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MARCELLY SANTANA MASCARENHAS

USO DA TECNOLOGIA CRISPR/Cas9 NA EDIÇÃO DE GENES E VALIDAÇÃO DE VETORES PARA TOLERÂNCIA A ESTRESSES BIÓTICOS EM BANANEIRA (*Musa* spp.)

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título de Doutora em Biotecnologia.

Orientador: Prof. Dr. Edson Perito Amorim
Coorientadora: Dra. Cláudia Fortes Ferreira
Dra. Janay Almeida dos Santos-Serejo
Dr. Tiago Antônio de Oliveira Mendes

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Estadual de Feira de Santana, área de concentração em Biotecnologia com ênfase em Recursos Naturais da Região Nordeste, como requisito para obtenção do grau de doutor, tendo sido aprovada pelos membros signatários abaixo.

Feira de Santana, Bahia, 17 de outubro de 2024.

Orientador: Prof. Dr. **Edson Perito Amorim**

Embrapa Mandioca e Fruticultura

Membro: Prof. Dr. **Rogério Mercês Ferreira Santos**

Universidade Estadual de Feira de Santana

Membro: Profª. Drª. **Ariana Silva Santos**

Universidade Estadual de Santa Cruz

Membro: Profª. Drª. **Alessandra Selbach Schnadelbach**

Universidade Federal da Bahia

Membro: Profª. Drª. **Silvia de Oliveira Dorta**

CIRAD - França

Dedico
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RESUMO

As diversas doenças causadas por fungos, oomicetos, bactérias e patógenos virais, comprometem o desenvolvimento das plantas afetando sua produtividade final. Para superar esses desafios, os programas de melhoramento buscam métodos e técnicas para aprimorar o desempenho das culturas em cenários de estresses. A edição gênica, via CRISPR (*Clustered Regularly Interspaced Short Palindromic Repeats*), surge como uma ferramenta versátil com potencial para desenvolver culturas tolerantes aos múltiplos estresses. O primeiro capítulo deste trabalho objetivou desenvolver uma revisão sistemática da literatura gerada nos últimos doze anos sobre o uso da tecnologia CRISPR na edição de genes para tolerância a estresses bióticos. Buscou-se avaliar artigos depositados em diferentes bases eletrônicas, usando *strings* de busca e critérios de inclusão e exclusão predefinidos. Esta revisão demonstrou que o sistema CRISPR/Cas é aplicado em diversas espécies vegetais a fim de promover tolerância aos principais estresses bióticos. A maioria dos estudos foram desenvolvidos no continente Asiático, especificamente na China. A enzima Cas9 é usada na maioria dos artigos, mas enzimas como Cas12 (Cpf1) e Cas13 também podem ser usadas como uma ferramenta adicional para edição de genomas. A revisão também revelou vários genes editados por CRISPR e que as respostas das plantas aos fatores de estresse são mediadas por muitas vias de sinalização complexas. Além disso, a qualidade dos artigos inseridos nesta revisão foi atestada por meio de uma análise de risco de viés. No segundo capítulo, foram desenvolvidos duas construções/cassetes como produtos biotecnológicos contendo ou não promotor tecido-específico de raiz de bananeira, além de utilizar a tecnologia CRISPR/Cas9 para o *knockout* do gene *PDS* (Fitoeno desaturase) na cultivar Prata-Anã. Para isso, dois RNAs guias (gRNA) foram desenhados e dois vetores/construções foram confeccionados, suas partes foram isoladas, purificadas e transformadas em *Agrobacterium tumefaciens*. Células embriogênicas de bananeira Prata-anã foram utilizadas como explantes para transformação via *A. tumefaciens*. A partir desses métodos foi possível desenvolver um protocolo para construção e validação de vetores CRISPR/Cas para *knockout* de genes.

Palavras-chave: Melhoramento genético, CRISPR/Cas, Revisão sistemática, *Fitoeno desaturase*, banana.

ABSTRACT

The various diseases caused by fungi, oomycetes, bacteria and viral pathogens compromise the development of plants affecting their final yield. To overcome these challenges, breeding programs seek methods and techniques to improve crop performance under stress scenarios. Gene editing via CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) appears as a versatile tool with the potential to develop crops tolerant to multiple stresses. The first chapter of this work aimed to develop a systematic review of the literature generated in the last twelve years on the use of CRISPR technology in gene editing for tolerance to biotic stresses. We sought to evaluate articles deposited in different electronic databases, using search strings and predefined inclusion and exclusion criteria. This review demonstrated that the CRISPR/Cas system is applied to several plant species in order to promote tolerance to the main biotic stresses. Most studies were developed in the Asian continent, specifically in China. The Cas9 enzyme is used in most articles, enzymes such as Cas12 (Cpf1) and Cas13 can also be used as an additional tool for genome editing. The review also reveals several genes edited by CRISPR and that plant responses to stress factors are mediated by many complex signaling pathways. Furthermore, the quality of the articles included in this review was confirmed through a risk of bias analysis. In the second chapter, it was proposed to develop at least one construct/cassette as a biotechnological product containing or not a tissue-specific promoter from banana root, in addition to using CRISPR/Cas9 technology to knockout the *PDS* (Phytoene desaturase) gene in the Prata-Anã cultivar. For this, two guide RNAs (gRNA) were designed and two vectors/constructs were made, their parts were isolated, purified and transformed into *Agrobacterium tumefaciens*. Embryogenic cells of Prata-anã banana were used as explants for transformation via *A. tumefaciens*. From these methods it was possible to develop a protocol for construction and validation of CRISPR/Cas vectors for gene knockout.

Keywords: Genetic breeding, CRISPR-Cas, Systematic Review, *Phytoene desaturase*, banana.

SUMÁRIO

INTRODUÇÃO GERAL

A alimentação é a base da sobrevivência humana, a produção agrícola aliada a segurança alimentar desempenham um papel crucial no futuro da humanidade. Segundo projeções da FAO (2024), a população mundial tende a atingir 9,3 bilhões de pessoas em meados do século XXI, exigindo um aumento significativo na produção agrícola para suprir à crescente demanda por alimentos (MA e LIANG, 2021). O crescimento populacional, condições climáticas extremas, diminuição da disponibilidade de água e terras agrícolas, doenças e pragas em plantas, têm sido consideradas as principais limitações para a produção de alimentos (SINGH et al., 2022).

Nas últimas décadas, técnicas do Melhoramento Genético Vegetal, que incluem cruzamentos, mutações e seleção assistida por marcadores, tem sido usadas para aumentar o desempenho de plantas em cenários de estresse. Essas abordagens tradicionais ainda são úteis para desenvolver variedades resilientes a estresses bióticos e abióticos (SHELAKE et al., 2019). No entanto, os métodos convencionais de Melhoramento Genético são demorados e muitas vezes não produzem os resultados desejados (VARSHNEY et al., 2021).

Avanços na Biotecnologia como a tecnologia da transgenia e a interferência de RNA foram utilizadas para produzir variedades geneticamente modificadas (GM) com características aprimoradas, tais como resistência a pragas e insetos, tolerância a herbicidas, estresse abiótico ou resistência a doenças e biofortificação (SINGH et al., 2009; KUMAR et al., 2020). No entanto, as variedades GM estão sujeitas a obstáculos regulatórios e com aplicações limitadas (AHMAD et al., 2021). No Brasil, segundo a Comissão Técnica Nacional de Biossegurança (CTNBio) produtos gerados por mutagênese dirigida podem não ser considerados transgênicos se não houver inserção de DNA exógeno permanente no organismo final (BRASIL, 2018).

A recente disponibilidade de ferramentas de edição oferece ampla oportunidade para introduzir modificações direcionadas no genoma de forma eficiente para estudar os aspectos funcionais de vários componentes do genoma em diversas plantas e oferece caminhos potenciais para o melhoramento de culturas tolerantes ao estresse biótico (JAIN, 2015).

As ferramentas de edição de genoma fornecem um método para introduzir mutação direcionada, inserção/deleção (indel) e modificação de sequência precisa usando nucleases personalizadas em uma ampla variedade de organismos. Zinc Finger Nucleases (ZFNs), Transcriptional Activator-Like Effector Nucleases (TALENs) e Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas (CRISPR-associated nuclease), são as ferramentas de edição de genoma mais comumente usadas (AHMAR et al., 2020; BHUYAN et al., 2023).

De forma geral, essas nucleases Cas específicas de sequência causam quebras de fita dupla

(DSBs) no loco/locos genômico alvo, que é/são reparados pelas vias de reparo intracelular; classificadas em junção de extremidade não homóloga (NHEJ) ou reparo dirigido por homologia (HDR). NHEJ leva à introdução de indels e HDR pode ser usado para introduzir mutações pontuais específicas ou inserção de sequências desejadas (como *tags* ou novos domínios) por meio de recombinação (JAIN, 2015; CHENNAKESAVULU et al., 2022; HAMDAN et al., 2022).

O CRISPR/Cas realiza o processo de reconhecimento através da complementação de bases entre o RNA guia e a sequência alvo, e a seleção do sítio alvo só precisa estar em conformidade com os requisitos do motivo adjacente ao protoespaçador (PAM) de diferentes sistemas (DEBBARMA et al., 2019). Comparado com ZFNs e TALENs, o sistema CRISPR/Cas é simples, flexível, estável e eficiente. Esses recursos permitiram que o CRISPR/Cas substituísse rapidamente o ZFN e o TALEN como uma das principais técnicas de edição de genoma (LI et al., 2022).

A tecnologia CRISPR teve origem na adaptação de um mecanismo natural de defesa de bactérias e archaeas contra bacteriófagos e plasmídeos (HILLE et al., 2018). A técnica foi otimizada experimentalmente, sendo necessários apenas dois componentes principais para seu funcionamento: um RNA guia (gRNA) e uma nuclease Cas (JINEK et al. 2012). O gRNA direciona a enzima Cas que, por sua vez, cliva um alvo específico no DNA. A quebra da fita dupla de DNA desencadeia um mecanismo natural de reparo celular, resultando na edição genômica propriamente dita (PAWELCZAKet al., 2018).

O sistema de vetores que transportam genes também utilizam promotores para regular a expressão dos componentes CRISPR/Cas. O promotor constitutivo CaMV 35S, oriundo do vírus do mosaico da couve-flor (Cauliflower mosaic virus - CaMV) é muito utilizado em plantas uma vez que garante a expressão contínua de genes que regulam todos os tipos de células (PRAMANIK et al., 2021; KIM et al., 2024). Promotores tecido-específicos também podem ser utilizados e garantem a especificidade no tecido alvo ou em momentos do desenvolvimento da planta tendo uma regulação mais precisa da expressão, reduzindo as chances de mutações fora do alvo e o comprometimento de outros tecidos (XUN et al., 2021; RAHMAN et al., 2022).

Devido à simplicidade de programação, o sistema CRISPR/Cas vem sendo utilizado em plantas desde 2013, com o objetivo de introduzir mutações principalmente em genes que resultassem em um fenótipo distinto e imediatamente reconhecível, como o gene da Fitoeno Desaturase (*PDS*), para testar e otimizar a eficácia da técnica em diversas culturas (LI et al., 2013; DUTT et al., 2020; SIDDAPPA et al., 2023; THAKUR e MERU, 2023; PHAD et al., 2024). Outros estudos também foram realizados para promover a ativação, repressão e/ou nocaute de genes, e para alterar modificações epigenéticas relacionadas a diferentes estresses bióticos em várias culturas, como tomate (LI et al., 2023; LI et al., 2024; ZHANG et al., 2024), banana (TRIPATHI et al., 2019), arroz

(ZHANG et al., 2023; KIM et al., 2024), milho (GUO et al., 2022), soja (YU et al., 2022) e outras.

A bananeira (*Musa* spp.), incluindo os plátanos, é uma das principais culturas alimentares básicas cultivadas que alimenta mais de 500 milhões de pessoas em países tropicais e subtropicais (FAOSTAT, 2024). A cultura é afetada por vários fatores, especificamente estresses bióticos e abióticos (NASCIMENTO et al., 2020; ROCHA et al., 2021; SOARES et al., 2021). O desenvolvimento de variedades melhoradas de bananeira usando Melhoramento Genético convencional é um desafio devido à baixa variabilidade genética no germoplasma de *Musa*, diferentes poliploidias, meiose desbanlanceada, ciclo de produção longo e esterilidade da maioria das cultivares comumente cultivadas pelos agricultores (SILVA et al., 2001). A tecnologia CRISPR surge como uma ferramenta muito eficaz, que permite a transferência de características úteis de diferentes espécies ou entre uma mesma espécie, contornando os gargalos naturais do melhoramento e sendo aplicável no melhoramento da bananeira (TRIPATHI et al., 2019).

A edição de genoma por meio da tecnologia CRISPR em culturas propagadas vegetativamente como a bananeira foi relatada recentemente, tendo como alvo mutações no gene *PDS* (KAUR et al., 2018; NAIM et al., 2018; NTUI et al., 2020). No entanto, cabe ressaltar que no Brasil ainda não foram relatados estudos com CRISPR em bananeira. Sendo assim, pretende-se utilizar esse sistema como um estudo de caso para *knockout* do gene *PDS* e em seguida editar genes de resistência a fatores bióticos/abióticos na cultivar mais utilizada pelos agricultores brasileiros, a Prata-Anã, como alvo principal para transformação/edição. Uma vez que quase 70 % da variedade de banana plantada no Brasil é do tipo Prata e levando-se em consideração que esta variedade é suscetível à principal ameaça à bananicultura, a murcha de Fusarium, a necessidade de medidas imediatas para contornar este cenário é iminente.

Portanto, esse trabalho tem por objetivos: *i*) realizar uma revisão sistemática da literatura publicada nos últimos doze anos sobre o uso da tecnologia CRISPR na edição de genes para resistência a estresses bióticos em plantas; ii) delinear um protocolo de construção de vetores para uso na técnica de CRISPR-Cas9 no estudo de caso com o *knockout* do gene *PDS* em bananeira da variedade Prata-Anã. Esse estudo trará contribuições significativas para o desenvolvimento de cultivares de bananeira resistentes/tolerantes a estresses bióticos/abióticos utilizando a tecnologia CRISPR e servirá de base para a edição de novos genes em outras variedades de bananeira de interesse.

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CAPÍTULO 1

Use of CRISPR technology in gene editing for tolerance to biotic factors in plants: A systematic review

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current issues in molecular biology

MDPI

Review

Use of CRISPR Technology in Gene Editing for Tolerance to Biotic Factors in Plants: A Systematic Review

Marcelly Santana Mascarenhas [1], Fernanda dos Santos Nascimento [2], Anelita de Jesus Rocha [2], Mileide dos Santos Ferreira [2], Wanderley Diaciso dos Santos Oliveira [1], Lucymeire Souza Morais Lino [2], Tiago Antônio de Oliveira Mendes [3], Claudia Fortes Ferreira [2], Janay Almeida dos Santos-Serejo [2] and Edson Perito Amorim [2,*]

[1] Department of Biological Sciences, Feira de Santana State University, Feira de Santana 44036-900, BA, Brazil; marcelly.bio@hotmail.com (M.S.M.); diacisowanderley@hotmail.com (W.D.d.S.O.)
[2] Embrapa Mandioca e Fruticultura, Cruz das Almas 44380-000, BA, Brazil; feel.20@hotmail.com (F.d.S.N.); anelitarocha@gmail.com (A.d.J.R.); mileideferreira12@gmail.com (M.d.S.F.); lucymeire.lino@gmail.com (L.S.M.L.); claudia.ferreira@embrapa.br (C.F.F.); janay.serejo@embrapa.br (J.A.d.S.-S.); edson.amorim@embrapa.br (E.P.A)
[3] Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Viçosa, 36507-900, MG, Brazil; tiagoaomendes@ufv.br (T.A.d.O.M.)
* Correspondence: edson.amorim@embrapa.br; Tel.: +55-75-3312-8058; Fax: +55-75-3312-8097

Abstract: The objective of this systematic review (SR) was to select studies on the use of gene editing by CRISPR technology related to plant resistance to biotic stresses. We sought to evaluate articles deposited in six electronic databases, using pre-defined inclusion and exclusion criteria. This SR demonstrates that countries such as China and the United States of America stand out in studies with CRISPR/Cas. Among the most studied crops are rice, tomatoes and the model plant *Arabidopsis thaliana*. The most cited biotic agents include the genera, *Xanthomonas, Manaporthe, Pseudomonas and Phytophthora*. This SR also identifies several CRISPR/Cas-edited genes and demonstrates that plant responses to stressors are mediated by many complex signaling pathways. The Cas9 enzyme is used in most articles and Cas12 and 13 are used as additional editing tools. Furthermore, the quality of the articles included in this SR was validated by a risk of bias analysis. The information collected in this SR helps to understand the state of the art of CRISPR/Cas aimed at improving resistance to diseases and pests to understand the mechanisms involved in most host–pathogen relationships. This SR shows that the CRISPR/Cas system provides a straightforward method for rapid gene targeting, providing useful information for plant breeding programs.

Keywords: biotic stress; CRISPR/Cas; plant diseases; phytopathogens; pests; plant genetic improvement

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1. Introduction

Biotic stresses caused by pests and pathogens such as viruses, bacteria, fungi, oomycetes, nematodes, and insects are largely responsible for low productivity in various crops [1]. In addition, the continuous increase in several new pest species makes the control of these pathogens challenging [2]. Microorganisms have specific characteristics and are classified into groups. Biotrophic microorganisms depend on the living plant to feed and complete their life cycle; necrotrophs, during their feeding habit, kill the host plant, and hemibiotrophs initially depend on the living plant (behaving like biotrophs) in order to survive and complete their cycle with a necrotrophic phase; where the host is degraded [3,4].

The plant and the pathogen are intertwined in a battle of recognition and evasion where a multilayered defense system, including pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), has evolved in plants to fight invading pathogens for survival [5]. In general, PTI uses pattern recognition receptors to

monitor PAMPs on the cell surface. Meanwhile, ETI relies on leucine-rich repeat repeat receptors with a nucleotide-binding domain to recognize pathogen effectors inside the cell [6–8].

Thus, understanding the molecular mechanisms of pathogen–host interactions, especially the identification of key targets related to defense responses in plants, would offer a great opportunity to design broad-spectrum and durable resistance in various crops [5,9,10]. Hence, plant breeding programs are looking for effective and long-lasting techniques to improve crops. However, some challenges, such as the complex inheritance of the vast majority of agronomic traits and the strong genotype–environment interaction, are still challenging [11].

Currently, three types of genome editing tools are widely used by researchers, including zinc finger nuclease (ZFN) [12], transcription activator-like effector nuclease (TALEN) [13], and CRISPR-clustered/associated regularly interspaced short palindromic repeats (CRISPR/Cas) [14]. ZFN and TALEN have not been widely used due to high costs and failures. The CRISPR/Cas system (which includes Cas9, Cas12, and Cas13) from a prokaryotic organism has transformed the field of gene editing with high efficiency and easy handling and application. Compared to the previous two generations of genome editing techniques, the CRISPR/Cas system is flexible, simple, stable, and easy to transform. These resources allowed for ZFN and TALEN to be replaced by CRISPR/Cas, which has become one of the main genome editing techniques.

CRISPR is composed of CRISPR RNA (crRNA) (transcribed from the spacer sequences) and transactivating crRNA, or single chimeric guide RNA (sgRNA) (formed by the fusion of crRNA and tracrRNA) for targeting and the specificity of targeting [14,15]. The Cas9 protein-RNA complex (from *Streptococcus pyogenes*) is formed by the combinations of the crRNA spacer to a target sequence close to an adjacent motif of the proto-spacer (PAM—3 base pair (bp) motifs essential for spacer acquisition and target cleavage) [15–17].

Due to its ease of execution, the CRISPR/Cas system has become the tool of choice for gene editing in any species of interest. By generating a double-strand break (DSB) at the desired site by the Cas-gRNA complex, the host–cell repairs the DNA lesion via the non-homologous end joining (NHEJ) pathway, resulting in short insertions or deletions, consequently leading to gene knockouts. Another form of repair is the homology-directed repair (HDR) pathway, which is more precise and has a lower probability of error [18,19]. In plants, the system has been used to knock out all members or a single member of a multigenic family [20] and even several unrelated genes [21], with the NHEJ pathway being the most reported [22].

Several studies have been published to demonstrate the different genes that positively or negatively regulate resistance to various pests and pathogens in model plants and diverse crops, such as *Arabidopsis thaliana*, where genes such as *ZAR1*, *UGT71C3*, and *miR398b* have been studied [23–25], in rice, *SWEET14*, *eIF4G*, and *PRAF2* [26–28], in maize, *ZmACD6*, *Zmksl2*, and *JAZ15* [29–31], in tomato, *SlWRKY16*, *SlWAT1*, and *SlDMR6* [32–34], and in soybean, *Rfg1*, *Rpp1*, and *GmLMM1* [35–37].

In addition, CRISPR technology has evolved rapidly and has shown great potentialfor plant biology, especially with regard to CRISPR/Cas9 variants, such as CRISPR/Cas12 and CRISPR/Cas13, which offer better specificity for DNA and RNA, respectively. For precise changes in a single base, without causing double-strand breaks, reducing off-target mutations, base editing methods have been implemented [38,39]. Other improved delivery tools, such as the use of nanoparticles and viral vectors, allow for the efficient introduction of the CRISPR system into plant cells and the delivery of RNP (ribonucleoprotein) complexes, rather than DNA plasmids, is being used to improve efficiency and reduce off-target effects [40]. Other advanced techniques allow for the simultaneous editing of multiple sites in the genome, making it easier to modify multiple traits at the same time, and systems such as Prime Editing and CRISPR 3.0 are emerging, allowing for precise insertions and deletions without the need for DNA breaks [38,39,41].

Thus, off-target effects have been significantly reduced due to improvements in the specificity of the CRISPR/Cas system. In addition, these data facilitate reliability and safety, allowing for regulatory approval, coupled with strategies such as temporary editing, where CRISPR machinery is rapidly degraded after editing, as well aa the ability to edit multiple genes simultaneously, which facilitates the engineering of complex traits in plants, such as disease resistance and nutritional trait improvements [39–42].

In order to systematically gather and review current research on the use of CRISPR/Cas technology in gene editing for biotic stress tolerance, this study presents a systematic re- view (SR) of articles published in the last twelve years. It also aims to contribute to the SR previously carried out on the use of CRISPR/Cas technology in gene editing for tolerance to abiotic stresses [22]. Here, we describe how the technique has been applied to pest and pathogen resistance studies and the locations and crops, among other data, for which it is possible to detect the current research trend on the subject and its impact on crops.

1. Materials and Methods

To carry out this SR, the State of the Art through Systematic Review (StArt) software (version 3.0.3 Beta) was used, developed, and made available by the Software Engineering Research Laboratory of the Federal University of São Carlos, at https://www.lapes.ufscar.br/resources/tools-1/start-1, accessed on 15 August 2023.

The review was prepared following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [43], structured in a set of evidence-based items that help authors report a wide range of systematic reviews and meta-analyses and can be used in plant, animal, and health intervention areas. A PRISMA checklist was drawn up to minimize bias in this SR, available at https://doi.org/10.5281/zenodo.13869284 (accessed on 29 September 2024). The SR process using StArt occurred in three stages: planning, execution, and summarization.

1.1. Planning

In order to plan the SR, a protocol was developed, available at https://doi.org/10.5281/zenodo.13371943 (accessed on 26 August 2024), which includes a description of the SR, the research objectives, the main/guiding question, the research questions (Table 1), the search string, the source mechanism, the inclusion and exclusion criteria, and the definition of the types of study. The question guiding the SR was based on the Population Intervention Comparison Results strategy [44] (Table 1). Thus, this SR aims to answer the following research question: how has CRISPR/Cas technology been used in gene editing in plants for biotic stress tolerance over the last twelve years?

Table 1. Description of the PICOS strategy used to develop the RS research questions on the use of CRISPR/Cas technology to edit tolerance genes/resistance to biotic stresses from studies published in the last 12 years.

Description	Abbreviation	Components of the Question
Population	P	Agricultural varieties under biotic stresses
Interest/Intervention	I	Gene editing in plants using CRISPR/Cas technology for disease resistance
Comparison	C	Plant breeding methods
Outcome	O	Editing genes that confer resistance to biotic stresses in plants
Study type	S	Scientific articles and literature reviews

After drafting the main research questions, secondary questions were elaborated (Table 2).

Table 2. Guiding questions for this SR on the use of CRISPR/Cas technology to edit tolerance genes/resistance to biotic stresses from studies published in the last 12 years.

Research Questions
1. In which country was the study performed?
2. What culture is the article about?
3. Which biotic agent is addressed in the study?
4. Which genes are reportedly associated with disease and pest resistance in plants?
5. Which nuclease is used in conjunction with the CRISPR tool?
6. What methodology is used to use CRISPR?
7. What method is used to prove the effectiveness of the tool?
8. What techniques/tools are associated with CRISPR/Cas9?
9. What transformation method was used?
10. Which explant was used to transform the plants?
11. What are the main vectors used to express Cas9 and/or gRNA in plants?
12. Were any unusual phenotypic characteristics observed in the plants after genetic transformation? Which ones?
13. What is the characteristic obtained after mutating the plant?

1.2. Execution

Searches were performed in different electronic databases such as Pub Med Central, Springer, Scopus, Web of Science and sites such as Google Scholar and CAPES Periodicals Portal. For the Google Scholar, Springer, PubMed Central, CAPES Periodicals, Web of Science and Scopus databases, the following keywords were used: ("CRISPR/Cas9" OR "CRISPR-Cas9" OR "CRISPR-Cas in plants") AND ("plant resistance" OR "plant disease resistance") AND ("plant disease" OR "biotic factors" OR "disease resistance" OR "plant pathogens" OR "pests" OR "plant parasite").

For the Web of Science and Scopus databases, another search string was also used, with the following keywords: ("CRISPR" OR "CRISPR/Cas9" OR "CRISPR-Cas9" OR "CRISPR-Cas in plants") AND ("biotic factors" OR "pathogen resistance" OR "phytopathogen resistance" OR "plant disease resistance" OR "disease resistance" OR "plant resistance" OR "pest resistance" OR "parasite resistance"), seeking to include as many studies as possible.

The Boolean connectives "AND" and "OR" were used to differentiate search terms and group synonymous terms, respectively. The search results in each database were imported into the BIBTEX, MEDLINE, or RIS formats, compatible with the StArt software. The bibliographic survey was performed from January 2013 to July 2024.

To select the articles, the title, abstract, and keywords were analyzed. Articles that met the terms of the search sequence and did not deviate from the proposed theme were accepted and submitted to the extraction stage. At this stage, only articles that answered the research questions (Table 2) previously established in the SR protocol were accepted as an inclusion criterion. Exclusion criteria were also used to extract the following articles: theses, dissertations, manuals, book chapters, review articles, papers not written in English, papers without a clear contribution, papers published prior to 2013, or papers that were off-topic.

1.3. Data Summarization

The data obtained from the scientific articles was summarized in tables, graphs, word clouds, and bibliometric maps. The graphs were constructed using the R version 4.4.1 statistical environment [45], using the ggplot2, reshape2, and ggpubr packages. The bibliometric analyses were performed according to the metadata of the selected articles using the VOSviewer_1.6.17 program [46] to verify the networks of interactions between keywords and between authors and co-citations. Word clouds containing the journals used to publish the articles, genes edited, tools, and software used to support the CRISPR/Cas

tool over the last twelve years were generated online and free of charge (https://www.wordclouds.com/, accessed on 18 November 2023), based on the frequency of the data.

1.4. Risk of Bias Analysis

To assess the risk of bias, the adapted Cochrane Collaboration Tool [47] was used. The methodological quality was analyzed by three authors (MSM, FdSN, and AdJR), and the articles selected in the extraction stage were subjected to four questions (Table 3) in order to further reduce data bias. These are essential questions that confirm whether editing using CRISPR/Cas was effective, reaching the target site or not.

Table 3. Questions to evaluate the methodological quality of the articles included in the SR on the use of CRISPR/Cas technology to edit tolerance genes/resistance to biotic stresses from studies published in the last 12 years.

Risk of Bias
1. Has off-target analysis been performed?
2. Has the pathogen been inoculated?
3. Has phenotypic analysis been performed after mutation in the plant?
4. Does the article answer at least 50% of the research questions?

Systematic errors in scientific studies that cause distortions in the results can happen; it is complex to state whether a study is biased or not, but systematic errors in scientific studies can be estimated and minimized through a careful evaluation of its methodological quality. Rigorous practices such as protocol development, the use of PICOS strategy, PRISMA checklist, and the others described above, significantly reduce the risks of bias.

The risk of bias can be classified as low, moderate, or high when the study presents negative responses ("no") of up to 25%, between 25 and 75%, and greater than 75%, respectively.

3. Results

3.1. Bibliographical Survey

Initially, 9513 studies related to the proposed topic were identified from the search strings in the selected electronic databases, which are widely used in plants. Google Scholar showed 5880 studies, Pub Med Central showed 1421, CAPES Periodicals Portal showed 509, Scopus showed 819, Web of Science showed 574, and Springer, 310. Although the Web of Science and Scopus databases use two search strings, Google Scholar contributed 61.8% of the articles submitted, which is justified by its broad search spectrum. From this total, 376 were detected as duplicates by the StArt software.

After analyzing the title, abstract, and keywords, 7623 studies were rejected and 734 were submitted to the extraction stage. The texts were read in full, resulting in 296 accepted articles (Figure 1). These selected articles met the inclusion/exclusion criteria because they are related to the theme of this SR, which aimed to include as many studies as possible on the use of CRISPR/Cas technology in the editing of tolerance/resistance genes to biotic stresses in the last 12 years; then, the information was deposited in the Supplementary Materials (Table S1) for consultation.

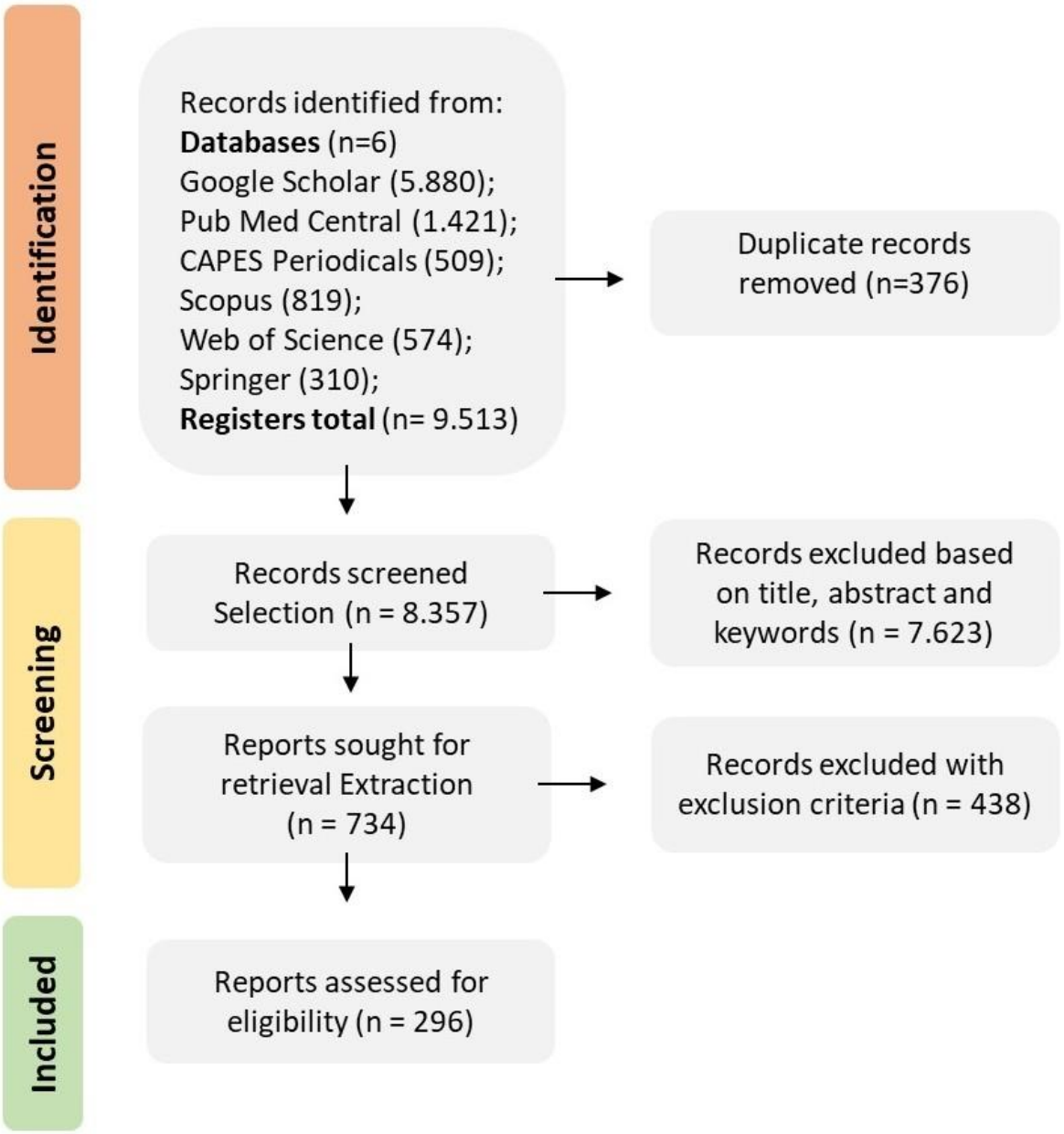

Figure 1. A PRISMA flow diagram with the respective stages of the process of selecting studies for inclusion/exclusion in the systematic review of the CRISPR/Cas technology used to edit genes for tolerance/resistance to biotic stress in plants according to the databases [43].

The studies evaluated covered the period from January 2013 to July 2024, with 2021 considered the year with the highest number of publications as to CRISPR/Cas technology in the editing of genes related to resistance to biotic stressors, contributing 21.6% of the articles. The other years had 16.6% (2022), 16.2% (2020), 11.5% (2023), 10.5 (2024), 9.8% (2019), 7.4% (2018), 3.7% (2017); for the years 2016, 2015, 2014 and 2013, less than 2% were obtained.

Considering the frequency of authors and all the keywords in the articles selected in the extraction phase, bibliometric maps were developed to represent the co-occurrence of these words (Figure 2). The size of the circles represents the number of times these words were repeated; the larger the circle, the more times the author and journal were cited. Colors indicate different groups of authors and keywords and the thickness of the lines the correlation between these words. The thicker the line, the higher the occurrence of the term.

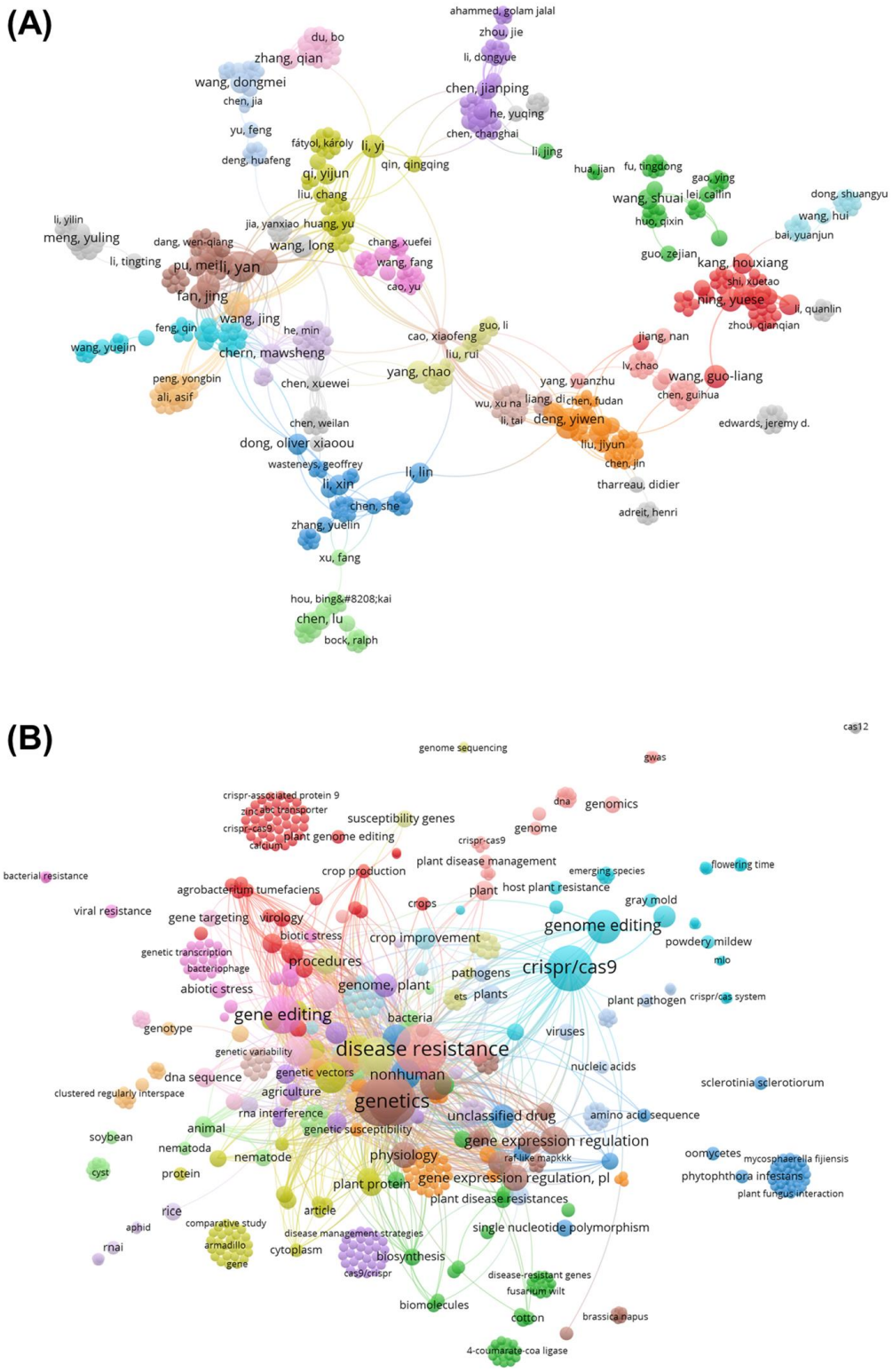

Figure 2. Bibliometric indicators of the collaboration network between authors and keywords of the selected articles on CRISPR/Cas technology and biotic factors. (**A**) Collaborators who have published the most on CRISPR/Cas and biotic stresses in the last 12 years. (**B**) Keywords of the selected articles on CRISPR/Cas technology used for gene editing of tolerance/resistance to biotic stresses in plants during the extraction phase of this systematic review. Different colors for each circle indicate collaboration between groups.

Twenty clusters were formed and identified using different colors, according to the degree of similarity between the authors' works (Figure 2A). Authors such as Yan Li, Jing Fan, Long Wang, Yuese Ning, Yi Li, Chao Yang, Liang Guo Wang, Qian Zhang, Jianping Chen, and Jing Wang are responsible for a large bibliographic contribution. These data demonstrate a trend in centralized research related to Chinese authors with not much exchange of information between Chinese researchers and those from the rest of the world. The links or distances infer the correlation between these authors and their collaboration on other works. Some small grouped but isolated nodes can be observed, but they show minimal contribution to the studies performed by the authors included in these groups.

For the keywords, approximately 92 nodes and 12 clusters were observed, which defined the main research themes in this area. The most relevant groups according to the size of each circle refer, in order of relevance, to the following words: disease resistance, genetics, CRISPR/Cas9, gene editing, and genome editing. These words form core groups associated with several other terms of collaboration with a theme that constitutes the smaller groups formed, for example, by the terms vectors, crop breeding and regulation of gene expression. Words such as Cas12, RNAi, bacterial resistance, and genomic sequencing appear in isolation, which indicates lower frequency and low correlation with other studies (Figure 2B).

3.2. Plant of Origin and Plant Cultures Edited Using CRISPR/Cas Technology

Of the 296 research papers, 158 (59.8%) originated in China, 30 (11.4%) in the United States of America (USA), 12 (4.5%) in Germany, 7 (2.7%) in South Korea, 5 (1.9%) in Canada and Pakistan, 4 (1.5%) in Spain and Saudi Arabia, 3 (1.1%) in India, Israel, Japan, and the Netherlands, and 2 (0.8%) in Australia, the Philippines, Sweden and the United Kingdom. The other countries only had one article submitted, which represents just 0.4% of the publications on the subject (Figure 3).

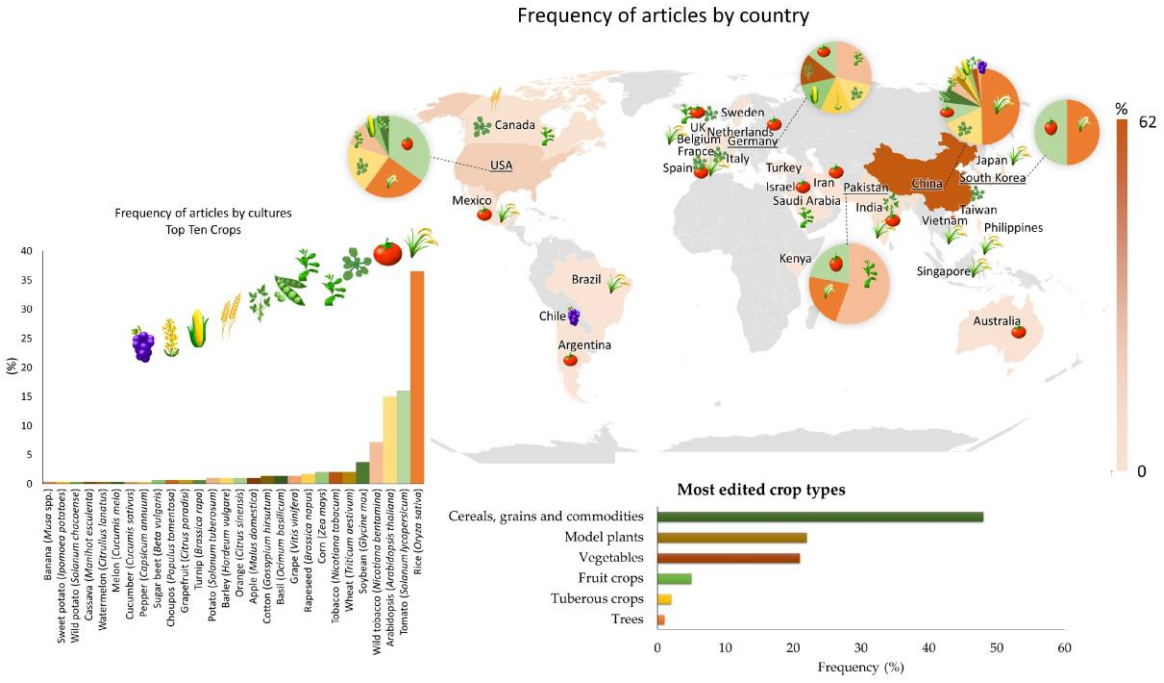

Figure 3. Frequency of articles according to country of publication and crop edited by CRISPR/Cas technology for plant disease tolerance/resistance. More than one plant species per article was considered in calculating the frequency.

China and the USA are the countries that produce and disseminate the most scientific knowledge on the subject. However, all continents, except for Antarctica, have contributed to this area of research. The articles identified 28 plant species used for gene editing related to resistance to biotic factors. Overall, the types of crops most edited by the CRISPR technique include cereals, grains and agricultural commodities, with 48% of the studies represented mainly by rice, followed by studies with model plants, represented mainlyby *Arabidopsis*. Other studies have included vegetables (21%), fruits (5%), tubers (2%) and trees (1%) (Figure 3).

Rice (*Oryza sativa*) was the most studied crop, present in 36.5% (109) of the studies, followed by tomato (*Solanum lycopersicum*) with 16% (48), *Arabidopsis thaliana* with 15% (45), wild tobacco (*Nicotiana benthamiana*) with 7.2% (22), soybean (*Glycine max*) with 3.8% (12), wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*), and corn (*Zea mays*), with 2% (6), rapeseed (*Brassica napus*) with 1.7% (5), and grape (*Vitis vinifera* L.), basil (*Ocimum basilicum*), and cotton (*Gossypium hirsutum*) with 1.4% (4). The other species had a frequency of less than 1% of the studies (Figure 3).

3.3. Biotic Stresses in Plants

The biotic agents cited in the literature were bacteria, fungi, viruses, oomycetes, insects, and nematodes, accounting for 51.8, 28.3, 10.5, 5.7, 2.7, and 1%, respectively. The genera *Xanthomonas* (75) and *Pseudomonas* (41) account for 92% of the studies on bacteria. For fungi, the most studied genera were *Magnaporthe* (54), *Botrytes* (19), *Fusarium* (15), *Sclerotinia* (7),and *Verticillium* (6) (Figure 4).

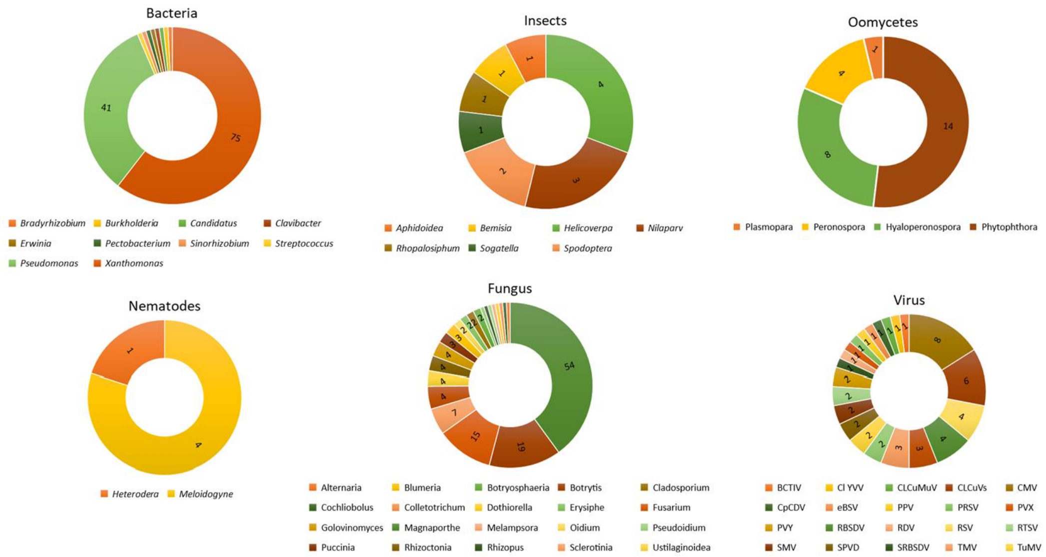

Figure 4. The most-studied biotic agents (bacteria, insects, oomycetes, nematodes, fungi, and viruses) in the last twelve years for resistance/tolerance to plant diseases using CRISPR/Cas technology. More than one biotic agent per article was considered in calculating frequency.

The most-cited viruses were cucumber mosaic virus (CMV) (8), cotton leaf curl virus (CLCuVs) (6), rice streak virus (RSV), rice black-streaked dwarf virus (RBSDV) (4), tomato yellow leaf curl virus (TYLCV) (3), and tobacco mosaic virus (TMV) (3). The oomycete *Phytophthora* (14), followed by *Hyaloperonospora* (8) and *Peronospora* (4), were the most covered. The insect genera *Helicoverpa* (4), *Nilaparvata* (3), *Spodoptera* (2), *Sogatella* (1), *Rhopalosiphum* (1), *Bemisia* (1), and *Aphidoidea* (1), and the nematode genera *Meloidogyne* (4) and *Heterodera* (1), were also observed in the studies (Figure 4).

As a result, the diseases most frequently covered were bacterial leaf blight (BLB), bacterial leaf streak (BLS) in rice, and bacterial spot in tomatoes. For fungi, brusone in rice, gray mold in tomatoes, and Fusarium wilt in various crops, among other diseases, were the most commonly observed (Figure 4).

3.4. Types of Explants

The explants used for plant transformation via CRISPR/Cas varied according to the plant species. Callus, cells, cotyledons, embryos, epicotyl, hypocotyl, anthers, inflorescence, leaf disks/leaves, plants, protoplasts, roots, and seeds were found as transforming materials (Figure 5).

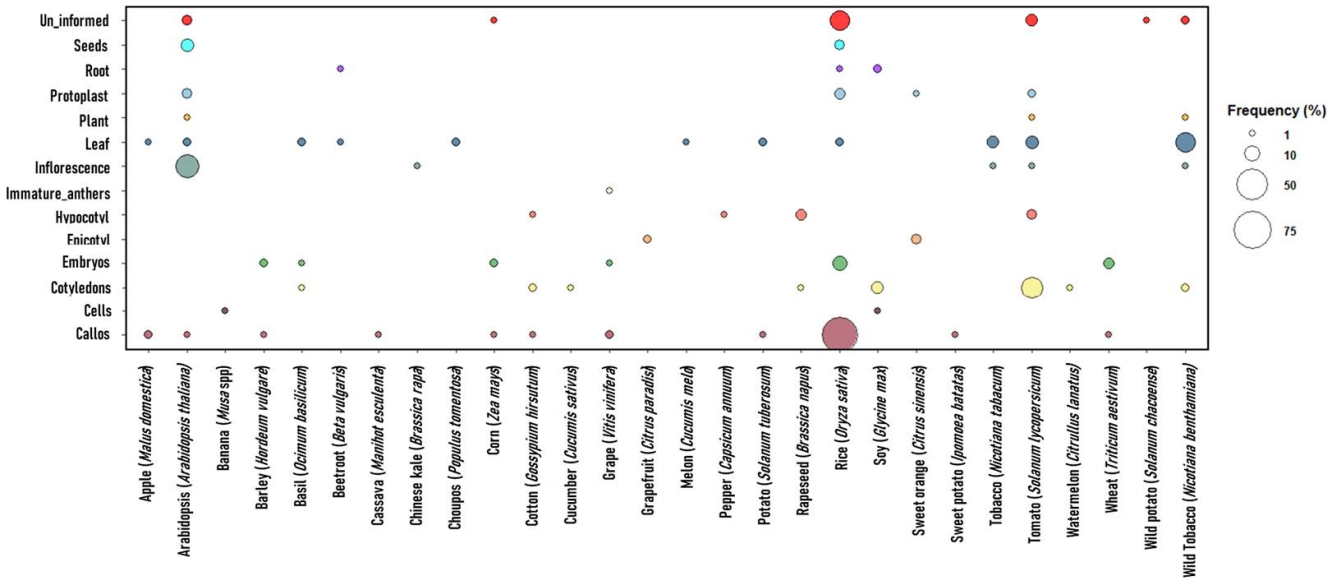

Figure 5. Explants used for the transformation of the different plant species covered in studies on gene editing via CRISPR/Cas for tolerance/resistance to biotic stress in the last 12 years. The colors of the circles represent each explant and the size of the circumference the frequency of each explant in different crops.

For the rice crop, transformation via CRISPR/Cas was mainly performed using embryogenic calli, with a frequency of over 50%. Embryos, protoplasts, seeds, leaves, and roots were also used as transforming sources, but with a frequency of less than 10%. In tomatoes, the most commonly used explants were cotyledons, with a frequency of more than 10% of the studies performed on this crop, followed by leaves (<10%). In *Arabidopsis*, the inflorescence (>10%), seeds, protoplasts, leaves, and plant (<10%) were used as explants. In wild tobacco, the leaves (>10%), inflorescence, plant, and cotyledons (<10%) were used as the main transformation explants (Figure 5).

3.5. Plant Disease Resistance/Susceptibility Genes

A word cloud designed from the genes cited in the papers as potential targets for resistance/susceptibility to plant diseases. The sucrose efflux transporter gene (*SWEET14*) appears prominently in the center of the word cloud and was the most cited in the papers, especially those related to resistance to bacteria *Xoo* in rice, followed by N Requirement Gene 1 (*NRG1*), *Pi21* resistance genes, LateraL Organ Boundaries 1 (*CsLOB1*), Mildew resistance locus o 1 (*SlMlo1*), Dependent Glycosyl Transferases (*UGT76b1*), and the *Xa7* resistance gene. The gene families *WRKY* (14), *SWEET*/*OsSWEET* (12), *UGT* (7), *Xa* (7), and *Solyc* (5) have also been extensively studied. In the articles selected for this SR,337 genes related to tolerance/resistance to biotic factors were covered; some papers used CRISPR/Cas technology to edit more than one gene (Figure 6).

Figure 6. Word cloud of CRISPR/Cas edited genes in different plant species related to tolerance/resistance/susceptibility to biotic stresses.

3.6. Auxiliary Methods to CRISPR/Cas

The methodological strategies most used in the studies collected to validate and support the CRISPR/Cas tool were PCR (27.4%), sequencing (26.5%), qPCR (22.9%), transgenics (8.4%), RNA-seq (3.9%), Western blotting (3.5%), transcriptomics (1.8%), virus-induced gene silencing (VIGS) (1.0%), bimolecular fluorescence complementation (BiFC) assay (1.0%), LC-MS/HPLC liquid chromatography analysis (0.9%), Northern blot (0.7%), microscopy (0.6%), metabolomics (0.4%), histochemistry (0.3%), and proteomics (0.2%) (Figure 7). The other methods accounted for less than 0.1% of the studies.

PCR, sequencing, and qPCR techniques were mainly used to demonstrate the efficacy of the CRISPR/Cas tool and detect on- and off-target mutations.

Certain types of software were also used to complement CRISPR/Cas-related analyses. The CRISPR-P version 2.0 software appears in 16.2% of the articles as an auxiliary method to CRISPR/Cas to predict target sites and/or mutations. Other widely used software/programs included BLAST, DSDecode, Cas-OFFinder, CCTop, CRISPR-PLANT, NCBI, CRISPR-GE, CRISPRdirect, SnapGene, ClustalW, CRISPR Design, CHOPCHOP, ClustalX, RNAfold, Geneious, CRISPOR, RNA Folding Form, and TIDE (Figure 8).

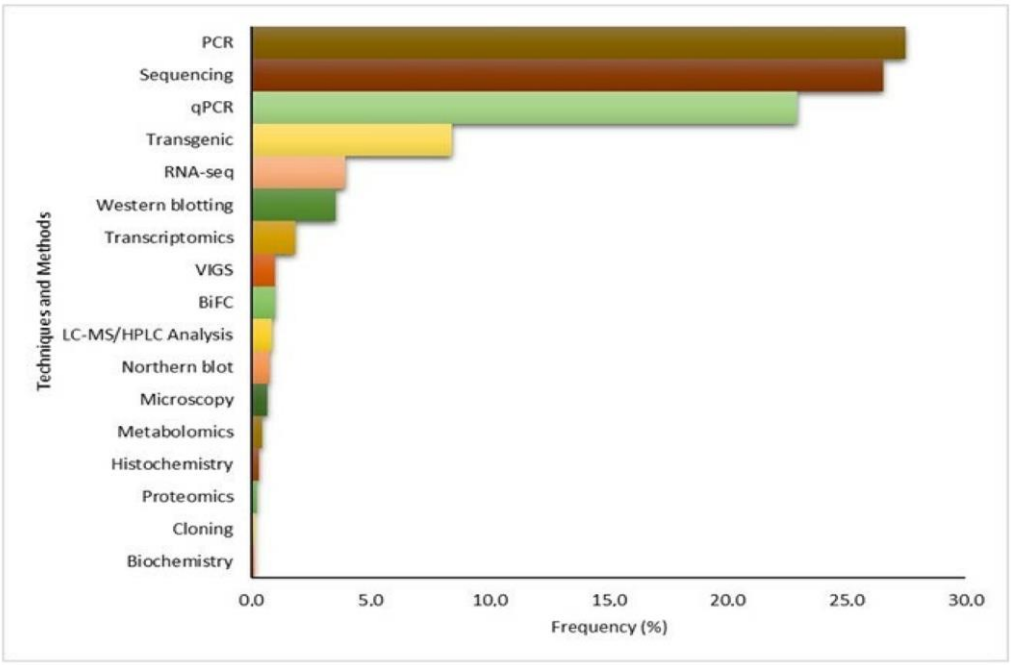

Figure 7. Auxiliary tools and analyses used with the CRISPR/Cas technique for validation and comparison between knockout with control and/or the overexpression of mutants identified in articles on tolerance/resistance to biotic stresses in the last 12 years.

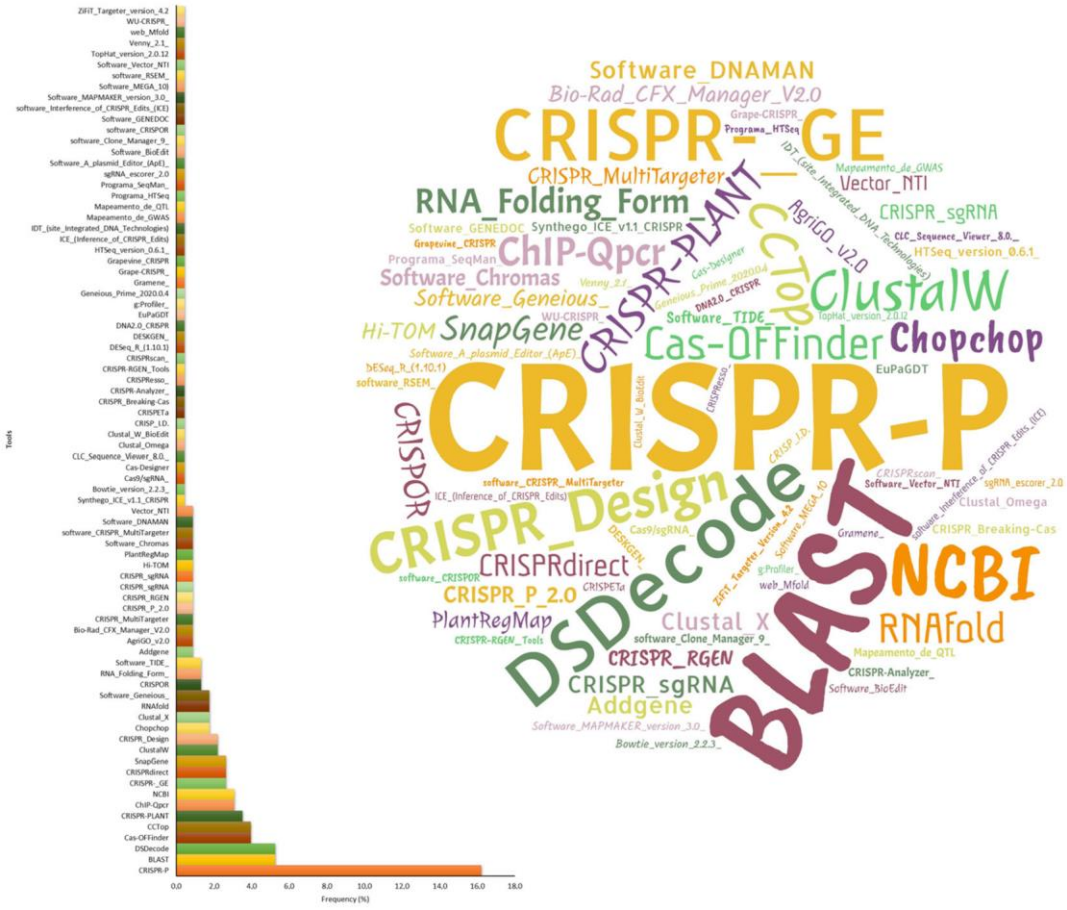

Figure 8. Frequency and word cloud of tools and software that help the CRISPR/Cas technique to search for specific target sites identified in studies on tolerance/resistance to biotic stresses in the last 12 years.

3.7. Use of CRISPR/Cas Technology

Most of the CRISPR/Cas methods used in the 296 studies selected for this SR had already been validated by other authors. The method used by Ma et al. (2015) [48], Xing et al. (2014) [49] and Wang et al. (2015) [50], and showed great reproducibility, being used in 24.7% of the studies (Table S2) to precisely edit plant genomes, deleting regions responsible for unwanted characteristics or inserting gain-of-function mutations.

For the CRISPR tool to be effective as Cas, endonuclease must be used. Of the studies collected, 98.3% (291) used Cas9 as an accessory to this editing system. Other endonucleases such as Cpf1, formerly known as Cas12a (2) and Cas13 (3), were also mentioned, but they were not very common.

Several vectors have been used to express Cas and/or single guide RNA (gRNA), but the most commonly cited is pCAMBIA and pYLCRISPR/Cas. The most widely used delivery method for introducing the gene of interest into plant cells was carried out by *Agrobacterium tumefaciens* (286) and *Agrobacterium rhizogenes* (6), occurring mainly via electroporation and heat shock.

3.8. Phenotypic Analysis and Characteristics Obtained after Mutation

Considering the agronomic characteristics and visible symptoms of the disease after mutation of the plants, 60.2% of the studies indicated that the phenotype was preserved, 13.7% inferred that the plants showed unusual characteristics after mutagenesis, such as dwarfism, albinism, and more aggressive symptoms of the disease, such as wider lesions than would be characteristic, and 26.1% of the articles did not perform this type of analysis or did not record having carried it out (Figure 9).

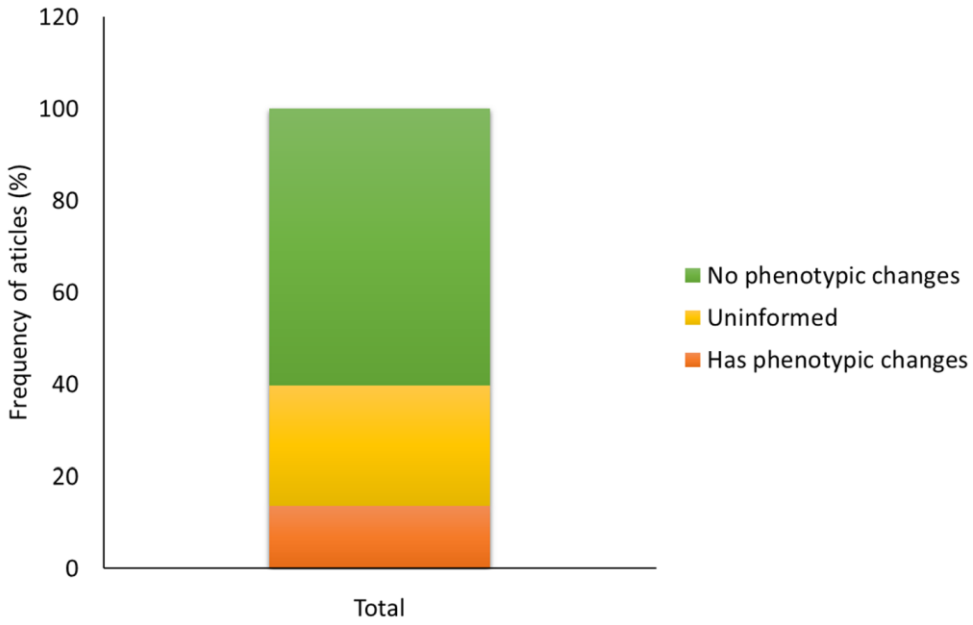

Figure 9. Frequency of articles that performed a phenotypic analysis of plants after mutation and the pathogen inoculation test.

Greater resistance to plant diseases was observed in approximately 70% of the studies and higher plant susceptibility after gene mutation was noted in 28% of the studies, indicating that these genes are related to plant defense/immunity response (Table S2).

3.9. Sources of Bias in the Included Studies

To assess risk of bias in individual studies, an adaptation of the Cochrane risk of bias tool protocol was performed, which is composed of domains; according to the reviewers' judgment, the study/outcome is classified as having a high, low or unclear risk of bias. The domains assigned to this SR are important and necessary questions in studies related to gene editing by CRISPR/Cas technology. Thus, questions such as "Was phenotypic analysis performed after mutation in the plant?", "Was off-target analysis performed?" were used to classify the methodological quality of the selected articles. And three authors did these analyses independently to avoid potential biases.

Based on the classification defined for the risk of bias and the questions designed to measure the risk, it can be inferred that 98.6% of the articles presented a low risk of bias (Figure 10). Only six articles did not answer question 3 ("Was phenotypic analysis performed after mutation in the plant?") and presented a high risk for this question. Three studies had an uncertain answer; however, the other questions were answered, which does not invalidate these studies from contributing to this SR. For question 1 ("Was off-target analysis performed?"), only two studies did not answer. The other questions were answered in full, confirming the good methodological and bibliographical quality of this study (Table S3).

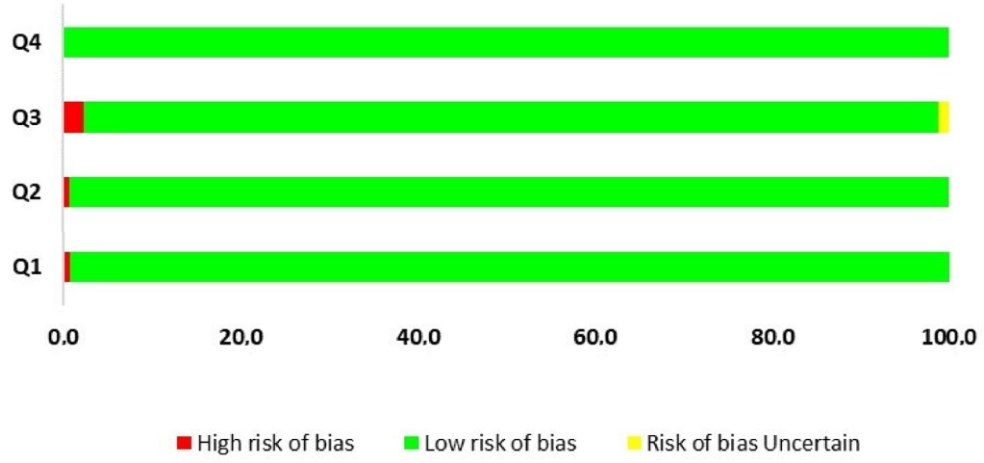

Figure 10. Risk of bias analysis based on the following questions: "Q1: Was off-target analysis performed? Q2: Was the pathogen inoculated? Q3: Was phenotypic analysis performed after mutation in the plant? Q4: Does the article answer at least 50% of the research questions?".

4. Discussion

4.1. Bibliographic Survey

This SR presents a compilation of data extracted from articles carefully selected between 2013 and 2024, with the aim of expanding knowledge on the use of CRISPR/Cas technology in plant gene editing for resistance to biotic stresses. The application of the CRISPR/Cas system in plants began in 2013 [51–54]; however, until 2015, the works consisted mainly of preliminary studies and the validation of techniques and protocols. Literature reviews were rejected to avoid bias, and letters to the editor and non-peer-reviewed

articles were also disregarded. For this reason, and to obtain more recent studies on the subject, articles from the last twelve years were considered.

The year 2021 saw the largest bibliographic contribution on the subject, which may be related to the increased demand for food in the world and the negative effects of the COVID-19 pandemic [55], which led to an 18% increase in production in 2021 and an 11% increase in 2022 [56], stimulating agribusiness and studies focused on the genetic improvement of crops in order to minimize food shortages. The amount of data obtained on the subject in recent years reveals its importance and the need for investment in this area of research aiming to provide returns for the population and rural producers. Furthermore, these data reveal that technology is evolving rapidly and could contribute to overcoming food shortages for exponentially growing populations [57].

The biometric analysis demonstrated that the keywords "disease resistance" or "CRISPR/Cas9" present in the search string are also the most cited words in the selected articles and indicate that, over the last twelve years, more than 5000 studies have focused on this topic.

Keywords such as "viral resistance", "DNA", "genomics", "oomycetes", "soybean", and "*Sclerorotinia sclerotiorum*" appear in isolation despite being related to the topic; this is because such words are found mainly in the body of the text and not in the titles, abstracts, and keywords of the selected articles.

The authors who have produced the most studies on the subject are from research institutions located mainly in China and the USA. The Rice Research Institute and Key Lab for Major Crop Diseases located at Sichuan Agricultural University in China is responsible for a major contribution to gene-editing work using CRISPR [58–60].

4.2. Study Sites and Edited Crops

Most of the studies included in this SR originated in China (140), which is in line with the data on agricultural production. Despite having less than 10% of the world's productive land, the country ranks first in the production of cereals, cotton, fruit, vegetables, meat, poultry, and fishery products, as well as accounting for 25% of the world's grain production [61]. This makes the country a major contributor to crop improvement studies using the CRISPR/Cas tool.

The studies performed in the USA were also representative (27). The country is the third largest food producer in the world and the first when it comes to exporting corn and soybeans, the main agricultural commodities [61]. Countries such as Germany, South Korea, Canada, Pakistan, Spain, and India have also contributed to studies on the subject.

Rice is the second-most-produced food crop in the world and the first-most-cultivated in China, which accounts for 30% of world production [61]. It is a monocot considereda model, because its genome is small and easy to manipulate when compared to other crops, which justifies the large number of studies (107) using CRISPR/Cas technology as a gene-editing tool for improving this crop [62–65].

In addition to rice, 27 other plant species have been covered in gene-editing studies in this SR. Tomato is the second-most-cited crop (47 articles) and the sixth most important crop economically, with a production of more than 100 million tons per year [61]. The model crops, *Arabidopsis thaliana* and *Nicotiana* sp., were also well cited in the selected papers; this may be related to the large amount of information already validated on these species and because their genomes have already been sequenced [66–69].

4.3. Biotic Stresses

Biotic stressors such as pathogens, insect pests, and weeds reduce the yield and quality of agricultural production. In high-yielding crops such as wheat, rice, corn, potatoes, and soybeans, losses can range from 17.2% in potatoes to 30% in rice [70]. Several diseases affect rice cultivation. Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is considered one of the most important bacterial diseases of rice. Irrigated or rainfed areas are common for growing this species and favor the development of the disease due

to the abundance of water facilitating the dispersion of the pathogen, or through the high availability of nitrogen [62,71–75].

Bacterial leaf streak of rice caused by *X. oryzae* pv. *oryzicola* (*Xoc*) is also another disease that has been widely covered in the studies collected [76–78]. The genus *Xanthomonas* has been the most studied in gene editing via CRISPR/Cas in the last twelve years [75]. The studies seek to understand the mechanisms involved in plant defense against pathogens in order to make them resistant/tolerant to diseases.

Brusone is the main fungal disease of rice, caused by *Magnaporthe oryzae*, which establishes itself in the plant under favorable environmental conditions and causes damage to grain quality, plant height, and the number of tillers [79]. Rice is grown and consumed worldwide and is a staple food for around 2.5 billion people [61], so it is necessary to understand the biology of these pathogens to develop strategies to control these diseases.

Pseudomonas syringae was another pathogen that was mentioned frequently [80–83]. This bacterium is found in a wide variety of plants and penetrates host tissues through lesions or structures such as stomata [84]. The species has been widely used to elucidate questions about plant immunity and bacterial pathogenesis. In the selected articles, the bacterium is mainly present in studies with the model plant *A. thaliana* [66,85].

Tomatoes are an economically essential vegetable worldwide and their production is also threatened by many pathogens [86–88]. Gray mold, caused by *Botrytis cinerea*, rarely occurs in the field; however, in protected environments, humidity becomes a problem, favoring the development of the fungus, which infects the plant through wounds and causes the rapid rotting of the fruit, resulting in harvest losses. Other biotic agents have also been addressed in the studies, such as fungi (such as *Fusarium*), CMV viruses, CLCuVs, and the *Phytophthora* oomycete.

4.4. Types of Explants

The genetic transformation of plants is based on the insertion of transgenes into totipotent plant cells, which then regenerate into fertile plants. Small fragments of living tissue isolated from a plant specimen, called explants, are used [89]. The explants used for transformation via *Agrobacterium* or bioballistics can vary depending on the plant species, including calli, embryos, protoplasts, inflorescence, leaves, hypocotyls, epicotyls, and cotyledons [2,62,90–95].

When transformation occurs by electroporation, protoplasts undergo membrane destabilization after being subjected to high voltage, resulting in temporary pores in the cell membrane, allowing for the influx of DNA molecules that will integrate into the genome of the species to be mutated [96]. This type of method requires plants to be obtained entirely from protoplasts, which requires mastery of the production and regeneration of this type of explant, which is still a challenge in tissue culture [97].

The vast majority of the studies collected for this research used embryogenic calli and leaves as explants for various plant crops. In rice, the use of calli as an explant source is predominant [62,74,98–100]. Calli are formed practically from any fragment of the plant; in rice, seeds are commonly used to induce calli, which grow slowly as an amorphous cell mass through stimuli supplemented with specific phytohormones [89].

In tomatoes, transformations have been carried out mainly from cotyledon and leaf explants [81,93,101]. Most tissue culture tests in this species have been performed to achieve organogenesis over somatic embryogenesis [102]. In studies performed by Costa et al. (2000) [103], the tomato varieties ‘IPA-5’ and ‘IPA-6’ demonstrated favorable regeneration capacity (97 and 80%, respectively) from cotyledons when inserted into a supplemented Murashige and Skoog medium.

Studies with *A. thaliana* have mainly used flowers/inflorescences as a source of explants [92,104–106]. The floral immersion method is considered simple, fast, and efficient, and consists of immersing developing floral tissues in a solution containing *Agrobacterium tumefaciens*, sucrose, and detergent to transform the plants [107,108].

In tobacco, leaves were the most commonly used explants [68,109,110]. In direct somatic embryogenesis tests, using leaf tissue explants from different tobacco genotypes, different *Agrobacterium* strains, and different transformation methods, the transformation and regeneration rates varied [111,112]. The success of the transformation system involves the integration of the DNA into the host genome, the expression, inheritance, and stability of the exogenous DNA, as well as the regeneration of explants that depend largely on the genotype, origin, and age of the explant and plant growth regulators used to supplement culture media [113].

4.5. Plant Disease Resistance/Susceptibility Genes

The gene that stood out in CRISPR/Cas studies for resistance to biotic stresses was *SWEET14* (Figure 6). *SWEET* genes encode sugar transporter proteins and often function as susceptibility (S) genes, the recessive alleles of which provide resistance [114]. This gene has been extensively studied and reviewed in studies involving the bacterial pathogen *Xoo*, which causes bacterial rust in rice [114,115]. In the context of plant–pathogen interaction, transcription activator-like effectors (TALEs) of the pathogen function in diverting the nutritional resources of rice, inducing the expression of *OsSWEET14* and thus causing susceptibility [72,114]. The activation of *SWEET14* by the pathogen results in an increase in the amount of sucrose available in the phloem apoplast, providing a source of nutrition for the pathogen promoting its proliferation [116].

The main strategy when using CRISPR/Cas9 in relation to the *SWEET14* gene is to mutate the coding region of *OsSWEET14* to test whether its disruption will result in broad-spectrum resistance to *Xoo* strains in rice [72,117] or the disruption of the TALE-binding elements of *Xoo* in rice harboring the recessive resistance allele in order to defuse the arms race between the effectors of the pathogen and their host targets [26,118,119]. Inhibition or *SWEET14* editing can reduce the plant's susceptibility to the pathogen, a potential strategy for the development of resistant cultivars.

Other genes, such as *NRG1*, *Pi21* resistance genes, *CsLOB1*, *SlMlo1*, dependent glycosyl transferases (*UGT76b1*), and the *Xa7* resistance gene, were also reported with considerable frequency in the studies (Figure 6). The *NRG1* genes are close homologs of the Activated Disease Resistance 1 family of leucine-rich repeat domain proteins (NLRs), the function of which is still unclear, so some studies have reported their functional analysis through CRISPR/Cas9 in *Arabidopsis* [120–122]. The *Pi21* gene belongs to the set of R genes that encode NLRs. It is resistant to rice brusone and is, therefore, the target of CRISPR/Cas9 rice-breeding programs to obtain mutant varieties [75,123–125].

Plants can prevent pathogen attacks through induced systemic resistance (ISR) and acquired systemic resistance (SAR). What differentiates them are the types of induction in the plant. SAR is activated through disease-causing organisms and relies on salicylic acid(SA) and genes, whereas beneficial microbes induce ISR and are independent of SA [126].The two forms of resistance are activated from different defense signals when the plant is attacked by pathogens [127].

4.6. CRISPR/Cas Technology for Gene Editing

This SR sought to identify the most commonly used protocols for gene editing via CRISPR over the last twelve years. Among the editing methods used, the protocols proposed by Ma et al. (2015) [48], Xing et al. (2014) [49] and Wang et al. (2015) [50] were the most cited, respectively. The three protocols seek to edit various target genes in dicots and monocots using a multiplexing system, using one to several binary vectors and the Cas9 endonuclease. These results corroborate the findings of [22] in a systematic review of gene editing using CRISPR technology to edit genes tolerant to abiotic stresses.

Different CRISPR/Cas systems have been widely used to generate DSBs at target genomic sites in various plant species. Among the two classes of CRISPR immune systems, Class 2 is simpler than Class 1 and therefore easier to use for the development of genome editing tools [128]. Thus, three Class 2 effectors, Cas9, Cas12, and Cas13, have been

extensively used for targeted DNA and RNA cleavage. The Cas9 endonuclease was the most widely used in the articles in this SR (259 studies), followed by Cas13 (3), and Cas12 (2). The effectors Cas9 and Cas12 are DNA-directed endonucleases, while Cas13 is an RNA-directed endonuclease [129].

As evidenced by Jinek et al. (2012) [14], Cas9 nucleases are guided by an RNA hybrid consisting of a crRNA and a tracrRNA. However, most Cas9 genome editing applications use an sgRNA that is designed by fusing crRNA and tracrRNA into a single RNA molecule for Cas9 to cleave DNA [130,131]. Normally, CRISPR/Cas9 requires a target site of 17 to 20 bp directly adjacent to a 5′-NGG PAM sequence (motif adjacent to the protospacer) to be effectively recognized by sgRNA [15,132]. Several authors have used Cas9 [68,133–136], and although several Cas9 orthologs have been discovered [137], Cas9 from *Streptococcus pyogenes* (SpCas9) is the nuclease that has been used the most for different genome manipulation experiments due to its high efficiency and simple NGG PAM sequence requirements [129].

The Cas12 endonuclease was identified in this SR with the aim of knocking out Xa13 [138] and PRAF2 [28] to improve resistance to bacterial rust caused by *Xanthomonas oryzae* pv. *Oryzae*. Cas12 is a class II type V endonuclease that was developed from *Prevotella* and *Francisella* [139]; it cleaves at a distal position of the PAM, generating a staggered break of the DNA double-strand, and recognizes a PAM region rich in T 5′-TTN-3′ [140] and proved to be an efficient alternative in editing these genes. Cas13 cleaves single-stranded RNA [141], and in the studies observed it was used to interfere against RNA viruses in plants, also presenting itself as a viable alternative to the use of Cas9 [142–144].

Cas9 and gRNA are regulated by appropriate promoters within a vector. The cauliflower mosaic virus (CaMV35S) is a constitutive promoter widely used for its strong expression in various plant tissues, being effective for mutations throughout the organism. The ubiquitin promoter (UBI), also constitutive and commonly used in monocots, has efficient and stable expression, especially in recalcitrant cultures. In addition, specific tissue promoters can also be used to induce mutations in plants. These allow for more controlled editing, limiting the expression of the system to specific sites, which reduces off-target effects, but can make mutations in the whole organism less efficient. The choice of promoter is crucial for the efficiency of the mutation due to to the objectives of gene editing, such as the need for localization or plant-wide expression, species compatibility, expression and efficiency, and the risks of off-site effects [145–147].

Several vectors have been used to express Cas and/or sgRNA, among the most cited being pCAMBIA (46) [63,73,148] a popular vector due to its easy handling, stability, and the existence of a variety of selection and reporter genes [149], and the pYLCRISPR/Cas9 vector (40) [150,151], which is a CRISPR/Cas9 system efficient in multi-locus gene knockout [48]. Other vectors, such as pHEE401E [152], also had considerable frequencies (Table S2). The most widely used delivery method for introducing the gene of interest into plant cells was carried out by *Agrobacterium tumefaciens* (286) and *Agrobacterium rhizogenes* (6). This is considered a powerful tool for delivering genes of interest to a host plant due to the efficiency of transformation, the low operating cost, and the simplicity of the transformation and selection protocols [153].

Although *Agrobacterium* mediated delivery is very efficient, it also has some disadvantages, such as the need for long periods of tissue culture to recover transgenic plants, the low frequency of stably transformed plants, the narrow range of genotypes within a crop species that can be transformed, and the limitations of the host range of certain *Agrobacterium* species [154]. The delivery of CRISPR/Cas reagents to plants can be carried out by several methods. The most common in addition to *Agrobacterium tumefaciens* mediated transformation include particle bombardment (biobalistics) and protoplast transfection [155,156].

Particle bombardment is useful for recalcitrant plant species, but it can cause physical damage to cells and random DNA integrations. Protoplast transfection, on the other hand, allows for the delivery of ribonucleoproteins (RNPs), reducing the risk of exogenous DNA integration, but the regeneration of complete plants from protoplasts can be challenging in some cultures [155,156]. Additional delivery methods of the CRISPR/Cas system, such as the use of nanoparticles and pollen magnetofection, can be an alternative for more precise and efficient delivery [157].

4.7. Auxiliary Methods to CRISPR/Cas

The main methodological strategies used in the studies collected to validate and support the CRISPR/Cas tool were PCR, sequencing, and qPCR techniques (Figure 7); these were mainly used to prove the efficacy of CRISPR/Cas-mediated editing and detect on- and off-target mutations (Figure 7). The PCR technique is an essential tool in molecular biology that allows for the amplification of nucleic acid sequences (DNA and RNA) through repetitive cycles in vitro, simulating what occurs in vivo during DNA replication [158].

PCR followed by sequencing has been reported in many studies; however, Zischewski et al. [159] highlight that a disadvantage of screening only potential pre-selected off-target sequences is the risk of overlooking mutations at other loci in the plant genome.In contrast, the use of the unbiased whole-genome-sequencing approach is the most com- mon detection method in plants, allowing for the identification of off-target effects in a less restricted way [160].

Different prediction software were also used to detect off-target effects (Figure 8). The CRISPR-P software was reported in 16.2% of the articles as an auxiliary method to CRISPR/Cas, aimed at predicting target sites and/or mutations. Other software/programs, such as BLAST, DSDecode, Cas-OFFinder, CCTop, CRISPR-PLANT, and CHOPCHOP, also had considerable frequencies. A major concern in CRISPR/Cas9 system applications is its off-target effects that occur when Cas9 acts on untargeted genomic sites and creates cleavages that can lead to adverse outcomes [161].

The tools identified in this SR aid in silico prediction and are generally free online software that can be properly accessed via the Internet. The prediction algorithms of these software are mainly based on sgRNA sequences, so the results of these methods are generally biased toward sgRNA-dependent off-target effects. For epigenetics and chromatin organization experiments, off-target prediction by these in silico tools needs additional experimental validation [161].

4.8. Phenotypic Analysis and Characteristics Obtained after Mutation

In 60.2% of the articles, the phenotype was preserved, with no unusual or unexpected characteristics occurring after mutagenesis. Sixty-one percent exhibited greater resistance to plant diseases and 29% greater susceptibility after editing (Figure 9). This is because most studies are focused on knocking out/silencing genes or knocking in/overexpressing a gene to study and demonstrate its functions. Thus, the technique that cuts double-stranded DNA and generates a DSB will be repaired by the NHEJ repair mechanism; this can be carried out for a specific and individual gene without other side effects [162].

In other articles, the CRISPR/Cas technique has been used to knock-in the overexpression of an individual gene. In this sense, it is possible to edit the genome by cutting the DNA sequence at a specific site, and then, through HDR, a foreign DNA sequence (target gene) will be inserted at this cleavage site [162]. In this way, position effects can be avoided because CRISPR/Cas can be used to precisely insert a foreign gene into a specific location within a genome without interrupting other genes.

In this sense, the overexpression of the OsbHLH6 gene in transgenic rice plants caused responsive gene expression to jasmonic acid and increased susceptibility to the pathogen *Magnaporthe oryzae* [63]. Similarly, the overexpression of the GmLMM1 gene in Nicotiana benthamiana severely suppressed the production of reactive oxygen species triggered by

microbe-associated molecules (bacterial flg22) and the pattern-induced cell death of the oomycete *Phytophthora sojae* [163].

Thus, the use of the CRISPR/Cas technique associated with gene knockout/silencing or gene knock-in/overexpression has contributed to the elucidation of various plant–pathogen interaction pathways in many pathosystems, without causing unwanted phenotypic changes, such as citrus canker caused by *Xanthomonas citri* subsp. *citri* in citrus [2], BLS of rice caused by *Xoc* and *Xanthomonas campestris* pv. *campestris* [73,164,165], *Phytophthora sojae* in soybeans [144], and *Botrytis cinerea* in tomatoes [81].

4.9. Sources of Bias in the Included Studies

The aim of SRs is to gather and synthesize data on a given topic that meets pre-established eligibility criteria and methods are used to reduce the chances of data bias [166]. The Cochrane Collaboration Tool was developed to assess the risk of bias of the studies to be included in the SRs and is widely used in health studies [47], which is why the method was adapted to the needs of this SR.

The tool aims to make the process clearer and more precise, free from errors that compromise the quality of the research. Therefore, possible limitations of the primary studies must be carefully assessed so that the results and conclusions obtained are reliable. It is not possible to determine the "quality" of a study without any kind of criteria; it is necessary to observe the design, the conducting of the research, and the analysis and presentation of the results so that the studies are not underestimated or overestimated [47,167].

In order to minimize errors in the choice of studies collected for this SR, inclusion/exclusion criteria, the PRISMA checklist, and questions on the topic (Table 3) were used to confirm whether the use of CRISPR/Cas technology was efficient in gene editing through off-target analysis. Inoculation tests of the pathogen and phenotypic analysis were also considered, as well as articles that answered at least 50% of the research questions (Table 1).

Literature reviews were excluded from the research, as many papers are cited repeatedly in the reviews, overestimating the data. Manuscripts that did not answer at least 75% of the risk-of-bias questions were considered high-risk and were not included in this SR. Only nine articles presented a risk equal to 25% for not answering one of the four questions, which is considered a low risk of bias, and two articles presented moderate risk, which means they answered only 50% of the questions. The articles selected for this SR are highly qualified and the methodologies used are reliable.

5. Final Considerations and Future Perspectives

The growing demand for food is a challenge for society in the face of population growth, changes in consumption patterns, environmental changes, and dealing with pathogens that cause plant diseases and pests. Meeting this demand is based on the need to guarantee global food security.

Biotic and abiotic stresses cause major losses in agricultural production, which calls for novel strategies to subsidize plant tolerance, as conventional practices are insufficient to meet the current and future food needs of the population. The use of the CRISPR/Cas tool can accelerate plant breeding by rapidly modifying genomes in a predictable and accurate way. Due to its efficiency, simplicity, and versatility, CRISPR/Cas has become a popular tool for genome editing and has been widely used in improving the resistance of various crops [57]. The development of disease-resistant varieties with good yields and quality isa fundamental strategy to guarantee global food security and generate employment and income for farmers.

This SR included 296 papers in which plant genes were edited via CRISPR/Cas to confer resistance to plant diseases and pests. We identified that Cas9 endonuclease is widely used in studies; however, this is not the only "molecular scissors" that can help the CRISPR editing system; the use of other enzymes such as Cpf1 (Cas12a), and Cas 13 has

been reported in CRISPR studies for editing genes related to plant resistance and could be applied more frequently in future studies.

Genes related to tolerance/resistance to biotic stresses were identified in this SR and the CRISPR/Cas system can be used for gene knockout, gene insertion and gene replacement, resulting in the loss of function, knockdown or activation of mutants, which can lead to the generation of tolerant/resistant plants to the various pathogens. However, some issues are still far from being clarified and serve as a starting point for future studies, such as the fact that the main genes that control important traits of crops have not been identified, which limits the application of CRISPR/Cas in plant breeding; and pathogens continue to modify their genome through evolution to break the already available resistance gained by editing the CRISPR/Cas gene. Thus, it is necessary to design new variants in a short period of time and insert them into the plants. In addition, many genes are represented by multigene families, making it difficult to produce a resistance phenotype by eliminating a single gene, and it is necessary to develop more precise CRISPR/Cas tools to perform multiplex genome editing.

Regarding the methods used for editing, gRNAs were designed with different target sequences to direct Cas9 to specific corresponding sites; however, proper care is important when designing gRNAs, as unwanted targets are a major limitation, and to reduce these challenges, tools and software such as CRISPR-P, CRISPR-GE, BLAST, among others, are used. Among the methods used for mutation detection, PCR and sequencing are the most reported methods that can detect unwanted targets. Explant regeneration in most plants is still a challenge because it is labor-intensive and poses a limitation in CRISPR/Cas-based gene editing.

The information provided in this SR was based on articles with methodological quality confirmed by a risk of bias analysis, which determined that most of the included studies were at low risk of bias. Among the most-studied crops, rice, tomatoes, and the model plant *Arabidopsis thaliana* stand out. Among the most studied genera of biotic agents are *Xanthomonas*, *Magnaporthe*, *Phytophthora* and cucumber mosaic, belonging to the group of bacteria, fungi, oomycete and viruses, respectively.

Although the use of CRISPR/Cas technology has revolutionized plant breeding in recent years, there are still many challenges to be overcome; its off-target alterations are themain bioethical concern, namely whether they can lead to ecological imbalance, geneticdrift, fatal diseases, or a chimeric phenotype in animals or even in humans. Another concern is whether GMOs produced by CRISPR/Cas9 can change the natural ecosystem by changing the mating potential of living organisms. Agricultural foods produced by CRISPR also face the same challenges as GMOs and may be prevented from being consumed insome countries. Despite these concerns, plants developed CRISPR/Cas can also becomesafe and GMO-free by using ribonucleoproteins (RNPs), i.e., without exogenous DNA. This will also help overcome the hurdles scientists face in commercializing biotech crops. To date, around 128 plant cultivars such as corn, soybeans, cotton, wheat, and sugar cane have been genetically edited, mainly for resistance to insects and/or herbicides, and have been approved by the National Technical Biosafety Commission [168].

Studies on gene editing with CRISPR/Cas for resistance to biotic agents are only beginning. The results obtained so far not only show that this technology offers precise modifications to the plant genome and has been successfully used to confer resistance to diseases and pests, but are also essential mainly to understand the function of genes related to various pathways of plant–pathogen interaction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb46100659/s1, Table S1: The selection of articles on the use of CRISPR/Cas technology associated with biotic stress tolerance in plants in the last twelve years. Table S2: Methods used for genome editing based on CRISPR/Cas technology and characteristics obtained by the plant after mutation in studies on biotic stresses in the last twelve years. Table S3: An assessment of the risk of bias of 296 articles selected for a systematic review on CRISPR/Cas technology used in gene editing in biotic stress tolerance published in the last twelve years. References [169–384] are cited in the Supplementary Materials.

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CAPÍTULO 2

Construction and validation of CRISPR/Cas vectors for editing the *PDS* gene in banana (*Musa* spp.)

Artigo - Construction and validation of CRISPR/Cas vectors for editing the *PDS* gene in banana (*Musa* spp.), aceito para publicação na revista científica Current Issues in Molecular Biology.

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Article

Construction and validation of CRISPR/Cas vectors for editing the *PDS* gene in banana (*Musa* spp.)

Marcelly Santana Mascarenhas [1], Fernanda dos Santos Nascimento [2], Luana Maria Pacheco Schittino [3], Livia Batista Galinari [3], Lucymeire Souza Morais Lino [2], Andresa Priscila de Souza Ramos [2], Leandro Eugenio Cardamone Diniz [4], Tiago Antônio de Oliveira Mendes [3], Claudia Fortes Ferreira [2], Janay Almeida dos Santos-Serejo [2] and Edson Perito Amorim [2,*]

[1] Department of Biological Sciences, Feira de Santana State University, Feira de Santana 44036-900, BA, Brazil; marcelly.bio@hotmail.com (M.S.M.)
[2] Embrapa Mandioca e Fruticultura, Cruz das Almas 44380-000, BA, Brazil; feel.20@hotmail.com (F.d.S.N); lucymeire.lino@gmail.com (L.S.M.L.); andresa.ramos@embrapa.br (A.P.d.S.R); claudia.ferreira@embrapa.br (C.F.F.); janay.serejo@embrapa.br (J.A.d.S-S.); edson.amorim@embrapa.br (E.P.A.)
[3] Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Viçosa, 36507-900, MG, Brazil; luana.schittino@ufv.br (L.M.P.S.); livia.galinari@ufv.br (L.B.G.); tiagoaomendes@ufv.br (T.A.d.O.M.)
[4] Embrapa Soja, Rodovia Carlos João Strass, Londrina 86085-981, PR, Brazil; leandro.diniz@embrapa.br (L.E.C.D.)
* Correspondence: edson.amorim@embrapa.br; Tel.: +55-75-3312-8058; Fax: +55-75-3312-8097

Abstract: Bananas and plantains are important staple food crops affected by biotic and abiotic stresses. The gene editing technique via Clustered Regularly Interspaced Short Palindromic Repeats associated with the Cas protein (CRISPR/Cas) has been used as an important tool for development of cultivars with high tolerance to stresses. This study sought to develop a protocol for the construction of vectors for gene knockout. Here we use the phytoene desaturase (*PDS*) gene as a case study in Prata-Anã banana by nonhomologous end junction method (NHEJ). *PDS* is a key gene in the carotenoid production pathway in plants and its knockout leads to easily visualized phenotypes such as dwarfism and albinism in plants. *Agrobacterium* mediated transformation delivered CRISPR/Cas9 constructs containing gRNAs were inserted into embryogenic cell suspension cultures. This is the first study to provide an effective method/protocol for constructing gene knockout vectors demonstrating gene editing potential in a Brazilian banana variety. The constitutive (CaMV 35S) and root-specific vectors were successfully assembled and confirmed in transformed *Agrobacterium* by DNA extraction and PCR.The specificity of transformation protocols makes it possible to use the CRISPR-Cas9 technique to develop Prata-Anã banana plants with enhanced tolerance/resistance to major biotic and abiotic factors.

Keywords: Bananas; Phytoene desaturase; CRISPR technology; Vector; Promoter; Prata-Anã; Knockout.

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1. Introduction

Bananas and plantains are the most widely grown fruits globally owing to their socioeconomic and nutritional importance. Bananas are one of the main economic resources in several countries, particularly in South America. In 2023, Brazil ranked fifth among the world's largest producers, with a production of 6.8 million tons contributing approximately USD$ 2.0 billion to the fruit agribusiness market. Regarding world production, approximately 135.1 million tons were produced and harvested on 5.9 million ha in 2023 [1].

Banana production is severely restricted by various pathogens, pests, and environmental factors that can hinder its cultivation [2-4]. Diseases caused by Fusarium

oxysporum f. sp. cubense (Foc), Mycosphaerella musicola, M. fijiensis, Ralstonia solanacearum, and banana streak *virus* (BSV), as well as major pests such as the banana rhizome borer (*Cosmopolites sordidus*), and the nematodes *Meloidogyne* spp. and *Radopholus similis* are major challenges for global banana and plantain production [5-7]. Abiotic stresses, such as water deficit and salinity, also threaten agricultural production worldwide, reducing yields and impacting plant growth, physiology, and reproduction [8, 9]. Therefore, using banana varieties that are resistant to diseases, pests, water deficit, and salinity becomes one of the most effective ways to mitigate these negative impacts on fruit production [10]. In addition, bananas are parthenocarpic fruits, which makes classical genetic improvement through crosses extremely laborious since parthenocarpy implies in low seed production.

Genome editing is a tool that allows the manipulation of genetic material to induce mutations in regions of interest, quickly and precisely, resulting in an organism with a desirable characteristics. Its application in crop plants has increased interest primarily because it simplifies regulatory steps [11].

Recent advances in new technical tools can potentially accelerate banana plant breeding to resist main biotic and abiotic factors. The banana genetic breeding program (BGBP) at Embrapa Cassava and Fruit has been investing in research that provide molecular tools to support the development of more resistant/tolerant cultivars. One of these techniques is CRISPR-Cas9 editing [12], which has been successfully used in *Musa* spp. [13-15].

The CRISPR/Cas9 system has been widely used in various plant species to induce mutations in the genome, allowing the study of gene functions for crop genetic improvement. This technique enables editing of genome parts by cutting, replacing, or adding sequences to the DNA of a given genotype [16, 17]. Hence, editing is typically performed using plasmid vector systems that carry genes, which when integrated into the host genome can encode the expression of the necessary products such as a nuclease, typically Cas9, and guide RNA (gRNA). Specific promoters, such as CaMV 35S for constitutive expression, or tissue-specific promoters are also used to regulate the expression of CRISPR/Cas components. In addition, the vectors include a transformant selection marker gene, which confers resistance to antibiotics or herbicides and facilitates the identification of the transformed cells. Eventually, reporter genes such as β-glucuronidase (*GUS)* or Green Fluorescent Protein (*GFP)* are used to monitor the efficiency of the transformation [18-21].

To support the use of CRISPR/Cas9 to increase tolerance for biotic and abiotic stresses in bananas, we propose the knockout of an easily visible gene, such as phytoene desaturase (*PDS*). *PDS* is one of the limiting enzymes in carotenoid biosynthesis, and knockout of the *PDS* gene directly affects photosynthesis, which subsequently leads to albinism and plant growth retardation [13].

This albino phenotype caused by the knockout of the *PDS* gene is easy to visualize and this step, called proof of concept, is critical to start a case study and identify any bottlenecks in the use of CRISPR-Cas9 technology in plants. The proof of concept with gene editing (CRISPR/Cas9) in banana is unprecedented in Brazil, but some work has already been conducted in other countries as a way of evaluating/efficient the technique [13, 14, 22]. Here, we developed an efficient CRISPR/Cas9 vector construction protocol in banana using gRNAs for the phytoene desaturase (*PDS*) gene. The two CRISPR/Cas9 constructs developed, one with a constitutive promoter and the other with a root-specific promoter, were delivered in embryogenic cell suspension cultures of the banana cultivar Prata-Anã (AAB). This is the first work with gene editing using this cultivar, the main banana variety planted in Brazil.

2. Materials and Methods

2.1 Plant material

Suspensions of embryogenic cells from the male inflorescence of the banana cultivar Prata-Anã (AAB) [23] were used as explant sources for genetic transformation via CRISPR-Cas9. The cells were grown and kept in the dark at 27 ± 2 °C on an orbital shaker at 120 rpm and subcultured every 10 days for maintenance at the Plant Tissue Culture Laboratory of Embrapa Mandioca e Fruticultura, Cruz das Almas, Bahia, Brazil (12°40′48.03″S and 39°05′20.91″W). The Prata-Anã cultivar was selected since it is the main banana variety planted and cosumed by Brazilians, however, it is susceptible to Fusarium wilt (Foc) and water deficit.

2.2 Identification of the PDS gene in banana and design of gRNAs

To search for the *PDS* gene in bananas, the complete genome sequences of *Musa acuminata* (Ma08_t16510.2) and *Musa balbisiana* (Mba08_g16040.1) were downloaded from the SouthGreen-Banana Genome Hub database (https://banana-genome-hub.southgreen.fr/). After identifying the conserved *PDS* regions of *M. acuminata* and *M. balbisiana*, specific primers of the banana *PDS* gene [15, 24] were used to rule out allelic variations in the target sites in the cultivars used in our study, and a fragments of 994 bp, 2166 bp, and 332 bp of the *PDS* gene, were sequenced. Genomic DNA was extracted from the leaves of the Bucaneiro (AA), Zebrina (AA), and Prata-Anã (AAB) genotypes, as described in Doyle and Doyle (1990) [25], with modifications proposed by Ferreira et al. (2019) [26]. The bands of interest were identified, selected, and purified using the PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The coding sequences (CDS) from the pair-end sequencing of the *PDS* genes of the Bucaneiro (AA), Zebrina (AA), and Prata-Anã (AAB) banana genotypes were aligned using the Seqassem software (SeqAssm, Sequentix, Klein Raden, Germany) [27] and then aligned with the sequences of the *PDS* gene of *M. acuminata* and *M. balbisiana* using the Clustal Omega software (Clustal Omega, version 1.2.2, EMBL-EBI, Hinxton, Cambridgeshire, UK) to identify conserved regions.

Subsequently, for the design of the gRNAs, the sequences resulting from the alignment were selected based on the positioning of the PAM sequence (Proto-spacer Adjacent Motif 5'-NGG-3); the PAM sequence is required for recognition by the Cas9 endonuclease. The gRNA off-target analysis was carried out by comparing the 20 nt gRNA target sequences in the *PDS* gene with the *M. acuminata* and *M. balbisiana* gene sequences using BLASTN (https://www.ncbi.nlm.nih.gov/) from the SouthGreen-Banana Genome Hub platform and the CRISPOR software (http://crispor.tefor.net/) which also look into consideration the intrisic clivage pattern of the Cas.

2.3 Construction of CRISPR/Cas9 vectors

The construction of the vectors considered the available information on promoter regions, terminators, and restriction enzyme sites: LB (left border), 35S, U6, T7, Cas9, and RB (right border), of twelve pre-existing vectors, and the sequences that were most repeated among the selected vectors were considered to be conserved and used to assemble the new vectors, V1 and V2 (V1: with the constitutive promoter_CaMV35s and V2: *Musa* spp. root-specific promoter – Prom_*Musa*_Embrapa_005).

The first vector had a CaMV 35S promoter and four parts (Part 1 + Part 2 + Cas9 + Part 3) (Table 1). The second vector has a patented banana root-specific promoter (Prom_Musa_Embrapa_005 - patent number: BR 10 2023 010195 0) and is also made up of 4 fragments (Part 1 + Part 4 + Cas9 + Part 3) (Table 1). To construct the binary plant transformation vectors containing Cas9 genes, gRNA, and the other parts, a pDIRECT-22C vector was used. The vectors were designed in the Benchling online software, available at https://www.benchling.com/crispr (acessed on 16 Febrary 2024) to obtain vector maps and restriction enzyme predictions for digestion and validation.

Table 1. Composition of the parts used in constructing the CRISPR/Cas 35S vectors and the specific banana root promoter.

Construction of CRISPR/Cas9 vectors CRISPR/Cas9	
Parts	**Composition**
Part 1	LB_U6 promoter_gRNA BsaI_U6 gene termination signal
Part 2	35s_T7_promoter_constitutive (gRNA with U6 promoter)
Part 3	OCS 3' terminator + RB
Part 4	35s + T7 + gRNA with U6 promoter (promoter banana)
Cas9	Bba_K1218011

LB = left border, RB = Right border, U6: RNA polymerase III; T7: expression - gRNA, OCS: terminator

The vectors were assembled using the manual Master Mix from the GeneArt™ Gibson Assembly® HiFi kit (Invitrogen/ThermoFisher), which allows several DNA fragments to be joined in a single isothermal reaction.

2.4 Transformation and validation of CRISPR/Cas constructs/vectors in Escherichia coli and assembly by Gibson Assembly

To make the *E. coli* DB3.1 and DH5-α cells competent, they were inoculated into liquid Luria-Bertani (LB) culture medium and incubated at 37 °C overnight under 150 rpm agitation. The culture was centrifuged at 5000 rpm for 5 min, the supernatant was discarded, and the pellet obtained was resuspended in 1 mL of 0.1M $CaCl_2$. After this process, the material was centrifuged at 5000 rpm for 5 min (4 °C), the supernatant was discarded, and the pellet was resuspended in 150 µL of 0.1M $CaCl_2$.

After making the cells competent, the parts of the vector were transformed into *E. coli* by heat shock. For each part, 3 µL of the vector containing the respective fragment was added to a 50 µL aliquot of competent cells, which were kept on ice for 30 min. After this period, the inoculum was subjected to a temperature of 42 °C for 45 s. The sample was immediately returned to the ice for 5 min. 1 mL of LB medium without antibiotics was added, and the tube was incubated at 37°C (150 rpm) for approximately 1 h. The material was then centrifuged for 5 min at 5000 rpm, the pellet resuspended in 50 µL of the supernatant and plated on solid LB medium plus kanamycin (50 mg/mL) [28].

Transformed *E. coli* cells were confirmed by extracting plasmid DNA and digesting the parts of the vectors. For Part 1, the restriction enzymes *XbaI* and *HindIII* were used. For Part 2, the *PvuII* enzyme was used, the Cas9 was digested with *EcoRV* and the pDIRECT-22C vector with the *NheI* and *KpnI* enzymes. Digestions were carried out according to each manufacturer's instructions. Once digested, excised, and purified, all parts were quantified and followed the Gibson assembly protocol. The two assembled constructs were transformed in *E. coli* DH5-α using 3 µL of the Gibson Assembly reaction and again confirmed through DNA extraction and PCR.

2.5 Transformation/transfection and validation in Agrobacterium

The protocol for transforming *Agrobacterium tumefaciens* by electroporation was adapted from Höfgen and Willmitzer (1988) [29] and standardized under the conditions described in this section.

To prepare competent cells, 1 mL of *A. tumefaciens* culture grown overnight was inoculated into 1 L of LB medium and incubated at 30°C under agitation. Cell growth was observed until the logarithmic phase (OD_{600} 0.5–0.6), pelleted by centrifugation at 2600 xg for 10 min at 25°C. The pellet was resuspended in 10 mL of ice-cold 10% glycerol and centrifuged again. The 500 µL of 10% glycerol was added to the new pellet, and the competent cells were distributed in 50 µL aliquots. The bacteria were plated on a selective LB medium containing kanamycin (50 mg/mL).

A 3 µL (100–200 ng) of plasmid DNA extracted from transformed *E. coli* DH5-α was added to 50 µL of *Agrobacterium* competent cells and gently homogenized to transform the two constructs. The cells were transferred to an electroporation cuvette (2 mm) and taken to the electroporator, where 2.5 kV, 25 µF, and 400 Ω pulses were applied. A 1 mL

Super Optimal Medium with Catabolic Repressor (SOC) was immediately added. The material was transferred to microtubes and kept on a shaker at 200 rpm for 2–3 h at 30 °C. Subsequently, it was centrifuged and resuspended in 100 μL of its supernatant, plated on solid LB medium with kanamycin (50 mg/mL) and kept at 28 °C for 42–72 h. Confirmation of *Agrobacterium* transformant cells was carried out via plasmid DNA extraction and PCR using vector-specific primers.

2.6 *Confirmation of the integration of CRISPR/Cas constructs*

For plasmid DNA extraction, 5 mL of the *Agrobacterium* inoculum was centrifuged at 10000 rpm for 2 min, the supernatant was discarded and 200 μL of ice-cold solution I (Table S1) and 10 mg/mL RNAse at 10% v/v, were added. The material was incubated for 10 min at room temperature. After this period, 200 μL of ice-cold solution II (Table S1) was added. The tubes were gently homogenized and kept on ice for 5 min. Subsequently, 200 μL of ice-cold solution III (Table S1) was added, and the tubes were gently homogenized and kept on ice for 5 min. The material was centrifuged at 10000 rpm for 10 min, and 400 μL of the supernatant was collected and poured into new microtubes. Briefly, 800 μL of ice-cold isopropanol was added, homogenized, and samples kept at -20 °C for at least 1 h.

After this period, it was centrifuged at 10000 rpm for 15 min, and the supernatant was discarded. Ice-cold 70% ethanol (500 μL) was added to the pellet, the sample was centrifuged at 10000 rpm for 15 min, and the supernatant was discarded. The pellet remained at room temperature (or in a dry bath at 45 °C), followed by resuspension in approximately 20 μL of nuclease-free water and kept in a freezer (-20 °C). DNA quantification was performed on Quibit and 1% agarose gels in TAE 1X (adapted from Sambrook et al., 1989).

Plasmid DNA extracted from *Agrobacterium tumefaciens* transformed with constructs 1 and 2 was subjected to PCR using primers Vc9_Fw and Vc9_Rv, as well as Vp3C9_Fw and Vp3C9_Rv (Table 2), to confirm parts 1, 2, 3, 4, vector, and Cas9.

Table 2. Specific primers for confirmation of constructs 1 and 2 assembled by Gibson Assembly after transformation in *Agrobacterium tumefaciens*.

Name	Seq 5'-3'	pb	%GC	Ta	Amplicon
Vc9_Fw	CTACCCTCCGCGAGATCATC	20	60%	45°C	1912pb C1
Vc9_Rv	CGACCTCATCCACAATGTTGC	21	52.4%	45°C	1587pb C2
Vp3C9_Fw	GCGTTACCTTCCAAATACGTG	21	47.6%	51°C	988pb
Vp3C9_Rv	CGCACGGTGAAACAGAAC	18	55.6%	51°C	988pb

The samples were amplified in a Veriti thermal cycler (Applied Biosystems, Waltham, MA, USA) with programming adapted from Cellco (Cellco Inc., Germantown, MD, USA). The primer sequences and annealing temperatures are shown in Table 2. The amplification products were separated through electrophoresis on a 1% agarose gel at 70 V in TAE buffer for 45 min in ethidium bromide. They were then visualized and photographed under ultraviolet light on an L-Pix Touch documentation system (Loccus, Cotia, Brazil).

2.7 Delivery of the CRISPR/Cas9 plasmid to Prata-Anã cells in suspension.

The plant embryogenic cell suspension culture (ECS) was diluted to 33% (v/v) in liquid Dichlorophenoxyacetic acid (2, 4-D) medium supplemented with acetosyringone (AS) 200 μM. The four treatments were arranged in 24-well plates. For treatment 1 (T1), 200 μL plant cell (PC) and 1 mL liquid 2,4-D culture medium were added. For treatment 2 (T2), 200 μL of PC and 1 mL of *Agrobacterium* ($OD_{600;\ 1.2un}$) with empty vector was added. Treatment 3 (T3) involved addition of 200 μL PC and 1 mL of *Agrobacterium* transfected

with construct 1 (V1_CaMV35s), whereas treatment 4 (T4) involved addition of 200 µL PC and 1 mL of *Agrobacterium* with construct 2 (V2_Musa_root specific promoter).

The plates were incubated for 6 h at 25 °C and 25 rpm on a rotary shaker in the dark. The mixture of plant and bacterial cells was transferred to a sterile 50 µm polyester mesh (4 cm^2) deposited on three sterile filter papers to remove excess liquid medium. The polyester membrane containing the cells was transferred to co-cultivation plates containing 10 mL of solid 2, 4-D medium (pH 5.3) supplemented with AS and incubated at 26 °C in the dark for 6 days.

After this period, the membranes were transferred to plates with solid 2, 4-D culture medium supplemented with timentin (200 mg/L) and kanamycin (50 mg/mL) and incubated in the dark at 25 ± 2 °C for 30 days. The membranes were then transferred to BAP (6-benzylaminopurine) + AIA (indoleacetic acid) medium, plus timentin and kanamycin, where they remained for 15 days in the dark at 25 ± 2 °C temperature. The plates were transferred to a 16 h/8 h photoperiod at 25 °C and subcultured every 30 days if necessary.

3. Results

3.1 Design of the gRNAs

By aligning the CDS resulting from the sequencing of the *PDS* genes of the Bucaneiro (AA), Zebrina (AA), and Prata-Anã (AAB) banana genotypes with the *PDS* gene sequences of *M. acuminata* (Genome A) and *M. balbisiana* (Genome B), the gRNAs could be selected. The off-target activity of the four gRNAs was assessed using BLASTN on the SouthGreen-Banana Genome Hub platform. All the gRNAs showed 95% nucleotide homology with the banana *PDS* gene (Figure 1).

```
Buca    AACTTACCATGAAAAGTTGCAGGGTGATGAACTGATGATTTTAGAACTGGGACATGCAAT    496
Zebr    AACTTACCATGAAGAGTTGCAGGGTGATGAACTGATGATTTTAGAACTGGGACATGCAAT    485
Prat    AACTTACCATGAAGAGTTGCAGGGTGATGAACTGATGATTTTAGAACTGGGACATGCAAT    462
Ma08    AACTTACCATGAAGAGTTGCAGGGTGATGAACTGATGATTTTAGAACTGGGACATGCAAT    1055
MbIT    AACTTACCATGAAGAGTTGCAGGGTGATGAACTGATGATTTTAGAACTGGGACATGCAAT    1010
        ************* ************************************************

Buca    AAAAACAGAAGGAACTGTACCATAAAGAACACTAACCTATATTAAAATATTTTGAGATTG    556
Zebr    AAAAACAGAAGGAACTGTACCATAAAGAACACTAACCTATATTAAAATATTTTGAGATTG    545
Prat    AAAAACAGAAGGAACTGTACCATAAAGAACACTAACCTATATTAAAATATTTTGAGATTG    522
Ma08    AAAAACAGAAGGAACTGTACCATAAAGAACACTAACCTATATTAAAATATTTTGAGATTG    1115
MbIT    AAGAACAGACGGAACTGTACCATAAAGAACACTAACCTATATTAAAATATTTTGAGATTG    1070
        ** ****** ************************************************

Buca    TTTAAGTCTTCTAGTTCGGTTTGCAACTTATGAATTGACAGAAAATAAGTTAGTCCGGAA    616
Zebr    TTTAAGTCTTCTAGTTCGGTTTGCAACTTATGAATTGACAGAAAATAAGTTAGTCCGGAG    605
Prat    TTTAAGTCTTCTAGTTCGGTTTGCAACTTATGAATTGACAGAAAATAAGTTAGTCCGGAG    582
Ma08    TTTAAGTCTTCTAGTTCGGTTTGCAACTTATGAATTGACAGAAAATAAGTTAGTCCGGAG    1175
MbIT    ATTAAGTCTTCTAGTTCAGTTTGCAACTTATGAATTGACAGAAAATAAGTTAGTCCGGAG    1130
         ***************** ******************************************

Buca    CACTGATGCATTCAAAATAGACAAAATATACAATTTGAAGCAATCATTGTACATGCTACT    676
Zebr    CACTGATGCATTCAAAATAGACAAAATATACAATTTGAAGCAATCATTGTACATGCTACT    665
Prat    CACTGATGCATTCAAAATAGACAAAATATACAATTTGAAGCAATCATTGTACATGCTACT    642
Ma08    CACTGATGCATTCAAAATAGACAAAATATACAATTTGAAGCAATCATTGTACATGCTACT    1235
MbIT    CATTGATGCATTCAAAATAGACAAAATATACAATTTGAAGCAATCATTGTACATGCTACT    1190
        ** ************************************************************

Buca    AATGCCGGGCCATGCTCTCTGGGACATACCATGTTGGTGTGGCACCATCATGAATTATTT    736
Zebr    AATGCCGGGCCATGCTCTCTGGGACATACCATGTTGGTGTGGCACCATCATGAATTATTT    725
Prat    AATGCCGGGCCATGCTCTCTGGGACATACCATGTTGGTGTGGCACCATCATGAATTATTT    702
Ma08    AATGCCGGGCCATGCTCTCTGGGACATACCATGTTGGTGTGGCACCATCATGAATTATTT    1295
MbIT    AATGTCGGGCCATGCTCTCTGGGACATACCATGTTGGTGTGGCACCATCATGAATTATTT    1250
        **** ************************************************************

Buca    ACCTCATTGAGATTGTTGTTATAGACACTAGATAACACTTCTGCAAATTAGTGGTGGAGC    796
Zebr    ACCTCATTGAGATTGTTGTTATAGACACTAGATAACACTTCTGCAAATTAGTGGTGGAGC    785
Prat    ACCTCATTGAGATTGTTGTTATAGACACTAGATAACACTTCTGCAAATTAGTGGTGGAGC    762
Ma08    ACCTCATTGAGATTGTTGTTATAGACACTAGATAACACTTCTGCAAATTAGTGGTGGAGC    1355
MbIT    ACCTCGTTGAGATTGTTGTGATAGACACTAGATAACACTACTGAAAATTAGTGGTGGAGC    1310
        ***** ************** ******************** *** ****************

Buca    TATTTACATAAATATTTTTATGCCTTGACCAATTTTATAATTTTTTGGCAGGAATATTTT    856
Zebr    TATTTACATAAATATTTTTATGTGTTGACCAATTTTATAATTTTTTGGCAGGAATATTTG    845
Prat    TATTTACATAAATATTTTTATGCCTTGACCAATTTTATAATTTTTTGGCAGGAATATTTG    822
Ma08    TATTTACATAAATATTTTTATGCCTTGACCAATTTTATAATTTTTTGGCAGGAATATTTG    1415
MbIT    TATTTACATAAATATTTTTATGCCTTGACCAATTTTATAATTTTTTGGCAGGAATATTTG    1370
        ********************  ************************************
```

Figure 1. Partial alignment resulting from the sequencing of the *PDS* genes of the banana genotypes Bucaneiro (AA), Zebrina (AA), and Prata-Anã (AAB) and sequences of the *PDS* gene of *Musa acuminata* and *Musa balbisiana* for gRNA design. The blue arrows indicate the selected gRNAs. Yellow highlights represent the gRNA nucleotides, and green highlights indicate the PAM sequences.

Two gRNAs were selected (indicated by blue arrows) to maximize the chances of mutations and deletion of large fragments as the gRNAs targets two different exons of the *PDS* gene in Prata-Anã, which contains both genomes (genome A and B). The gRNA1 (GAACTGATGATTTTAGAACTGG) and gRNA2 (GACCAATTTATAATTTTTTGG) were integrated into the constructs.

3.2. Construction of CRISPR/Cas vectors

The parts of the constructs (Part1_LB_U6pro_sgRNABsaI_U6term; Part2_35s_T7_promotor_const; Part3_OCSterm_RB; Part4_35s_T7_promotor; Cas9) were synthesized, the sequences in common with various vectors were compared, and those with the highest level of conservation were selected to create the new vectors.

After selecting the sequences and constructing the parts, the maps of the vectors (Figure 2) for gene editing in bananas via *Agrobacterium* transformation were drawn up.

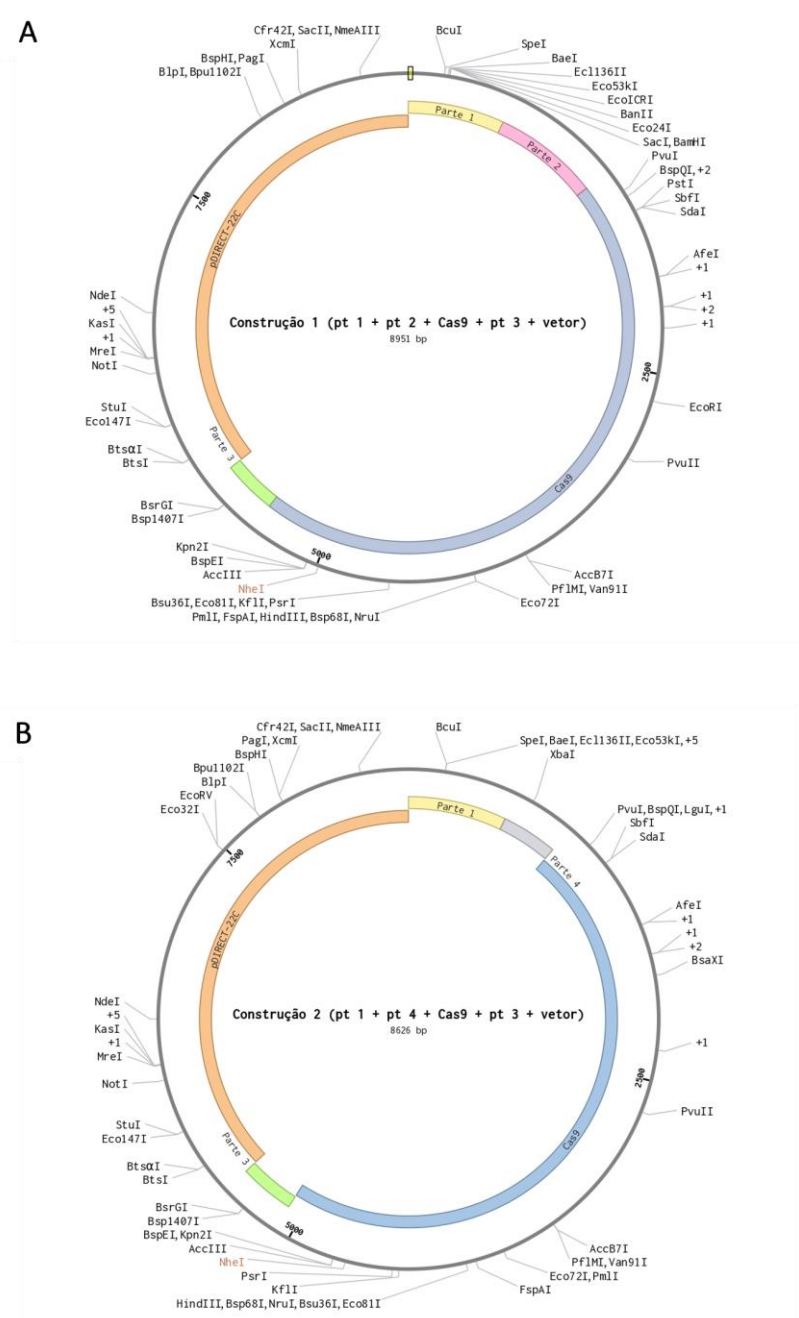

Figure 2: Maps of the vectors with CaMV 35S promoter and Part 1 + Part 2 + PCR Cas9 + Part 3 + Vector pDIRECT-22C (A) and vector with root-specific promoter with Part 1 + Part 4 + PCR Cas9 + Part 3 + Vector pDIRECT-22C (B) for use in banana cisgenesis via *Agrobacterium* transformation. The outer part of the vector contains the possible restriction enzymes for digesting the vectors.

3.3. Standardization of protocol for processing and digestion of parts

The competence of *E. coli* DB3.1 and DH5a and transformation by heat shock were standardized and confirmed by DNA extraction and digestion of the parts. Parts 1, 2, 3, 4, and Cas9 were transformed into *E. coli* DH5-α and pDIRECT-22C into *E. coli* DB3.1 to multiply the plasmids. The transformant colonies underwent a plasmid DNA extraction process (miniprep). The Part 1 miniprep was digested with the restriction enzymes XbaI (Cellco) and HindIII (Promega), and a 699 bp fragment was released (Figure 3). The Part 2 miniprep was digested with the restriction enzyme PvuII (Jena Bioscience), releasing a fragment of 697 bp (Figure 3).

Cas9 is one of the essential parts for Gibson's assembly of the vector and is largely responsible for the functioning of the CRISPR/Cas system. After transformation and selection in a medium containing the antibiotic kanamycin (50 mg/mL), plasmid DNA was extracted and digested by the EcoRV restriction enzyme, resulting in the release of a 5115pb fragment (Figure 3).

The pDIRECT-22C vector is also an important element for assembling the vector of interest by Gibson Assembly, as it contains overlap and is widely used for gene expression in plants. This vector contains a gene for resistance to the antibiotic kanamycin. Therefore, it was also transformed into *E. coli* DB3.1 and selected in a culture medium containing the antibiotic kanamycin (50 mg/mL). Plasmid DNA was extracted, and the resulting sample was digested using the restriction enzymes NheI and KpnI (Cellco), releasing a 5400 bp fragment (Figure 3), which, like the other fragments, was excised and purified.

Parts 3 and 4 were not cloned in the vector; hence, digesting and purifying them was not necessary; they were just resuspended in 20 μL of nuclease-free water to a final concentration of 25 ng/μL.

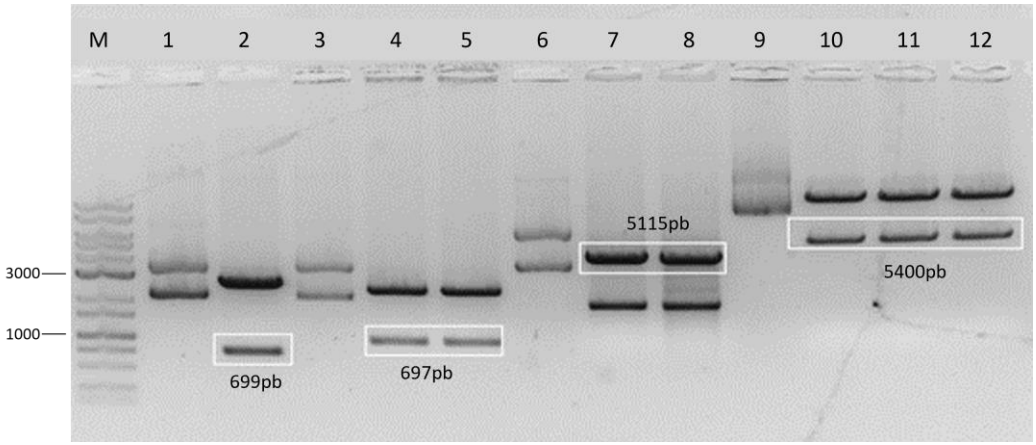

Figure 3: Digestion of the parts and vectors of interest in Gibson Assembly. Mw: 1 kB Plus NeoBio molecular weight marker (CV-1000 kB); 1: miniprep from part 1 (undigested control); 2: 699 bp fragment excised from part 1; 3: miniprep from part 2 (undigested control); 4 and 5: 697 bp fragments excised from part 2; 6: Cas9 miniprep in pUC57 (undigested control); 7 and 8: 5115 bp fragments excised from Cas9; 9: pDIRECT-22C vector miniprep (undigested control); 10, 11, and 12: 5400 bp fragments excised from pDIRECT-22C.

3.4. Quantification of vector parts and assembly by Gibson Assembly

Parts 1, 2, 3 and 4, previously digested, excised and purified Cas9 and pDIRECT-22C, were assayed by fluorimetric quantification using a Qubit 4 fluorometer according to the manufacturer's protocol.

The assembly protocol suggests a concentration of 0.08 pmols of each of the inserts in the reaction. To assemble the Gibson Assembly, the necessary amount, in μL, of each part was calculated (Table 3), combining these fragments in a single enzymatic reaction. Accurate quantification and the use of the correct molar proportions between the fragments and the vector are critical for the efficient assembly by Gibson Assembly.

3.5. Transformation and confirmation of constructs in Agrobacterium tumefaciens

After standardizing and confirming the protocol developed, the two assembled constructs (V1_CaMV35s and V2_Musa_Root) were transformed into *Agrobacterium* using approximately 3 μL (100–200 ng) of plasmid DNA extracted from the transforming *E. coli* strains previously selected for kanamycin resistance and through the electroporation

transformation protocol. The constructs were confirmed through DNA extraction (Figure 4) and PCR (Figure 5).

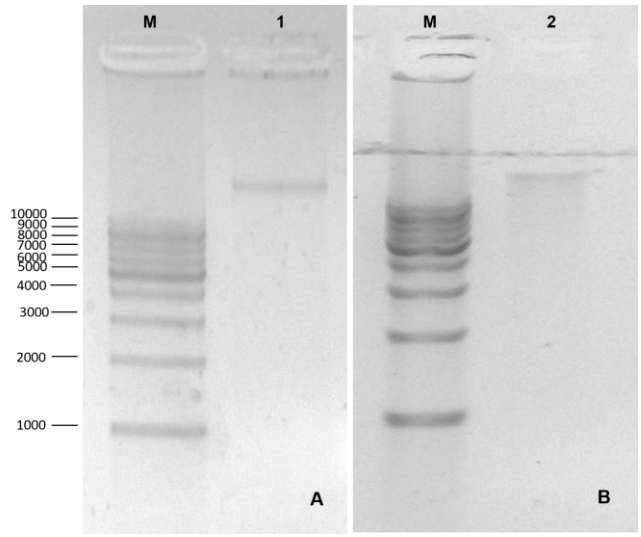

Figure 4: Extraction of plasmid DNA from the constructs inserted into *Agrobacterium tumefaciens*. M: molecular weight marker 1kB DNA Ladder - MMK-105S (Cellco); 1 and 2: amplicons with more than 10000 bp equivalent to construct 1 (a) and 2 (b).

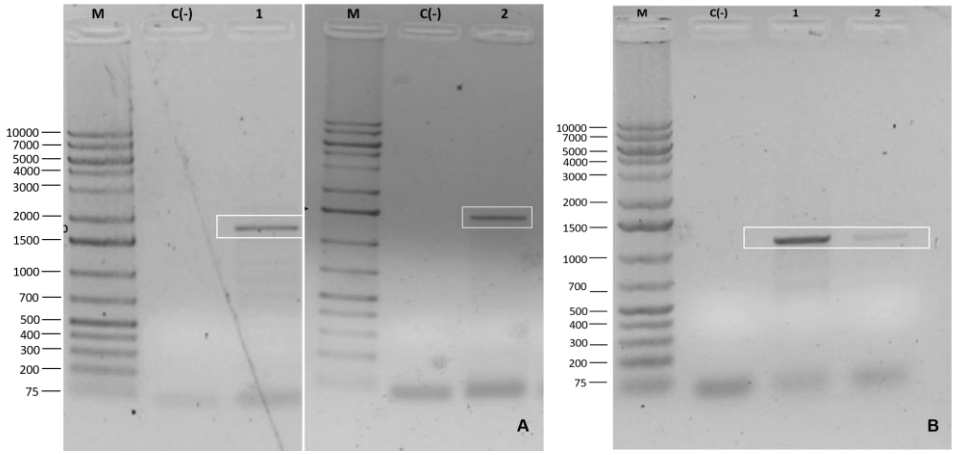

Figure 5 PCR amplification of the *Agobacterium tumefaciens* strain transformed with constructs 1 and 2. (a) PCR with primer set VC9_Fw and VC9_Rv (M: molecular weight marker 1kB Plus DNA Ladder - MMK-130S (Cellco); C(-): negative control of the reaction with water; 1 and 2: 1912 bp amplicons at 45 °C for constructs 1 and 2. (b) PCR for primers Vp3C9_Fw and Vp3C9_Rv; M: marker; C(-): negative control; 1 and 2: 1000 bp amplicons at 51 °C.

3.6. *Delivery of the CRISPR/Cas9 plasmid in banana cv. Prata-Anã*

Mutants were generated by delivering the two CRISPR/Cas9 constructs (V1_CaMV35s and V2_Musa_Raiz) into a suspension of embryogenic cells from the Prata-Anã (AAB) cultivar using transformation via *Agrobacterium*. The treatments were organized into 24-well plates, with treatments comprising as follows: T1 consisting of the plant cell (PC) and liquid 2,4-D culture medium; T2 consisting of PC and empty vector; T3 consisting of PC and Construction 1; and T4 consisting of PC and Construction 2 (Figure 6).

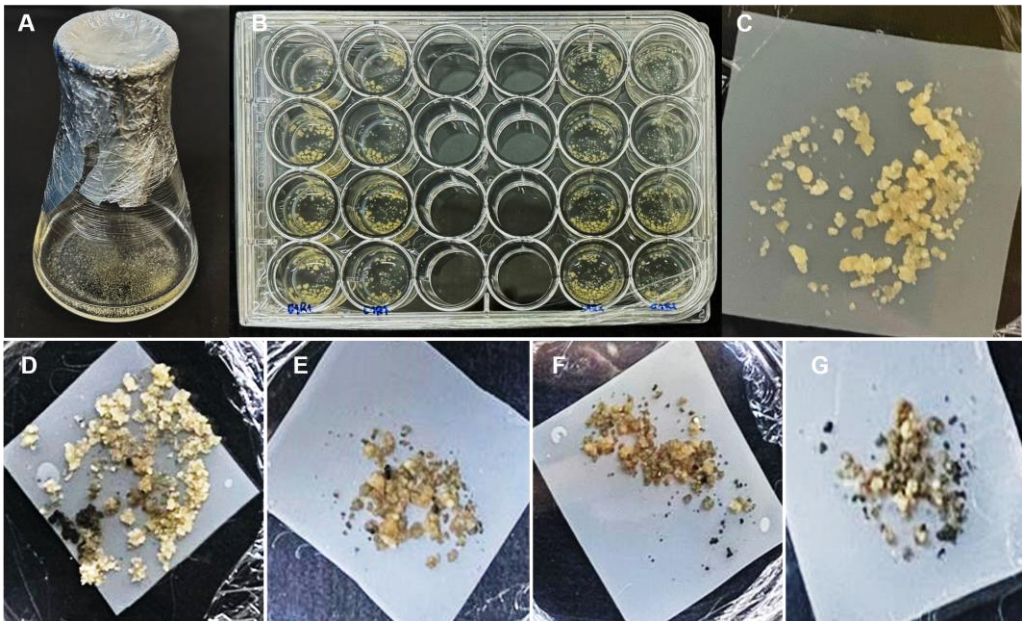

Figure 6. Process of genetic transformation and regeneration of the Prata-Anã banana. (a) Embryogenic cell suspension used for transformation with the CRISPR/Cas9 plasmid; (b) embryos in 2, 4-D culture medium and transforming *Agrobacterium*; (c) embryos in polyester membrane and 2, 4-D+AS culture medium (T1) after 0 days and the different treatments (d, e, f, g) with BAP+AIA+Kan+Timentin culture medium (d: T1, e: T2, f: T3, g: T4) after 70 days of transformation, kept at 25 °C with a 16 h/8 h photoperiod.

The germinating plant cells were transferred to jars or test tubes with solid BAP + AIA medium for regeneration.

4. Discussion

Banana production is severely affected by biotic and abiotic factors. Among the biotic factors, fusarium wilt (STR4 and TR4) is the most destructive. Considering abiotic factors, water deficit, also brought by climate changes, not only leads to losses in production, but also contributes to intensify biotic stresses already at play [30]. Given this scenario, adequate technologies are essential to develop new banana genotypes endowed with durable and broad-spectrum tolerance or resistance. Traditional breeding cannot always produce new genotypes that deal with rapid changes. In the case of banana plants, classical breeding is even more time-consuming owing to the inherent parthenocarpy of the species, which leads to low seed production.

Gene editing technology makes it possible to modify the target DNA at a specific location precisely and quickly, inducing specific genetic variations but maintaining the genetic identity of elite cultivars [31, 32]. Developing well-defined methods and protocols using this technology allows reproducibility, consistency and reliability, versatility, minimization of editing errors, facilitation of systems delivery to the site of interest, and promotion of resource savings.

CRISPR/Cas vector systems are well-designed genetic constructs made up of a Cas9 enzyme or its variants, such as Cas12a (Cpf1), Cas13, and others, responsible for cleaving the DNA and RNA respectively at the site of interest, which allows for precise editing, as well as a guide RNA that directs the enzyme to the target of the mutation. The system also includes promoters that can be constitutive, tissue-specific, or inducible; insertion sequences, selection markers, cloning, and replication elements are also part of this system [33-35].

The 35S promoter from the cauliflower mosaic virus (CaMV 35S) is widely used in plants to ensure the continuous expression of genes that regulate all cell types. Its

application is well reported in several crops such as rice [36-39], tomato [40, 41], and soybean [42, 43], with successful cases for the expression of Cas9. However, tissue-specific promoters are activated only in the designated tissues or at specific moments in development, with precise regulation of expression, reducing the chances of off-target mutations and the involvement of other tissues [44, 45].

Krasnyanski et al. (2001) [46] used two promoters derived from a binary vector to transform tomatoes. The *uidA* gene was driven by the CaMV 35S/AMV or the E-8 promoter specific for fruit ripening. The authors successfully expressed the gene with both promoters, and the specific promoter was not expressed in other tissues, only in the fruit.

Although the CaMV 35S promoter is widely used, growing efforts are needed to explore tissue-specific promoters to achieve more efficient and targeted expression. This study developed two vectors using the CaMV 35S promoter and the root-specific promoter (Prom_Musa_Embrapa_005 - patent number: BR 10 2023 010195 0). The root-specific promoter only activates the transcription of genes locally in root cells, ensuring that their essential functions, such as nutrient and water absorption, hormone production, substance transport, food storage, interaction with microorganisms and other functions, are performed optimally [33, 47]. This type of promoter can act by recognizing specific regulatory elements, interacting with transcription factors, environmental signals or tissue restriction [48, 49].

Diseases such as *Fusarium* wilt and nematodes are transmitted through the soil. Using a root-specific promoter associated with the CRISPR/Cas system offers an advantage for increasing disease resistance in plants as it is a more precise and efficient approach to the local defense response. When the system is activated in the roots, genes related to stress signaling or the synthesis of antimicrobial compounds, such as phytoalexins and specific proteins, as well as plant hormones, can be expressed and enable the development of localized resistance, minimizing off-target effects on other plant structures [48, 33, 44].

In soybean, the GmADR1 promoter was used to direct the expression of the GmCaM4 gene in root tissues, and the plants showed high resistance to salinity [44]. The maize *Chitinase A1* and *Phospholipid transferase* promoter (pZmCTA1 and pZmPLTP) were used to design a specific callus system with beneficial effects on hereditary mutations and a reduction in somatic mutations [50].

In Arabidopsis and soybean, the GmPRP2 promoter was tested for its expression pattern, and it was concluded that this is a preferential root promoter and can be used to improve functions related to plant roots, such as nutrition, tolerance, or resistance to biotic and abiotic stressors [51]. The TIP2 promoter and 18 other promoter sequences were evaluated for specificity in banana and *Nicotiana tabacum* (tobacco) roots. These promoters could be used in new CRISPR/Cas constructs, expanding the options for controlling diseases, pests, and other stresses [52].

The use of specific promoters in the CRISPR/Cas system has been explored and their successful integration into the CRISPR/Cas system, confirmed; the choice of promoters depend on the tissue to be studied and the objective of the experiment [53, 54]. The authors successfully confirmed the integration of promoters into a CRISPR/Cas9 system to facilitate research focused on plant breeding using specific or constitutive promoters for a better understanding of the mechanisms surrounding the expression of genes of interest and for validation using transformed plants.

CRISPR/Cas vectors allow the efficient delivery of essential elements such as the Cas protein, gRNA and other components. The Cas9 protein belongs to type II class II and was developed from *S. pyogenes*. Cas9 nucleases are guided by CRISPR RNAs (crRNAs), which resemble transactivating crRNAs (tracrRNAs) and facilitate the formation of the ribonucleoprotein complex [34]. However, most Cas9 genome editing applications use the gRNA molecule, designed by fusing crRNA and tracrRNA into a single RNA molecule.

In most cases, CRISPR/Cas9 requires a target site of 17 to 20 base pairs (bp) directly adjacent to a 5'-NGG PAM sequence (motif adjacent to the protospacer) to be effectively recognized by the gRNA, as used in this study [55, 56].

Promoters control the delivery of these genes, increasing the precision of gene editing. Different vectors can be found for different organisms. pCAMBIA is a binary vector that is widely used owing to its versatility, high efficiency of *Agrobacterium* mediated transformation in mono- and dicot species, and easy manipulation in the laboratory [57, 14]. The authors analyzed several vectors already available in the literature to construct the final vector. The regions to make up the new vector were chosen based on the degree of homology between the nucleotide sequences of each structure. pCAMBIA was found in most of the works and has a high degree of conservation, which justifies its use.

In the present study, the cloning process with the pDIRECT-22C vector and genetic construction was carried out in *E. coli.* Once Gibson Assembly had assembled the DNA vector in an *in vitro* reaction, the plasmids were transferred to *A. tumefaciens*. This vector is often used for cloning, as it supports multiple gRNAs and multiplex manipulation of genes. It also has antibiotic resistance genes such as kanamycin and ampicillin, for selection of transformed cells with the vector insert [58, 59].

Various plant transformation methods can be used to apply the CRISPR/Cas system. The most common is transformation mediated by *A. tumefaciens*, a bacterium capable of transferring its genetic material into the plant genome. The method has high efficiency, stability in delivering transgenes, less physical damage to DNA and can be used in different species. However, its application in monocots is limited [57, 60, 61].

Explants are small fragments of living tissue that can be removed from different parts of a plant, such as roots, stems, and leaves, and play an important role in the efficiency of transformation [62]. In bananas, stem apices, meristems and male inflorescences can be used as a source of explants. In this study, we chose to use embryos from male inflorescences due to the easy regeneration of transformed plants [63, 64]. When compared to other types of explants, such as leaves or roots, these cells offer a higher transformation rate and a lower frequency of somaclonal mutations due to their greater totipotency capacity [65-67]. The efficiency of *Agrobacterium* mediated transformation in embryogenic cells can be attributed to their greater cellular competence, allowing effective integration of the transferred DNA and resulting in more viable mutants [68, 69]. These data suggest that, although different explants can be successfully used in genetic transformation, embryogenic cells represent a superior option for transformation efficiency and plant regeneration, especially for recalcitrant species.

Knockout of the *PDS* gene in banana plants and other crops using CRISPR/Cas9 technology has been widely studied due to its function as a phenotypic marker of successful editing. Disruption of this gene causes a loss of function in carotenoid biosynthesis, resulting in albino plants, which allows direct assessment of transformation efficiency [15, 42]. Studies conducted on Cavendish banana cultivars used embryogenic cell suspensions to knock out the *PDS* gene, resulting in a high regeneration efficiency and expression of the albino phenotype [14]. These findings reinforce the importance of properly selecting explants to maximize the success of transformation and gene editing via CRISPR/Cas in plants.

The development of an efficient protocol for constructing CRISPR/Cas9 systems using constitutive promoters (such as CaMV 35S) and tissue-specific promoters, followed by *A. tumefaciens-mediated* transformation and knockout of the *PDS* gene in banana, represents a significant advance in plant biotechnology. This protocol allows precise gene editing, with the *PDS* gene acting as a visual marker to confirm the effectiveness of the editing as its interruption results in an albino phenotype in the plants.

Selecting explants with a high regenerative capacity is essential to ensure efficient integration of the CRISPR/Cas vector and the regeneration of viable plants. Using

constitutive promoters ensures broad and continuous expression of the Cas9 endonuclease throughout the plant. In contrast, specific promoters can regulate expression in target tissues, offering high precision in the edits. This approach highlights the importance of combining a robust vector construct with the careful selection of explants and promoters, resulting in an efficient methodology for plant breeding.

5. Conclusions

In this study, we developed a vector construction protocol, validated a construct/cassette as a biotechnological product, for knockout of the *PDS* gene in Prata-Anã banana using CRISPR/Cas9 technology. This study enables the continuation of recent research focused on the genetic improvement of bananas against biotic and abiotic stressors, such as Fusarium wilt and water deficit. The methods used so far to edit genes using CRISPR/Cas9 technology have proved successful in various crops. The consolidation of a constitutive and root-specific vector/promoter and the possibility of knocking out the *PDS* gene (proof of concept) in the Prata-Anã cultivar is unprecedented in Brazil. The vectors developed here will be used in future studies to knock out genes for resistance/tolerance to biotic and abiotic stresses in banana varieties of commercial interest. However, challenges such as variability in transformation efficiency, possibility of off-target effects, and limitations imposed by specific PAM sequences still need to be overcome. In the future, the integration of new endonuclease variants and accessory tools may improve the performance of the technique. This protocol can be used to optimize other methods of delivering the components of the CRISPR/Cas system, such as biobalistics, Ribonucleoproteins (RNPs), and protoplasts (transfection/electroporation), and can be used in vegetative propagated species for resistance/tolerance to biotic and abiotic factors, which will also have a significant impact on genetic improvements in agriculture by addressing the challenges of global food security.

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Supplementary materials

Table S1. Preparation of could solutions.

Solution 1	Solution 2	Solution 3
Tris HCl 1M; pH 8,0; 25mM (0,25 mL); EDTA 0,5M; pH 8,0; 10mM (0,2 mL); Glucose 500mM; 50mM (1,0 mL); Destilled water (8,55 mL); pH 8,0; Autoclave for 20 minutes.	Distilled water (8,0 mL); NaOH 2M (1,0 mL); SDS 10% (1,0 mL).	Potassium acetate 5M; pH 5,5 (29,45g/60 mL of H_2O); Acetic acid (11,5 mL); Destilled water (28,5 mL); pH 5,5.

CONCLUSÃO GERAL

Esta tese buscou investigar por Revisão Sistemática (RS) o uso da tecnologia CRISPR/Cas na edição de genes relacionados a resistência/tolerância a estresses bióticos em plantas nos últimos doze anos. Dados de 296 estudos realizados em diferentes espécies foram sistematizados e agrupados. Progressos significativos ao longo desses anos foram observados, consolidando sua posição como uma ferramenta promissora para o melhoramento genético de plantas. A combinação desta tecnologia com vetores bem delineados e *Agrobacterium* destaca a sua eficácia em múltiplas espécies, avançando assim na compreensão dos mecanismos de defesa das plantas.

A possibilidade de diversificação dos métodos de edição genética utilizando outras enzimas como Cas12a (Cpf1) e Cas13 ampliam as possibilidades de aplicação em pesquisas futuras. A complexidade dos mecanismos de defesa das plantas contra stresses bióticos ainda requer maior investigação, dadas as complexas interações entre patógenos e hospedeiros. Portanto, a exploração contínua desta tecnologia, combinada com novos conhecimentos sobre redes de defesa vegetal, podem fornecer soluções inovadoras para os desafios do stresse biótico e contribuir significativamente para o desenvolvimento agrícola sustentável.

Embora o uso da tecnologia CRISPR/Cas tenha revolucionado o melhoramento de plantas nos últimos anos, ainda há muitos desafios a serem superados, como efeitos fora do alvo, a eficiência da entrega do sistema CRISPR em células vegetais, questões éticas e regulatórias, bem como questões ambientais e de biossegurança. Muitos estudos estão sendo realizados sobre o assunto, no entanto, existem apenas alguns produtos comercializáveis.

Estudos sobre edição genética com CRISPR/Cas para resistência a agentes bióticos estão apenas começando. Os resultados obtidos até agora não só mostram que esta tecnologia oferece modificações precisas no genoma da planta e tem sido usada com sucesso para conferir resistência a doenças e pragas, mas também são essenciais principalmente para compreender a função de genes relacionados a várias vias de interação planta-patógeno e podem atuar na resistência de amplo espectro com base na edição multilocus.

As informações obtidas a partir da RS foram utilizadas para auxiliar a construção e validação de vetores CRISPR/Cas para edição do gene *PDS* em bananeira. Um protocolo de construção de vetores permite a continuação de pesquisas focadas no melhoramento genético de bananas contra estressores bióticos e abióticos. Os métodos usados até agora para editar genes usando a tecnologia CRISPR/Cas9 têm se mostrado bem-sucedidos em várias culturas.

A consolidação de um vetor/promotor constitutivo e específico de raiz e a possibilidade de knockout do gene *PDS* (prova de conceito) na cultivar Prata-Anã é inédita no Brasil. Os vetores aqui desenvolvidos serão usados em estudos futuros para *knockout* de genes para resistência/tolerância a agentes bióticos e abióticos em variedades de banana de interesse comercial. Este protocolo pode ser usado para otimizar outros métodos de entrega dos componentes do sistema CRISPR/Cas, como biobalística, ribonucleoproteínas (RNPs) e protoplastos (transfecção/eletroporação) o que trará um impacto significativo em melhorias genéticas na agricultura.