The resulting transformants, carrying the silenced gene, were used to infect *C. sativa*, allowing for the assessment of the effects of gene silencing on the plant's phenotype. Additionally, bioinformatics tools predicted the secretion of the GIP protein. The obtained results validate RNAi as a potential alternative tool for studying molecular factors and for controlling and managing *P. cinnamomi*.

4. Conclusión

In conclusion, throughout this thesis, it was possible to provide a more comprehensive perspective on gene editing tools (CRISPR/Cas9, RNA interference (RNAi), zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs)), as well as the role played by non-coding RNAs. Some processes of plant-pathogen interactions were also studied. Furthermore, some of the results demonstrate several advantages that can be achieved by taking advantage of genetic manipulation to face various challenges that modern agriculture faces.

Laboratory work on silencing the *GIP* and *NPP1* genes has demonstrated that silencing cassettes for the *P. cinnamomi GIP* and *NPP1* genes can be efficiently produced using a PCR-based cloning method, which offers a cost-effective alternative to other RNA interference mediators. Furthermore, successful transformation of *P. cinnamomi* zoospores by electroporation was achieved, and integration of the silencing construct into the genome was confirmed through genotypic testing, including SANGER screening and sequencing.

Castanea sativa plants infected with transformed *P. cinnamomi* exhibited a lower percentage of wilted leaves and root necrosis compared to those infected with *untransformed P. cinnamomi*. Regarding the GIP protein, subcellular localization predictions revealed that this protein is secreted through the classical ER-Golgi pathway. During plant infection, the pathogen secretes GIP proteins that bind to the plant's extracellular EGaseA, inhibiting its hydrolytic activity and thus suppressing the induction of defense responses. The localization of the GIP protein in plant extracellular EGaseA corroborates the prediction results. The analysis and characterization of the *NPP1* and *GIP* genes helped to better understand the infection process caused by *P. cinnamomi* in *Castanea sativa*.

Furthermore, this work demonstrated the feasibility of producing transformed *P. cinnamomi* strains, leading to a reduction in susceptibility to the disease because, by silencing the disease-causing gene, the protein essential for pathogenicity will be at lower levels or even absent.

This work contributed to the fundamental understanding of host-pathogen interactions and paved the way for potential methods of preventing and treating these types of infections. The transformation of *P. cinnamomi* through genetic silencing techniques corroborates other work already carried out that demonstrated the viability of RNA interference as a very effective tool in disease control and crop improvement.

As future objectives, we intend to characterize more genes involved in the infection processes of *Castanea sativa* crops; and produce more RNAi cassettes for silencing more genes. The use of RNAi cassettes to combat plant diseases can be a more ecological and sustainable alternative compared to the use of chemical pesticides. Reducing the susceptibility of plants to diseases can contribute to reducing the need to use chemical products to control pests. We still intend to analyze the effectiveness of the silencer cassette through RT-PCR and Next-Generation Sequencing (NGS). We believe this will allow a more detailed functional analysis of the infection process and the elucidation of molecular communication mechanisms between the pathogen and host during infection.

Additional research could be carried out, for example: (1) transform *P. cinnamomi* with a vector housing various silencer cassette for different genes involved in the infection mechanism and analyze their effect on plant phenotype; (2) analyze how the silencing of certain genes impacts the plant proteome, examining the interaction between *P. cinnamomi* and *Castanea sativa* using the marking of proteins with different fluorescent vectors.