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JULIA BIBIANA MERCHÁN GAITÁN

Regulación de plantas genéticamente editadas y selección del gen SAMT para la edición de *Carica papaya* buscando resistencia a Meleira.

VITÓRIA, ES

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Tesis doctoral presentada al Programa de Pós-Graduação em Biotecnologia do Centro de Ciências da Saúde da Universidade Federal do Espírito Santo, como requisito parcial para la obtención del título de Doctor en Biotecnología.

Orientadora: Profa. Dra. Patricia Machado Bueno Fernandes
Co-orientador: Prof. Dr. Silas Pessini Rodrigues

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UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
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Ata da 26ª sessão de Defesa de Tese do Programa de Pós-Graduação em Biotecnologia, do Centro de Ciências da Saúde da Universidade Federal do Espírito Santo, da discente JULIA BIBIANA MERCHÁN GAITÁN, realizada às catorze horas do dia nove de maio de dois mil e vinte e quatro, na sala de Webconferência do Programa de Pós-Graduação em Biotecnologia, tese intitulada "Regulación de plantas genéticamente editadas y selección del gen SOMT para la edición de *Carica papaya* buscando resistencia a Meleira/Regulamentação de plantas geneticamente editadas e seleção do gene SOMT para a edição de *Carica papaya* visando resistencia a meleira"**.** A sessão pública foi realizada em formato virtual, com transmissão por meio de videoconferência (https://meet.jit.si/pgbiotecnologiaufes). A presidente da Banca, Profª. Drª. Patricia Machado Bueno Fernandes (orientadora), apresentou os demais membros da comissão examinadora constituída pelos Doutores: Silas Pessini Rodrigues (coorientador); José Aires Ventura, membro titular interno; Roberto Tarazi, membro titular externo à Ufes; Juliany Cola Fernandes Rodrigues, membro titular externo à Ufes; Daisy de la Caridad Pérez Brito, membro titular externo à Ufes, em seguida, passou a palavra para a aluna que apresentou a sua proposta de Tese. Terminada a apresentação e a arguição do discente, a banca reuniu-se em separado e concluiu por considerar a doutoranda **APROVADA** na defesa de Doutorado. Eu, Patricia Machado Bueno Fernandes, que presidi a Banca de defesa, assino a presente Ata, juntamente aos demais membros e dou fé. Vitória, 09 de maio de 2024.

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DEDICATORIA

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RESUMEN

Este trabajo aborda la regulación de una de las tecnologías más modernas para el control de enfermedades, la técnica CRISPR (*Clustered regularly interspaced short palindromic repeats*) que está basada en acción de una proteína Cas y un ARNg que juntos pueden dirigirse a zonas específicas del ADN para modificarlos, a través de una revisión sistemática de los sistemas regulatorios de diferentes países. Se examinaron en los diversos enfoques adoptados por los países de la región de Latinoamérica para regular la tecnología CRISPR, teniendo en cuenta las oportunidades que ofrece, los posibles riesgos éticos y sociales asociados. Hasta ahora, en el Brasil se ha analizado el proceso de evaluación caso por caso de cultivos modificados genéticamente por el ente regulador CTNBio para el uso de nuevas tecnologías de fitomejoramiento y así determinar si se consideraría un OGM o mejoramiento convencional.

Este análisis se llevó a cabo, porque existe un gran interés en utilizar la tecnología CRISPR para desarrollar nuevos cultivares vegetales, especialmente plantas de relevancia para la fruticultura brasileña. El cultivo de *Carica papaya*, es de particular interés debido a las limitaciones en su producción, debido a la susceptibilidad de la planta al complejo de virus que causa la enfermedad de la meleira en la papaya, es económica y biológicamente relevante, ya que se han identificado virus en el látex vegetal, un fluido derivado de las laticíferas, que se dan en más de 20 familias de plantas y sobre las que existe un conjunto de información que señala a las laticíferas como sistemas de respuesta de las plantas a los virus. Por este motivo, se realizó una revisión detallada sobre el papel de los laticíferos de diferentes especies, examinando la capacidad del látex tanto pro y antiviral para proteger a las plantas, así como su potencial para ser utilizado como fuente natural de moléculas de defensa contra insectos vectores e infecciones fúngicas. También se evaluó el potencial uso de CRISPR en respuesta de control de virus que afectan a los laticiferos mediante una recopilación de artículos dirigidos a la edición de genes con una expresión específica o relacionada con la biología de los laticiferos, lo que confirma que es posible modular la función de los laticiferos mediante CRISPR.

Fue discutida la relación del complejo PMeV en la planta *C. papaya* L. y la importancia del clonaje del gen Salicilato O-metiltransferasa en la defensa contra este complejo viral, debido a su presencia en los mecanismos de defensa en la planta relacionados con laticíferos. Describiéndose avances experimentales en el desarrollo de un protocolo para silenciar el gen salicilato O-metiltransferasa de *C. papaya* mediante el *Golden Gate Assembly*, detallando el diseño de ARNg para la construcción y multiplicación de vectores de edición génica, pasos intermedios en el proceso de silenciamiento génico del gen SAMT para el control de la enfermedad de la meleira en papaya, dando lugar a las condiciones óptimas para utilizar la tecnología CRISPR en plantas de *C. papaya*.

Palabras clave: CRISPR/Cas9. Regulación. Latinoamérica. Latex. Complejo PMeV. Salicilato O-metiltransferasa.

REGULAMENTAÇÃO DE PLANTAS GENETICAMENTE EDITADAS E SELEÇÃO DO GENE SAMT PARA EDIÇÃO DE *CARICA PAPAYA* VISANDO RESISTÊNCIA À MELEIRA.

RESUMO

Este artigo aborda a regulamentação de uma das tecnologias mais modernas para o controle de doenças da plantas, a técnica CRISPR (*Clustered regularly interspaced short palindromic repeats*), que se baseia na ação de uma proteína Cas e de um gRNA que, juntos, podem atingir áreas específicas do DNA para modificá-las, por meio de uma revisão sistemática dos sistemas regulatórios de diferentes países. Foram analisadas as diferentes abordagens adotadas pelos países da região da América Latina para regulamentar a tecnologia CRISPR, levando em conta as oportunidades que ela oferece e os possíveis riscos éticos e sociais associados. Até o momento, no Brasil, o processo de avaliação caso a caso de culturas geneticamente modificadas pelo órgão regulador CTNBio para o uso de novas tecnologias de melhoramento genético é analisado para determinar se seria considerado OGM ou melhoramento convencional.

Essa análise foi realizada porque há grande interesse em usar a tecnologia CRISPR para desenvolver novos cultivares de plantas, especialmente plantas relevantes para a fruticultura brasileira. A cultura do mamão *Carica papaya* é de particular interesse por causa das limitações de produção, devido à suscetibilidade da planta ao complexo de vírus que causa a doença da meleira do mamão, é econômica e biologicamente relevante, pois os vírus foram identificados no látex da planta, um fluido derivado de laticíferos, que ocorrem em mais de 20 famílias de plantas e para os quais há um conjunto de informações que aponta para os laticíferos como sistemas de resposta da planta aos vírus. Por esse motivo, foi realizada uma revisão detalhada sobre o papel dos laticíferos de diferentes espécies, examinando a capacidade do látex pró- e antiviral de proteger as plantas, bem como seu potencial para ser usado como fonte natural de moléculas de defesa contra insetos vetores e infecções fúngicas. O uso potencial do CRISPR em resposta ao controle de vírus que afetam os laticíferos também foi avaliado por meio da coleta de artigos voltados para a edição de genes com expressão específica ou relacionados à biologia do laticífero, confirmando que é possível modular a função do laticífero usando o CRISPR.

Foi discutido a relação do complexo PMeV na planta *C. papaya* L. e a importância da clonagem do gene Salicylate O-methyltransferase na defesa contra esse complexo viral, devido à sua presença nos mecanismos de defesa da planta relacionados aos laticíferos. São descritos os avanços experimentais no desenvolvimento de um protocolo para silenciar o gene da salicilato O-metiltransferase de *C. papaya* usando o *Golden Gate Assembly*, detalhando o desenho de gRNA para a construção e multiplicação de vetores de edição de genes, etapas intermediárias no processo de silenciamento gênico do gene SAMT para o controle da doença da meleira em mamão, resultando em condições ideais para o uso da tecnologia CRISPR em plantas de *C. papaya*.

Palavras-chave: CRISPR/Cas9. Regulamentação. América Latina. Látex. Complexo PMeV. Salicilato O-metiltransferase.

REGULATION OF GENETICALLY EDITED PLANTS AND SELECTION OF THE SAMT GENE FOR EDITING CARICA PAPAYA FOR RESISTANCE TO MELEIRA.

ABSTRACT

This paper addresses the regulation of one of the most modern technologies for the control of plant pathologies, the CRISPR (*Clustered regularly interspaced short palindromic repeats*) technique, which is based on the action of a Cas protein and an gRNA that together can target specific areas of the DNA to modify them, through a systematic review of the regulatory systems of different countries. The different approaches adopted by countries in the Latin American region to regulate CRISPR technology were analyzed, taking into account the opportunities it offers and the possible associated ethical and social risks. So far, Brazil has analyzed the case-by-case evaluation process of genetically modified crops by the regulatory committee CTNBio for the use of new plant breeding technologies to determine whether it would be considered a GMO or conventional breeding.

This analysis was carried out because there is great interest in using CRISPR technology to develop new plant cultivars, especially plants relevant to Brazilian fruit growing. The *Carica papaya* cultivar, is of particular interest because of limitations in its production, due to the plant's susceptibility to the virus complex that causes papaya meleira disease, is economically and biologically relevant, as viruses have been identified in plant latex, a fluid derived from laticifers, which occur in more than 20 plant families and on which there is a body of information that points to laticifers as plant response systems to viruses. For this reason, a detailed review was conducted on the role of laticiferans of different species, examining the ability of both pro- and antiviral latex to protect plants, as well as its potential to be used as a natural source of defense molecules against insect vectors and fungal infections. The potential use of CRISPR in response to control viruses affecting laticifers was also evaluated through a compilation of articles targeting gene editing with specific expression or related to laticifer biology, confirming that it is possible to modulate laticifer function using CRISPR.

The relationship of the PMeV complex in the *C. papaya* L. plant was discussed and the importance of the cloning of the Salicylate O-methyltransferase gene in the defense against this viral complex are discussed, due to its presence in the plant defense mechanisms related to laticifers. Describing experimental advances in the development of a protocol to silence the salicylate O-methyltransferase gene of C. papaya by means of the *Golden Gate Assembly*, detailing the design of gRNA for the construction and multiplication of gene editing vectors, intermediate steps in the process of gene silencing of the SAMT gene for the control of meleira disease in papaya, resulting in optimal conditions for the use of CRISPR technology in *C. papaya* plants.

Keywords: CRISPR/Cas9. Regulation. Latin America. Latex. PMeV complex. Salicylate O-methyltransferase.

ESTRUCTURA DE LA TESIS

Esta tesis se presenta en formato de Artículo Científico, los dos primeros capítulos se encuentran en el formato como fueron publicados, por tal motivo las figuras y las tablas no se encuentran enlistadas. El tercer capítulo se realizó con las normas de la Associação Brasileira de Normas Técnicas (ABNT) NBR 10520 de 2023

LISTA DE FIGURAS

LISTA DE TABLAS

LISTA DE ABREVIATURAS Y SIGLAS

ASGT	AS glucosil transferasa
BAMT	Metiltransferasa del ácido benzoico (del inglés benzoic acid carboxyl methyltransferase)
BSMT1	Metiltransferasa dependiente de S-adenosil-l-metionina (del Inglés S-adenosyl-l-methionine-dependent methyltransferase)
CAND1	Culina Asociada y Neddilación Disociada 1 (del inglés Cullin Associated and Neddylation Dissociated 1)
Cas9	Proteína 9 asociada a CRISPR (del inglés CRISPR associated protein 9)
CDS	Secuencia codificante
CIAT	Centro Internacional de Agricultura Tropical
CIBio	Comissão Interna de Biossegurança
CNBS	Conselho Nacional de Biossegurança
CQB	Certificado de Qualidade em Biossegurança
CRISPR	Repeticiones palindrómicas cortas agrupadas y regularmente espaciadas (del inglés Clustered regularly interspaced short palindromic repeats)
CTNBio	Comissão Técnica Nacional de Biossegurança
DNA	Ácido desoxirribonucleico (del inglés Deoxyribonucleic acid)
DNAc	Ácido desoxirribonucleico complementario (del inglés Complementary deoxyribonucleic acid)
eIF4E	Factor de iniciación de la traducción 4E (del inglés translation initiation factor 4E)
FAO	Organización de las Naciones Unidas para la Agricultura y la Alimentación (del inglés Food and Agriculture Organization)
GEd	Editado genéticamente (del inglés Genetically edited)
GMO	Organismo modificado genéticamente (del inglés Genetically modified organism)
HcPro	Componente ayudante-proteinasa (del inglés Helper component-proteinase)
HIV-1	Virus de la inmunodeficiencia humana 1 (del inglés Human

	immunodeficiency virus 1)
HPV	Papilomavírus humano (del inglés Human papilomavirus)
HR	Respuesta hipersensible (del inglés hypersensitive response)
Hsp70	Proteína de choque térmico 70 (del inglés Heat Shock Protein 70)
ICA	Instituto Colombiano Agropecuario
INIA	Instituto Nacional de Investigación Agropecuaria
LAC	América Latina y el Caribe (del inglés Latin America and Caribbean)
LB	Luria Bertani
LMO	Organismo vivo modificado (del inglés Living Modified Organism)
LSP1	Pérdida de susceptibilidad a los potyvirus 1 (del inglés Loss-of-Susceptibility to Potyviruses 1)
MES	Metil esterasa
MeSA	Salicilato de metilo (del inglés Methyl salicylate)
NPR1	Receptor de péptidos natriuréticos 1 (del inglés Natriuretic Peptide Receptor 1
ODM	Mutagénesis dirigida por oligonucleótidos (del inglés Oligonucleotide-directed mutagénesis)
OERF	Agências de Registro e Inspeção
PAM	Motivo adyacente de protoespaciador(del inglés protospacer adjacent motif)
PCD	Muerte celular programada (del inglés Programmed cell death)
Pol III	ARN polimerasa III (del inglés RNA polymerase III)
PLCPs	Proteasas de cisteína similares a la papaína (del inglés Papain-like cysteine proteases)
PMeV	Papaya meleira virus
PMeV2	Papaya meleira virus 2
pSAMC	Plasmídeo Salicilato O-metiltransferasa de carica
RdRp	ARN polimerasa dependiente de ARN (del inglés RNA-dependent RNA Polymerase)
RNA	Ácido ribonucleico (del inglés Ribonucleic acid)
ROS	Especies reactivas de oxígeno (del inglés Reactive oxygen species)
SA	Ácido salicílico (del inglés Salicylic acid)
SABATH	Ácido salicílico, ácido benzoico, teobromina (del inglés Salicylic Acid, Benzoic Acid, Theobromine)

SAG	SA 2-O-β-D- glucósido
SAH	S-adenosilhomocisteína
SAM	S-adenosilmetionina
SAMT	Salicilato O-metiltransferasa (del inglés salicylic acid o-methyltransferase)
SAMCP	Salicilato O-metiltransferasa de *C. papaya*
SAR	Resistencia sistémica adquirida (del inglés systemic acquired resistance)
SARS-CoV-2	síndrome respiratorio agudo grave coronavirus 2 (del inglés Severe acute respiratory syndrome coronavirus 2)
TALEN	Transcripción activador-como efector nucleasa (del inglés Transcription activator-like effector nuclease)
UPS	Sistema de ubiquitina-proteasoma (del inglés Ubiquitin–proteasome system)
WTO	Organización Mundial del Comercio (del inglés World Trade Organization)
ZFN	Nucleasas de dedos de zinc (del inglés Zinc finger nucleases)

SUMARIO

INTRODUCCIÓN GENERAL

La edición CRISPR-Cas9 es una técnica de ingeniería genética que permite la modificación dirigida de genes en células vivas (Xuan *et al.,* 2017). Este método se basa en el uso de la enzima Cas9, que actúa como unas "tijeras moleculares" y corta el ADN en sitios específicos determinados por las guías de ARN. La edición de esta técnica se utiliza para realizar cambios específicos en el ADN de una célula que pueden utilizarse en muchos campos, como la agricultura y la biotecnología (Jiang y Doudna, 2017). Esta técnica tiene gran importancia en el control de enfermedades causadas por virus en plantas, ya que permite la edición precisa de secuencias genéticas específicas, reduciendo la necesidad de aplicar pesticidas y siendo beneficioso para el medio ambiente por la exposición a agroquímicos (Shahriar *et al.,* 2021). Aunque tiene un gran potencial para desarrollar nuevos productos, la tecnología CRISPR se enfrenta a barreras normativas. Antes de desarrollar cualquier nuevo cultivar vegetal, es importante comprobar que se puede aprobar su uso no solo en el país o países productores, sino también en toda la red de países consumidores.

En este sentido, se exploró los diversos enfoques adoptados por los países de la región para regular la tecnología CRISPR, mostrados en el capítulo 9 del libro recientemente publicado (Fernandes *et al.,* 2023, en: *Global Regulatory Outlook for CRISPRised Plants*), teniendo en cuenta las oportunidades que ofrece y los posibles riesgos éticos y sociales asociados. Examinando el impacto de estas regulaciones en la investigación científica, el desarrollo económico y el bienestar social en los países en desarrollo de América Latina. Además, se abordó cuestiones fundamentales como la necesidad de equilibrar el progreso científico con la protección de la salud pública y la biodiversidad, así como la importancia de promover un diálogo inclusivo en el que participen la comunidad científica, los responsables políticos y la sociedad civil, ayudando a garantizar una producción agrícola más estable y predecible, lo que es esencial para la seguridad alimentaria mundial (Elsharawy; Refat, 2023).

La regulación de la tecnología CRISPR en los países latinoamericanos es un tema de creciente relevancia científica, ética y legal (Entine *et al.,* 2021). Sin embargo, la

implementación de esta tecnología trae consigo desafíos éticos y sociales que requieren una cuidadosa consideración, especialmente en contextos donde los recursos y los marcos regulatorios pueden ser limitados (Kuiken; Kuzma, 2021). El uso de este método de edición genética se ha convertido en un foco de debate, ya que la adopción de esta tecnología plantea cuestiones sobre la equidad de la innovación científica, los riesgos asociados a su aplicación y la capacidad de los sistemas reguladores locales para supervisar y controlar su uso (Zarate *et al.,* 2023). En definitiva, la regulación de CRISPR en América Latina presenta un desafío multifacético que requiere un enfoque integral y colaborativo para garantizar beneficios éticos, equitativos en el desarrollo y aplicación de esta novedosa tecnología genética.

En Latinoamérica se aborda la regulación de los organismos editados de dos maneras: algunos países diferencian entre organismos editados y modificados genéticamente, mientras que otros no. Los países que en este momento tiene regulación de edición genética CRISPR son Argentina, Brasil, Colombia, Chile, Honduras y Paraguay que han implementado regulaciones en este ámbito. Sin embargo, algunos países como Ecuador y México tienen regulaciones restrictivas para los organismos genéticamente modificados (OGM) o aún no han adoptado regulaciones específicas para la edición genética. Esto plantea un gran problema en estos dos últimos países, debido a que, se halló el complejo de virus causante de la enfermedad de la meleira en papaya en Ecuador y México, generando conflicto en el desarrollo de una planta de papaya resistente a esta enfermedad mediante el uso de CRISPR (Fernandes *et al.,* 2023).

Este trabajo exploró la necesidad de desarrollar cultivares de papaya (*Carica papaya* L.) resistentes a la enfermedad de la meleira en papaya. La enfermedad de la meleira ha sido estudiada desde la década de 1980, debido al impacto que ha tenido en los países productores de papaya de América, como México, Ecuador y Brasil, siendo este último el segundo productor mundial de papaya (FAO, 2022). En 2021, Brasil exportó alrededor de 50.000 toneladas de papaya, un aumento del 15,1% en comparación con el mismo período del año anterior (FAO, 2022).

En estudios de la enfermedad de la meleira describe que puede ser transmitida por insectos vectores, como la chicharrita, que adquiere y transmite el PMeV en plantas de papaya de la variante mexicana (García-Cámara *et al.,* 2019). El virus también puede transmitirse por propagación vegetativa, pero no a través de semillas recolectadas de frutos enfermos (Abreu *et al.,* 2012; Tapia-Tussell *et al.,* 2015). El aspecto pegajoso de los frutos de papaya infectados después de que el látex se haya oxidado por exposición al aire ha dado lugar al nombre de enfermedad de la meleira de la papaya (Abreu et al., 2015; Antunes *et al.,* 2020).

El complejo PMeV está formado por dos virus, inicialmente se identificó como un virus ARN de doble cadena (Maciel-Zambolim *et al.,* 2003), al que se denominó papaya meleira virus (PMeV), un virus similar al Totivirae; sin embargo, otras investigaciones han propuesto clasificarlo en la familia Fusagraviridae (Wang *et al.,* 2016; Das et al., 2021) y el segundo es un virus monocatenario de sentido positivo llamado papaya meleira virus-2 (PMeV2) de la familia Umbraviridae (Antunes *et al.,* 2020; Maurastoni *et al.,* 2020).

La asociación de PMeV y PMeV2 parece residir principalmente en las laticíferas, donde modifica los niveles de potasio y el equilibrio osmótico, provocando la ruptura de las células que exudan látex translúcido rico en proteasa, causando daños en los frutos y en las hojas jóvenes mostrado en la figura 1 (Antunes *et al.,* 2020). Además, presenta síntomas extremadamente agresivos que aparecen sólo después de la floración (Abreu *et al.,* 2015). La interacción entre la papaya y el complejo PMeV es complicada e involucra un sistema inmune de múltiples capas (Antunes *et al.,* 2020) que afecta negativamente el rendimiento y la calidad del cultivo, causando síntomas devastadores y pérdidas considerables figura 1.

Figura 1. Plantación enferma de *Carica papaya* (a). Erradicación en plantaciones enfermas de *Carica papaya* (b). (Antunes *et al.,* 2020).

Como en la respuesta de otras plantas a la infección viral (Ray; Casteel, 2022), los genes modulados en las plantas afectadas por la enfermedad incluyen los implicados en las especies reactivas del oxígeno, la señalización del ácido salicílico, la degradación proteasomal y la fotosíntesis, que son las principales defensas de la planta contra la infección por el complejo PMeV figura 2. (Abreu *et al.,* 2015; Sa Antunes *et al.,* 2020; Rodrigues *et al.,* 2011).

Actualmente, no existen variedades de papaya resistentes a la enfermedad causada por el complejo PMeV, por lo tanto, se requiere un diagnóstico fiable mediante las técnicas actuales que demandan la síntesis de ADNc con diferentes temperaturas de desnaturalización, ya que se trata de dos virus (Maurastoni *et al.,* 2020). Dado que el complejo PMeV se identificó en el látex de plantas enfermas, se prestó mayor atención al papel de los laticiferos en las respuestas de las plantas a los virus.

En ese sentido, se revisó el papel del latex en la relación planta-virus (Merchán-Gaitán *et al.,* 2024). Varias pruebas demuestran que el látex contiene moléculas capaces de impedir o favorecer la replicación viral. En el mismo artículo, se recopilan los avances en el uso de la tecnología CRISPR para controlar los genes que suelen expresarse en el laticifero. Esto abre la posibilidad de hacer un latex más eficiente en su acción

antiviral. Debido a su relevancia, se abarcaron diversos estudios e investigaciones relacionados con la biología vegetal y la genética de los laticiferos, incluyendo la estructura del complejo PMeV, la biosíntesis del caucho natural, la inactivación de genes específicos y el desarrollo de variedades vegetales enriquecidas con caucho. También se analizó el papel potencial de los polifenoles en la lucha contra las enfermedades causadas por virus, citando estudios sobre la actividad antivírica de polifenoles vegetales y extractos de plantas, así como los retos que plantea la gestión de la meleira en la papaya. Además, se menciona estudios realizados sobre la actividad antiviral del látex de ciertas plantas contra virus como la gripe y el Chikungunya.

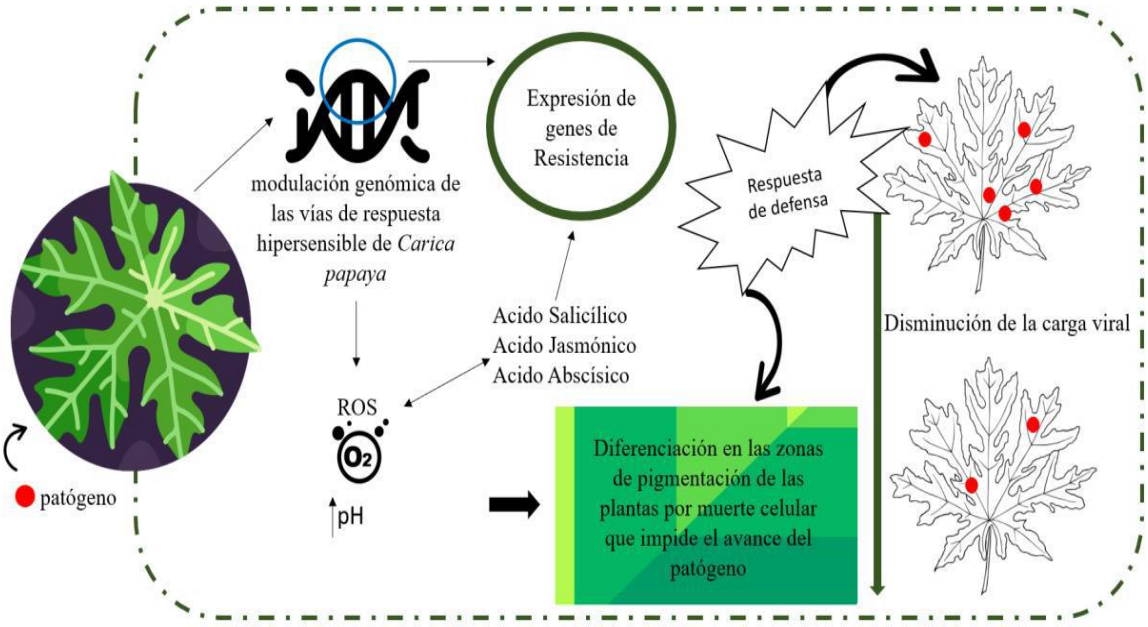

Figura 2. La interacción planta-patógeno provoca una modulación en el genoma de la planta, lo que da lugar a una respuesta de la vía del ácido salicílico (AS), que es uno de los principales compuestos asociados a su defensa. Otras respuestas a la modulación génica en respuesta al proceso infeccioso son un aumento de las especies reactivas del oxígeno (ROS), metabolitos primarios y secundarios, que pueden dar lugar a diferenciación en la pigmentación de las hojas por muerte celular como respuesta de defensa, ayudando a prevenir la propagación del proceso de infección viral y provocar una reducción de su carga en la planta infectada.

La implicación de los laticíferos en las respuestas de la papaya al complejo PMeV viene siendo analizada por nuestro grupo desde hace algunos años (Rodrigues *et al.,* 2009; Madroñero *et al.,* 2018; Soares *et al.,* 2017). De particular interés es el trabajo de análisis del transcriptoma de la interacción virus-planta de *Carica papaya*, el gen SAMT tiene una alta presencia en la etapa de posfloración de la planta en presencia de los sintomas causada por el complejo PMeV, incrementando los niveles de MeSA que está asociado a la susceptibilidad de la planta, teniendo una disminución de los niveles de ácido salicílico como se muestra en la figura 3 que generan baja señalización de los genes de defensa (Madroñero *et al.,* 2018). Por esta razón, el uso de este gen SAMT podría ayudar a controlar la enfermedad de la meleira en la papaya.

La relación que hemos establecido entre el efecto del gen SAMT y la modulación de los genes de defensa de los laticíferos mediante la realización de una revisión sistemática, que mostró la respuesta antiviral de los laticíferos en diferentes modelos de plantas porque exhiben una fuerte actividad metabólica (Merchán-Gaitán *et al.,* 2024). in situ, los laticiferos contiene orgánulos comunes de las células vegetales como el núcleo, en el que ocurre la síntesis de muchas funciones fisiológicas (El MoussaouI *et al.*, 2001; D' Auzac; Jacob; Chrestin, 2018), y donde se activan genes de respuesta de resistencia, que pueden verse afectados por la acción del gen salicilato metiltransferasa figura 3, generando la baja activación de los genes de defensa y contribuir a una mayor presencia de sintomas de la enfermedad de la meleira en la planta.

Para el uso de CRISPR es necesaria la búsqueda y selección del gen candidato a ser editado con la ayuda de herramientas bioinformáticas y experimentales, para controlar la enfermedad de la meleira en *C. papaya*. El gen escogido fue el Salicilato O-metiltransferasa (SAMT), que es una enzima de tipo III perteneciente a la familia de genes SABATH que recibió el nombre de los tres primeros miembros identificados funcionalmente, la metiltransferasa del ácido salicílico (SAMT), la metiltransferasa del ácido benzoico (BAMT) y la teobromina sintasa (D'Auria *et al.*, 2003). Desempeña un papel importante en las plantas, ya que codifica una proteína con actividad carboxilmetiltransferasa del ácido salicílico (AS), sintetizándolo en un metabolito

secundario llamado salicilato de metilo (MeSA), como se muestra en la figura 3. A bajos niveles de MeSA, aumentan los niveles de AS, presentes en la cascada de respuesta inmune de la planta a estreses bióticos y abióticos (Tieman *et al.*, 2010).

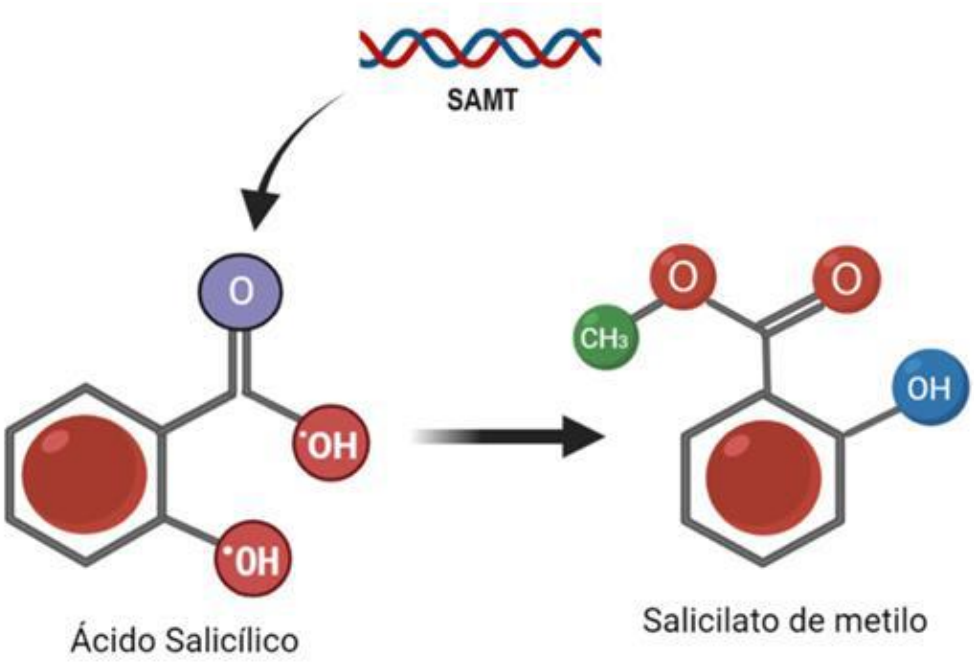

Figura 3. Síntesis del ácido salicílico por la enzima del salicilato O-metiltransferasa.

Dada la importancia económica de la papaya y los desafíos asociados a los métodos convencionales de control, el desarrollo de estrategias para el control de la infección viral a través de la modulación de genes vegetales sensibles al virus y la expresión en los laticiferos, representa un camino para el mantenimiento y la mejora de la producción de papaya, no sólo en Brasil sino también en otros países, al tiempo que promoverá la seguridad alimentaria, destacando el estado actual de la regularización de estas nuevas tecnologías de edición genética CRISPR en América Latina.

OBJETIVOS

OBJETIVO GENERAL

Revisar, desde el punto de vista regulatorio, la posibilidad de inserción de la tecnología CRISPR en países latinoamericanos. Examinar los sistemas de defensa de las plantas frente a los virus, centrándose en las células laticíferas, con el objetivo de precisar las respuestas pro y antivirales de las plantas para identificar genes potenciales para el control de la meleira en papaya. Finalmente, desarrollar un protocolo de edición del gen SAMT de *C. papaya* mediante CRISPR.

OBJETIVOS ESPECIFICOS

- Analizar la regulación de la tecnología CRISPR en los países latinoamericanos;
- Explorar la función del látex en plantas como respuesta a infecciones virales;
- A partir de información de las bases de datos de ARN y proteínas de nuestro grupo y de información de la bibliografía, identificar un gen candidato que pueda conferir tolerancia en plantas de *Carica papaya* L;
- Diseñar ARNg para la edición genética del gen SAMT en *Carica papaya* L. para ser utilizado con la tecnología CRISPR;
- Establecer la construcción de los ARN guía SAMCP1, SAMCP2, SAMCP3, SAMCP4 y SAMCP5 en el vector pKSE401;
- Validar experimentalmente la clonación de los plásmidos pSAMC1, pSAMC2, pSAMC3, pSAMC4 y pSAMC5 en la bacteria *E. coli*.

CAPÍTULO 1: Capitulo publicado en el libro (Global Regulatory Outlook for CRISPRized Plants) en el volume Genome Modified Plants and Microbes in Food and Agriculture. Elsevier. 2023. https://doi.org/10.1016/C2021-0-03034-0 FI = 8,9 Qualis C en Biotecnología.

CHAPTER 9 REGULATION OF CRISPR-EDITED PLANTS IN LATIN AMERICA

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9.1 Introduction

The Latin America and Caribbean (LAC) region is responsible for 13% of global agricultural and fish commodity production, while also accounting for 25% of the world's exports in these products. With abundant land and water resources, the region spans over 2 billion hectares, with approximately 38% of the available land dedicated to agriculture. The agricultural landscape in the region is characterized by a diverse range of farming structures. In the Southern Cone, especially in Argentina and Brazil, large- scale commercial farms geared toward exports dominate the agricultural sector. However, there are also approximately 15 million smallholder and family farms that contribute significantly to the region's food production (FAO, 2019). Moreover, LAC countries play an important role as major exporters of various agricultural products such as soybeans, maize, animal feed, sugar, coffee, and fruits and vegetables. In 2020 Brazil stood out as the largest agricultural and food exporter, generating approximately US$ 97 billion in export revenue. Mexico follows closely behind with US$ 43 billion in agricultural and food exports. Argentina, with US$ 39 billion, and Chile, with US$ 23.9 billion, also contribute significantly to the region's agricultural exports. Ecuador holds a prominent position as the world's largest producer and

exporter of bananas, with US$ 12.4 billion in export revenue. Additionally, Ecuador exports commodities such as coffee, cut flowers, and fruits like citrus, cacao, and mangoes. Peru also plays a noteworthy role in the agricultural export market, with US$ 11.1 billion in 2020 (Fig. 9.1) (USDA, 2021; OEC, 2023; ITA, 2022). The LAC region possesses a wealth of natural resources, which positions it as a significant player in global agricultural production and trade. The challenge for the future lies in maintaining growth and lower international prices, while ensuring that future agricultural growth is more sustainable and more inclusive than it has been in the past by using new gene editing technologies such as CRISPR–Cas9.

Figure 9.1 LAC countries and the exportation value (US $) of the main exporters: Brazil, Mexico, Argentina, Chile, Ecuador, and Peru

The story of the discovery and development of a biotechnology tool based on the microbial adaptive immune system, known as CRISPR-Cas9, is fascinating (Doudna and Charpentier, 2014). From laboratory benches to supermarket shelves, the perception of biotechnology by the general public has changed, and regulators have had to review their normative guides. Although several gene-editing technologies were developed in the last few decades, CRISPR-Cas9 is unique in its high versatility, accuracy, and ease of use and has truly revolutionized the field.

The clustered regularly interspaced short palindromic repeats (CRISPR) technology allows researchers to make precise changes (edits) in the DNA sequence easily and at a lower cost compared to other gene editing techniques. As an example, a gene editing experiment using meganucleases requires 4-5 years of work and costs more than 6000 dollars; one using zinc finger nucleases and oligonucleotide directed mutagenesis costs more than 30,000 dollars with 3 months of work; and one using transcription activator like effector nucleases (TALENs) requires 3-4 months of lab work and costs more than 10,000 dollars (Lacadena Calero, 2017). A CRISPR-Cas9 gene-editing experiment, on the other hand, only requires 2 3 weeks to be carried out and costs about 40 dollars (Lassoued et al., 2019).

Another powerful advantage of CRISPR is that it allows gene editing without the insertion or transfer of an exogenous gene from a nonsexually compatible organism. Therefore regulatory agencies may not consider the resulting genetically edited (GEd) organism to be equivalent to a genetically modified organism (GMO). Table 9.1 shows important points to consider when comparing GEd organisms and GMOs.

Like any disruptive innovation, CRISPR applications have raised moral and ethical issues. A number of international summits have been held with the active participation of regulators from the world’s major food producer and exporter countries to discuss this issue. In this context, each country has updated or is in the process of updating its regulatory framework.

In this chapter, we will address the status of the regulatory frameworks regarding CRISPR-derived gene-edited plants of the main agricultural players in Latin America, as well as their participation in international forums for discussion of genome-editing techniques and their impact on food production and food security.

In order to continue to meet the needs of the societies for which they are created, legislative systems must constantly adapt to technological advances. This is particularly true for techniques that are rapidly evolving, such as gene editing. We recognize that the regulatory developments for GEd plants are rapidly changing and will continue to evolve as more countries release their regulatory policies.

Table 9.1 Characteristics of genetically edited (GEd) organisms and genetically modified organisms (GMOs).

	GEd organisms	GMOs[a]	References
Origin of DNA	Nucleotides are deleted or inserted in the same DNA sequence	Genes synthesized in the laboratory or genes from another species are used	Jouve La Barreda (2020)
DNA location	Specific location in the genome	Random location in the genome	Bellver (2016)
Use of recombinant DNA (r-DNA)	Transiently—it can be removed from the final edited organism	Permanently—integration of r-DNA into the recipient genome	Duensing et al. (2018)
Detection	Undetectable—it is not possible to detect whether the genetic modification was performed by CRISPR or by conventional breeding	Detectable—techniques such as PCR, DNA sequencing, immunohistochemistry can detect the introduction of the exogenous gene	Miller and Hefferon (2021)
Research and development time	3–5 years	5–10 years	Jansing et al. (2019)
Costs	Low	High	Lacadena Calero (2017)
Regulation	Unregulated in some countries	Regulated—approval is dependent on a detailed and rigorous risk analysis	Kuiken and Kuzma (2021)

[a]GMOs are organisms that have undergone some modification or artificial transformation in their genetic material (DNA) by means of biotechnology, that is, genetic engineering techniques (Zhang et al., 2016). They have also been defined as organisms (plants, animals, or microorganisms) in which the DNA has been altered in a way that does not occur naturally by mating and/or natural recombination and the purpose of these modifications is to add new characteristics or alter some preexisting ones (World Health Organization WHO, 2014).

9.2 Gene-editing tools

The advancement of biotechnology has allowed the development of different gene-editing tools. The most widely known and most widely used technology today is CRISPR. CRISPR uses a guide sequence that, by forming base pairs with the target sequence in the DNA, allows the introduction or removal of one or more specific nucleotides of the DNA strand (Martinez-Oliva, 2020). The CRISPR-Cas9 system is powered by an RNA that serves as a guide molecule, directing the Cas9 enzyme's activity in recognizing the target sequence to be altered (Bellver, 2016; Cong et al.,2013).

However, there are other gene-editing techniques that are also important in molecular biology and biotechnological development. These include:

1. TALENs, a class of restriction enzymes that can be artificially engineered to cut a specific DNA sequence (Joung and Sander, 2013).

2. Oligonucleotide-directed mutagenesis, in which point mutations are performed at specific locations in the DNA sequence (Sauer et al., 2016).

3. Base editors, which chemically modify the target nucleotides directly during the genome and transcriptome editing process (Porto et al., 2020).

4. Zinc finger nucleases, artificial enzyme endonuclease used for gene targeting, which consist of a clivage domain and a zinc finger protein designed to cut a specific DNA sequence (Carroll, 2011).

Fig. 9.2 shows the outcomes and characteristics of GEd crops using different gene-editing techniques. Of particular note is the diversity of characters that can be achieved, from the change of fragrance to resistance to pathogens or climatic adversities.

It is worth noting that the use of these GEd tools, including CRISPR, has expanded in the market in recent years. The global expansion of the CRISPR market has been predicted to be more than US$19.45 billion by 2028 (ISAAA, 2022).

CRISPR constitutes a truly revolutionary advance in biotechnology and has already become an important gene-editing tool in animals and plants. However, how can the

use of this tool for genetic modification affect different countries? This is a very important question that must be examined in-depth, considering their legal, ethical, social, and commercial implications. Latin American countries (LACs) have established different legislative frameworks related to CRISPR-derived GEd plants in their territories, resulting in nonhomogeneity in commercial relations between these countries and global markets.

Laws regarding the application of biotechnological tools are a prerogative of each country, following international protocols and agreements. Although in most countries it may take many years for GMOs to be approved for commercial release, GEd organisms have, in general, been subjected to less stringent regulation (Niiler, 2018). Indeed, several LACs (Argentina, Brazil, Dominican Republic, Guatemala, Honduras, Paraguay, and Uruguay) joined the United States, Canada, and Australia in a joint statement to the World Trade Organization which promoted relaxed regulations for genome editing, stating that governments should “avoid arbitrary and unjustifiable distinctions” between GEd crops and conventionally bred crops (World Trade Organization [WTO], 2018).

The governments of most LACs have incorporated this new technology, adapting their economic models and legislative frameworks to allow the use of GEd organisms under different degrees of stringency (Table 9.2).

Table 9.2 Restrictions to genetically edited (GEd) organisms in Latin American countries

High	Low	In progress	No specific regulations	No information
Belize Bolivia Mexico Peru Venezuela	Argentina Brazil Chile Colombia Ecuador Guatemala Honduras Paraguay	El Salvador Panama	Costa Rica Dominican Republic Nicaragua Trinidad y Tobago Uruguay	French Guiana Guiana Suriname

Here, to make understanding simpler, we define countries with high restrictions as those that consider GEd organisms to be equivalent to GMO, or that have altogether banned the use of biotechnology in their territories. Countries with low restrictions are those that have legal frameworks that consider GEd organisms with the absence of exogenous genes to be equivalent to conventional organisms, therefore being deregulated. Countries that are still discussing the matter were considered as “in progress.” All other countries that did not meet the above criteria or that do not have published data were considered as “with no specific regulations.”

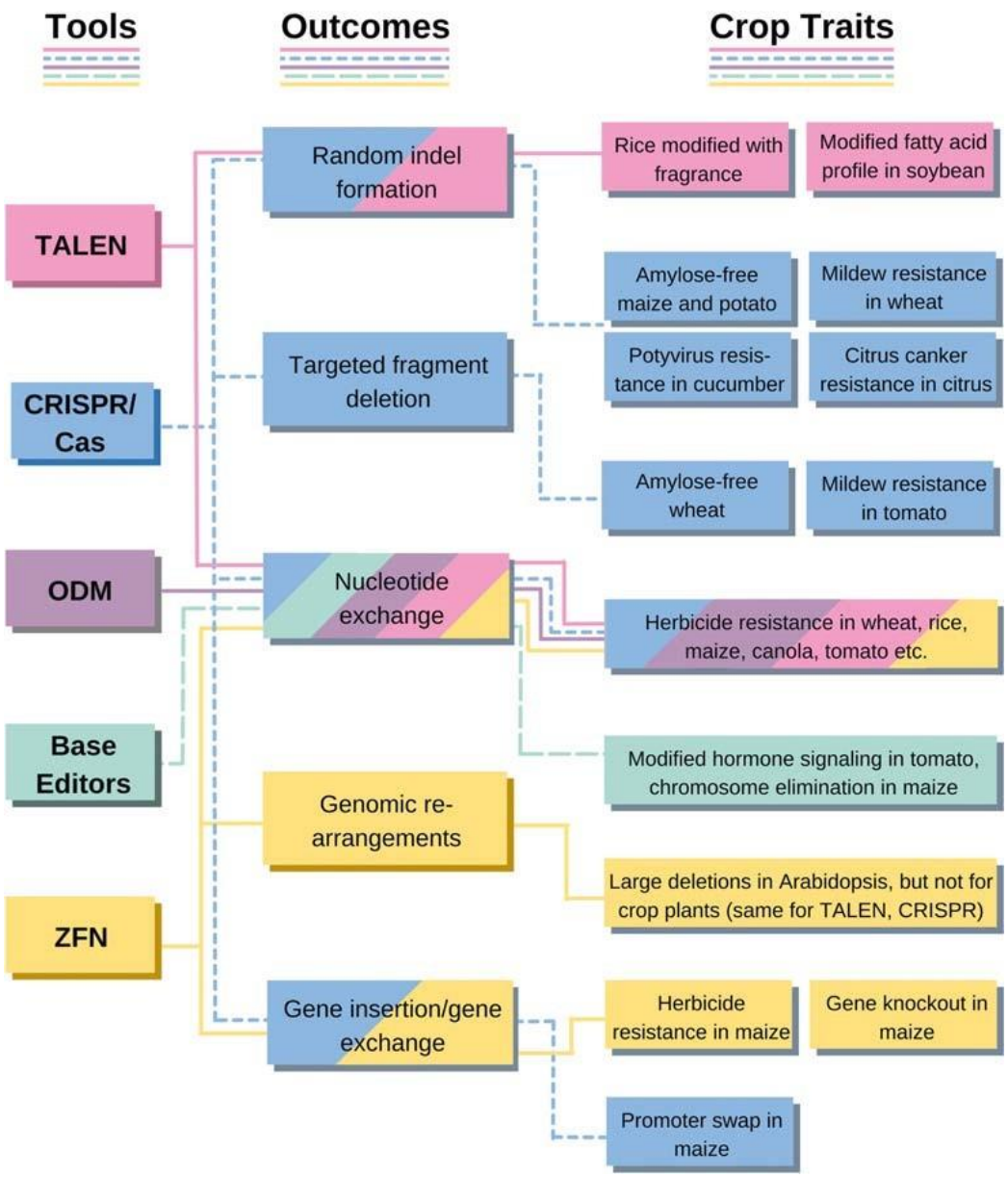

Figure 9.2 Genome-editing tools, their outcomes, and crop characteristics. Colored arrows and boxes link published crop trait examples with the associated genome-

editing tool and outcome. Modified from Jansing, J., Schiermeyer, A., Schillberg, S., Fischer, R., Bortesi, L., 2019. Genome editing in agriculture: technical and practical considerations. Int. J. Mol. Sci. 20 (12), 2888.

Next, we will detail the situation regarding the regulation of CRISPR-derived GEd plants in Latin America and the impact of these regulations at both local and global levels.

9.3 CRISPRized plants considered as conventional

In order to be considered deregulated, products from genetically edited organisms must demonstrate to the Advisory body of each country that it does not contain an exogenous gene or protein in the final product. Therefore the deregulated product is exempt from risk assessment. Argentina, Brazil, Chile, Colombia, Ecuador, Guatemala, Honduras, and Paraguay adopted the same regulatory criteria for gene editing. In general, in these countries, the institutions should present their products derived from new technologies of gene editing to an Advisory body, which will attest that there is no exogenous genetic material in the new trait (Gatica-Arias, 2020; HSF, n.d.; Scarpeline de, 2016).

Table 9.3 shows a summary of the legal frameworks for CRISPRized plants in LACs. It can be observed that different types of regulatory instruments, with different scopes, and with hierarchically distinct governmental decisions are present in these countries.

Argentina has addressed early on the importance of seeking to launch the use of gene-editing techniques, being one of the first countries to have a guideline on the procedures for products with gene-editing characteristics (Lema, n.d.). In 2012 E. Charpentier and J. Doudna turn CRISPR/Cas9 into “genetic scissors,” a fact that won them the Nobel Prize in 2020 (Doudna and Charpentier, 2014). Argentina’s rapid response to this important technological advance is impressive.

Argentina’s agricultural sector has benefited from the added value that the use of new genetic improvement techniques (such as CRISPR) offers to its economy (Whelan et al., 2020), increasing their yields, reducing production costs, and increasing the profitability of their crops, generating expectations among producers with crops of

higher organoleptic and nutritional quality offered to the community (Agrofy News, n.d.; Whelan and Lema, 2017).

Table 9.3 Latin American specific regulation for CRISPRized plants.

Country	Advisory body[a]	Pertinent laws, regulations, and documents	Date of issue
Argentina	CONABIA	Resolución 173/2015	12/05/2015
Brazil	CTNBio	Resolução Normativa 16	15/01/2018
Chile	SAG	Resolución 1523/2001	19/07/2017
Colombia	ICA	Resolución 29299/ 2018	01/08/2018
Ecuador	SINABIO	Decreto 752/2019, art. 230	12/06/2019
Guatemala	MAGA	Acuerdo Ministerial No. 271	20/09/2019
Honduras	SENASA	Acuerdo C.D. 008−2019	27/08/2019
Paraguay	CONBIO	Resolución 842/2019	10/07/2019

[a]*CONABIA*, Comisión Nacional de Biotecnología Agropecuaria—National Advisory Commission on Agricultural Biotechnology; *CONBIO*, Comisión Nacional De Bioseguridad Agropecuaria Y Forestal—National Commission of Agricultural and Forestry Biosecurity; *CTNBio*, Comissão Técnica Nacional de Biossegurança—National Biosafety Technical Commission; *ICA*, Instituto Colombiano Agropecuario—Colombian Agricultural Institute; *MAGA*, Ministerio de Agricultura Ganadería y Alimentación—Ministry of Agriculture Livestock and Food; *SAG*, Servicio Agrícola y Ganadero—Agricultural and Livestock Service; *SENASA*, Servicio Nacional de Sanidad Agropecuaria—National Agricultural Health Service; *SINABIO*, Sistema Nacional de Seguridad—National Biosecurity System.

Potato is an example of an important crop genetically edited by the CRISPR method that has contributed to the country's economy (Colman et al., 2017; González et al., 2020).

Chile was the second country in Latin America to publish regulations for the use of new gene-editing technologies. In 2017 the Ministry of Agriculture decided to regulate, on a case-by-case basis, gene-edited crops. According to the Chilean regulation, if the crop does not contain foreign DNA, they are considered conventional plants.

In Chile, seed developers and researchers can use genetic engineering technology for research and export only, even though Chile is the fifth largest producer of seeds in the world (Genetic Literacy Project [GLP], 2020). Therefore the evolution of CRISPR technology in the field will be a great facilitator in Chilean crop production. Unlike other LACs, the Brazilian legal framework has enacted a specific law, Biosafety Law No. 11,105/2005, to promulgate the control mechanisms for the use of GMOs and GMO derivatives in terms of their production, handling, research, commercialization, transport, transfer, and release into the environment, as well as in activities and

research projects in educational institutions, establishing standards for control and regulation in the use of these organisms for the purpose of the environment protection.

Also, Article 3, Section 1 describes textually, “it is not included in the category of GMOs those resulting from techniques that involve the direct introduction of hereditary material in an organism, as long as it does not involve the use of recombinant DNA/ RNA molecules or GMO,” and Section 2 states, “it is not included in the category of GMO derivatives a pure substance, chemically defined, obtained through biological processes and that does not contain GMO, heterologous proteins or recombinant DNA” (Presidência da República do Brasil, 2005). Therefore the Brazilian National Biosafety Technical Commission (CTNBio) published the Normative Resolution No. 16/2018 (Comissão Técnica Nacional de Biossegurança, CTNBio, 2018), aiming to delineate the evaluation process for the use of new plant breeding technologies and to determine what would be considered non-GMO. CTNBio performs a case-by-case analysis (Fig. 9.3). Briefly, the criteria used by CTNBio for the designation of non-GMOs are: (1) nonexistence of recombinant DNA/RNA; (2) presence of genetic elements that could be obtained by crossbreeding; (3) presence of induced mutations that could also be obtained by established techniques, such as radiation or chemical exposure; and (4) presence of mutations that could be naturally occurring (CTNBio Normative Resolution No. 16/2018 —Article 1 y 3rd).

Currently, several crops have been genetically edited using CRISPR technology in Brazil, constituting a great advance in agriculture and the economy. As a matter of fact, Brazil Embrapa Agroenergia developed the world’s first sugarcane with the CRISPR technique. These plants intended for bioethanol production were edited for drought tolerance and enhanced yield productivity (CTNBio, 2015).

Embrapa is also using CRISPR technology on maize and beans to obtain stress-tolerant plants (Almeida et al., 2020; Faria et al., 2017). Like Argentina and Brazil, Colombia also regulates genetically edited organisms separately from GMOs and defines them as non-GMOs. The purpose of this resolution was to classify and separate the final product of crops that have been genetically edited, such as those produced by the CRISPR technique, from GMOs.

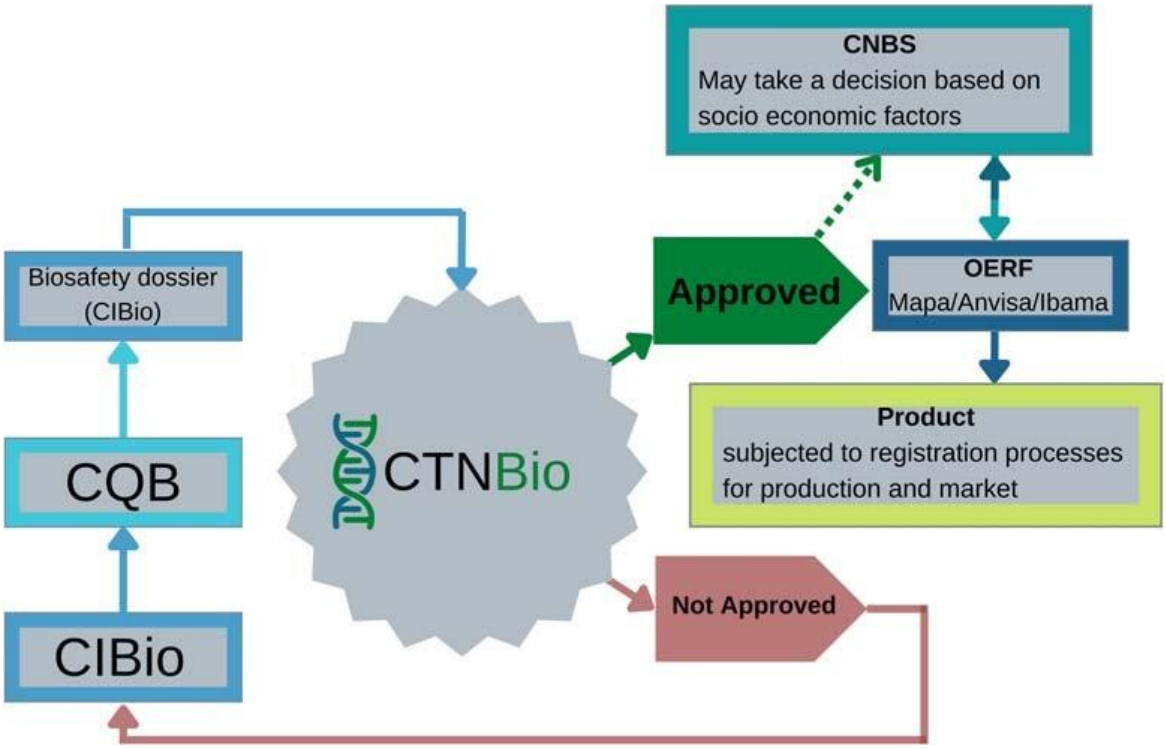

Figure 9.3 Brazilian National Technical Commission on Biosafety—CTNBio evaluation procedure according to Normative Resolution No. 16. CTNBio performs a case-by-case analysis: if the product is designated as a GMO, it will have to go through all the biosafety requirements, but if the product is designated as a non-GMO, it can follow the registration process. CNBS, National Biosafety Council; CQB, Biosafety Quality Certificate; CIBio, Internal Biosafety Commisions; OERF, Registration and Inspections Agencies. Reprinted from Nepomuceno, A.L., Fuganti-Pagliarini, R., Felipe, M.S.S., Molinari, H.B.C., Velini, E.D., de Campos Pinto, E.R., Dagli, M.L.Z., Filho, G.A., Fernandes, P.M.B., 2020. Brazilian biosafety law and the new breeding technologies. Front. Agric. Sci. Eng. 7, 204—210.

This is textually established in resolution 29299 of 2018, in which it is stated that “By which the procedure is established for the processing before the ICA of applications for a cultivar improved with innovation techniques in plant breeding through modern biotechnology, in order to determine whether the cultivar corresponds to a Living Modified Organism (LMO) or to a conventional one” (Instituto Colombiano Agropecuario [ICA], 2018). Also in paragraph 3.5 of Article 3 of the same resolution, it

refers to “Foreign genetic material: Gene, set of genes, DNA sequences that are part of a defined genetic construction and that have been introduced into the genome of an individual in a stable form, through modern biotechnology techniques, overcoming the natural physiological barriers of reproduction,” focusing on the final product whether there is an exogenous DNA or not, comparing them with the original crop to determine if it is a GMO or a conventional crop (non-GMO).

The regulatory commission of ICA and CTNBio must resolve the request for final modified products within 60 days, studying case by case, to establish whether they are defined as GMO or non-GMO. If the final product is defined as a GMO, it must carry out all risk assessment procedure and field tests, as required by the regulatory framework of decree 4525; however, if the product is defined as a non-GMO, it is regulated as a conventional crop and no risk assessment is necessary (Genetic Literacy Project GLP, 2019).

Gene editing in Colombia has made significant progress, particularly through the utilization of CRISPR technology. The resolution of legal challenges within various regulatory frameworks and the examination of patent requests have contributed to this advancement. Decision 486 of the Andean Community, to which Colombia is a signatory, which establishes the industrial property regime, specifically states in Article 3 the necessity of preserving and respecting the biological and genetic heritage, along with the traditional knowledge of native, Afro-American, and local communities, and it shall be considered when conferring protection to elements of industrial property. Consequently, the granting of patents for inventions derived from materials obtained from such heritage or knowledge is contingent upon compliance with the community standards, national regulations and the international legal system (Andean Community, 2000).

The CIAT—International Center for Tropical Agriculture has developed some CRISPRized plants, such as cocoa, cassava, and rice. Their goal is to associate the usual breeding technologies with the new genomic editing tools to incorporate stress tolerance traits into those crops.

It is interesting to mention the case of Ecuador, which declares itself free from transgenic crops and seeds. Nevertheless, seeds and genetically modified crops may be introduced only in those cases of national interest duly supported by the Presidency of

the Republic and approved by the National Assembly. Also, their consumption is allowed as long as properly labeled. As the Ecuadorian law does not mention the biotechnological production process where a transgenic or a GEd organism will be developed, it was possible to write a new article covering the new technologies of gene editing. In the end, the new product from CRISPRized plants will not undergo risk assessment in the case where foreign or recombinant DNA is not present (Gatica-Arias, 2020).

It is observed that up to 2018, all LACs that develop biotechnology or are biotech crops farmers have already made their decisions or are very close to this in relation to genetically edited plants. As a matter of fact, some important facts happened this year: CIAT hosted a workshop on genome editing, regulatory issues, communication, and public acceptance with the participation of members from the National Biosafety Commissions from Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Paraguay, Peru, Uruguay, Costa Rica, Guatemala, Honduras, Bahamas, Belize, Dominican Republic, Trinidad and Tobago, Canada, the United States, and Mexico (Gatica-Arias, 2020). In September, the Ministries of Agriculture from Argentina, Brazil, Chile, Paraguay, and Uruguay signed a declaration considering, among other topics that “Crops improved by gene editing have the potential of playing a key role to address the challenges faced by agricultural production, thereby helping increase the supply of food and other farm products in a sustainable manner” and for that “avoid non-science-based trade barriers to farm products improved by gene editing” (Riskesdas, 2018).

9.4 CRISPRized plants regulated as genetically modified organisms and countries that banned their use

Venezuela is probably the most restrictive country in Latin America in terms of the adoption of plant biotechnology. With the objective of developing seed production that benefits biological diversity and helps to preserve life on the planet, in 2018, Venezuela enacted the Law of Seeds (Ley de Semillas: Ley de Todos, La Asamblea Nacional de La República Bolivariana de Venezuela) (Ley de Semillas, 2018). This law is contrary to the production and use of LMOs and GMOs and is contrary to patents and breeder rights on seeds. However, in Article 9 of this law, it is clear that it deals with transgenic

seeds. Therefore in the case of GEd organisms that do not have exogenous genes, they could be outside the scope of the law. Nevertheless, Chapter I, article 11 item 21 of Law of Seeds defines GMO or transgenic as any living organism or in which it has a new combination of genetic material, obtained through the application of modern biotechnology, which might include GEd organisms.

Mexico has a long history of biotechnological research and development. Several Mexican institutions are working on GM plant projects with increased performance or tolerance to drought or diseases (USDA, 2018). However, due to the pressure from anti- biotechnology activists together with Maya farmers and honey producers, in 2017, Mexico suspended the planting of GEd corn and soybeans. Importation for feed and food use is still allowed (Garcia Ruiz et al., 2018). Although cultivation of other GM crops is allowed, the last environmental release permit for GMOs issued in Mexico was in 2018, as publicized in the National Information System on Biosafety. No record of authorization issued for gene-edited plants was found in the system (Gobierno de México, 2021). This demonstrates the tendency to reject biotechnology that is dominating public opinion in Mexico, despite having no scientific basis. This fact is reinforced by the decision of the state of Yucatan to unilaterally declare itself a GMO-free zone (USDA, 2018).

In 2000 Belize has placed a moratorium that prevents the liberation into the environment of GMOs that was finally ratified for cultivation or breeding purposes but not for research (Belize Agricultural Health Authority, n.d.; Gatica-Arias, 2020). Regarding plants edited by CRISPR or other techniques, there is no movement in this country.

Peru enacted Law No. 31111/2020 establishing, until December 31, 2035, a moratorium that prevents the entry and production in the national territory of living modified organisms for cultivation or breeding purposes, including aquatic ones, to be released into the environment (El Peruano, 2020). In this way, the Peruvian government extended the moratorium that was already in force. However, in 2019, the Ministry of the Environment (MINAN—Ministerio del Ambiente) discovered that GM crops had been used clandestinely in some regions of the country (Alcántara, 2019). Despite this, no movement toward new regulations for the use of GMOs or GEd organisms has been observed in Peru.

9.5 No specific regulation for genetically edited organisms

The use of GMOs is restricted in Bolivia, where only genetically modified corn and soybean are allowed to be planted (Morales et al., 2015). Views toward GMOs have also shifted. For example, Article 255, in 2009, which had banned GMOs has evolved into Supreme Decree No. 24676 described below, which has enabled the importation and use of GMOs. Bolivia: “Supreme Decree No. 3874, April 18, 2019 Exceptionally authorizes the National Biosafety Committee to establish abbreviated procedures for the evaluation of Soybean event HB4 and Soybean event Intact, intended for the production of Additives of Plant Origin Biodiesel.”

The following countries do not have specific regulations on genetically edited organisms: Costa Rica, Dominican Republic, Trinidad and Tobago, and Uruguay (Gatica-Arias, 2020). Although Uruguay does not have a specific regulation for GEd organisms, several investigations are under development at the Universidad de la República and Instituto Nacional de Investigación Agropecuaria (INIA) using the CRISPR/Cas9 technology to edit genes in soybean, mandarine, and tomato (Gatica-Arias, 2020).

9.6 Conclusions and future perspectives

Considering all data presented in this review chapter, it is clear that there is no consensus in LACs as to legislation to guide CRISPRized crops and related product analysis (Table 9.2). While some countries such as Brazil, Argentina, Colombia, and Chile, among others, have well-established rules and low restrictions and are already embracing CRISPRized products in their markets (see Table 9.4 for details), other countries have high restrictions, legislation being developed, or no specific regulations and even no information at all about how these countries are going to deal with products coming from gene-editing tools such as CRISPR. This lack of alignment between LACs regarding CRISPRized products may, in the near future, create significant trade barriers between importing and exporting countries of important

commodities, the main crops that are currently the focus of editing tools, considering the financial input that these crops add to national economies. Once this point is reached, and it will be, countries will be obliged to seek a joint understanding.

Table 9.4 CRISPRized plants released on the market.

Country	Plant	Description	Institution	References
Brazil	Maize (*Zea mays cerifina*)	Selected waxy maize increased its amylopectin yield up to 98% compared to conventional maize	Du Pont do Brasil SA—Pioneer Seeds Division	CTNBio (2018)
Argentina	Potato (*Solanum tubersoum* L.)	A polyphenol oxidase gene responsible for most of the protein activity in tuber was edited	National Institute of Agricultural Technology (INTA)	Instituto Nacional de Tecnología Agropecuaria (INTA) (2020)
Colombia	Rice (*Oryza sativa*)	Bacterial blight resistant	Colombian Agricultural Institute (ICA)	International Center for Tropical Agriculture (CIAT) (2019)
Chile	Apple (*Pyrus malus* L.)	Resistance to the fungus *Venturia inaequalis*	Institute of Agricultural Research (INIA) and Chilean Exporters Association (ASOEX)	ChileBIO (n.d.)

The reality is that CRISPRized crops and related products are here to stay for a variety of reasons, including added value to commodities and consumer crops; technology toolboxes that are more precise and relatively simpler to apply, avoiding the existing scientific bias of other methodologies; these crops can also overcome regulation restrictions and costs; and they are accessible to a larger number of research institutions, bringing “new players” to the field of product development and innovation. In the future, there will be an increasing number of CRISPR-modified crops and related products on market shelves. It is a path of no return, and the secret to the technology’s

success will be to align regulation, labeling, and how the message of CRISPRized crops and related products will be delivered to consumers.

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REVIEW THE ROLE OF PLANT LATEX IN VIRUS BIOLOGY

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Abstract: At least 20,000 plant species produce latex, a capacity that appears to have evolved independently on numerous occasions. With a few exceptions, latex is stored under pressure in specialized cells known as laticifers and is exuded upon injury, leading to the assumption that it has a role in securing the plant after mechanical injury. In addition, a defensive effect against insect herbivores and fungal infections has been well established. Latex also appears to have effects on viruses, and laticifers are a hostile environment for virus colonization. Only one example of successful colonization has been reported: papaya meleira virus (PMeV) and papaya meleira virus 2 (PMeV2) in Carica papaya. In this review, a summary of studies that support both the pro- and anti-viral effects of plant latex compounds is provided. The latex components represent a promising natural source for the discovery of new pro- and anti-viral molecules in the fields of agriculture and medicine.

Keywords: laticifer; plant-virus interactions; virus; papaya meleira virus

1. Introduction

Latex is a complex fluid produced by many plants and exuded when plant tissue is damaged through herbivory or physical damage. The color of latex produced by different species can vary from clear to yellow, white, or orange, and the quantity can vary from copious to almost undetectable [1,2]. When exposed to air, it rapidly coagulates, contributing to a reduction in attack by herbivores. This is an advantage for survival in environments with significant populations of herbivorous insects, such as the tropical regions of the planet [1]. The production of latex can vary between even closely related species, with the same species facing different conditions and different parts of the same plant, as shown in *Ficus carica* L. [1].

Latex's usefulness to plants is evinced by the fact that over 20,000 species produce latex from over 40 families [2], and it has apparently evolved independently on several occasions [2]. Tropical plant species as a group include a higher proportion of latex producing species than those from temperate regions [3], 14% compared to 6%. However, the purpose of latex was initially unclear. Various theories were put forward over the years, such as storage of nutrients or waste products or maintenance of water balance, but the available evidence offers no support for any of these [2]. For example, although the latex of *Euphorbia esula* contains carbohydrates, these are unavailable to the plant even under conditions of light starvation [4]. The first author to suggest latex as a defense mechanism was James [5], who noted how North American milkweeds produce copious amounts of distasteful latex, which offers "better protection to the plant from enemies than all the thorns, prickles, or hairs that could be provided". The importance of latex against insect herbivory is now well established, and a role against fungal pathogens has emerged [6].

With a few exceptions, latex is produced and stored in specialized cells called laticifers (Figure 1). The convergent evolution of latex production has led to a number of different laticifer structures, although they can be broadly divided into non-articulated and articulated [7]. Non-articulated laticifers originate from cells that elongate and push their way through other cells, branching without cell division but with nuclear division. Neighboring cells do not merge. This group includes the milkweeds (*Asclepias* spp.) and members of the Euphorbiaceae such as *Jatropha dioica*, whose laticifer network

is made up of only 5–7 huge cells. Articulated laticifers are formed from chains of cells that merge to form long tubes, and in some species, further merger takes place, forming loops or nets (anastomosing). Examples of articulated laticifers are those found in papaya plants (*C. papaya*), opium poppy *(Papaver somniferum*), and banana (*Musa acuminata*) [7].

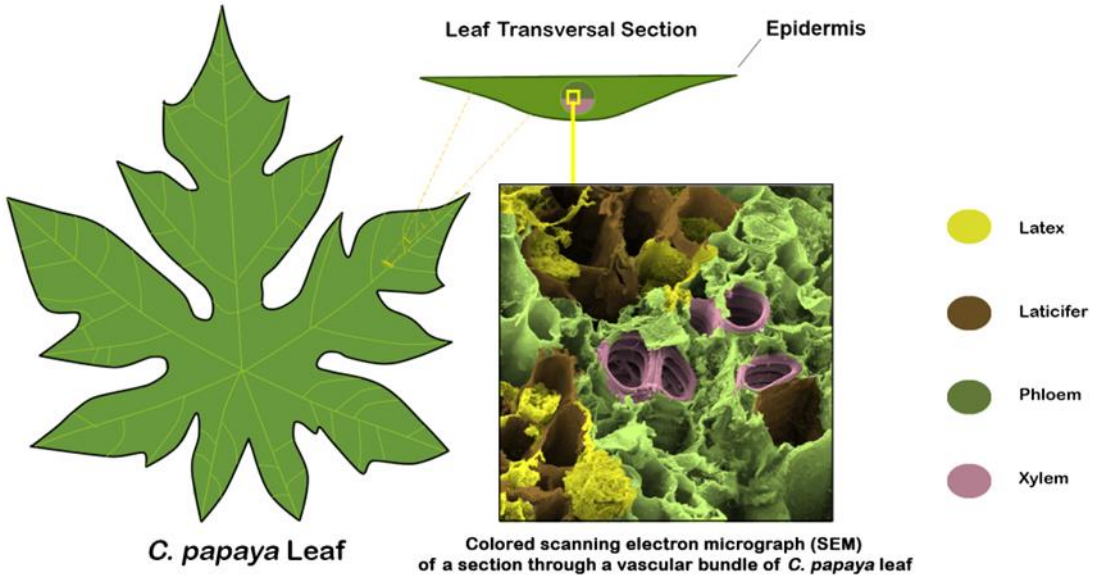

Figure 1. Schematic of the distribution of laticifers in a transversion section of *C. papaya* leaf.

There is evidence that some plants can at least tailor the contents of their laticifers depending on which part of the plant the laticifer resides in. For example, the white mulberry (*Morus alba*) has non-articulated, branched laticifers that apparently arise independently in different organs of the plant and are discontinuous. In those leaf petioles, two insecticidal chitinases were the most abundant proteins, while in latex from the trunk, an antifungal chitinase was most abundant. These and other variations in content suggest that the laticifers are primed to repel either herbivorous insects from fresh, unlignified leaves or fungal/microbial invaders from the lignified trunk [1].

Latexes contain a huge range of secondary metabolites, but the main ones are terpenes, phenolics, alkaloids, and cardenolides. These chemical compounds

contribute to environmental plant fitness but not to plant growth itself [1]. Some latex components have an identified, or putative, role in antiviral processes (Table 1). Plant latex also possesses a redox system and a protein-based mechanism that may operate to control virus infection in laticifers. Thus, the aim of this review is to compile information on plant latex's pro and anti-viral roles and discuss their potential use. In addition, the application of genetic editing to deepen the understanding of laticifer biology is also presented.

Table 1. Confirmed effects of plant laticifer components on viruses and potential interactions inferred from other plant tissues.

ANTIVIRAL INTERACTION			
Component	**Interaction**	**Model**	**Reference**
alkaloids	DNA and RNA synthesis inhibition and viral replication blockage	HIV-1, SARS-CoV-2	[1]
cysteine protease inhibitor	inhibition of viral cysteine proteases	potato virus Y in potato plants	[8]
cysteine/serine protease inhibitor	inhibition of viral proteases	tomato-spotted wilt virus in tomato	[9]
ubiquitin–proteasome system	degradation of viral proteins	potyvirus in *Arabidopsis thaliana*	[10]
phenolics and polyphenols	binding to the viral envelope	influenza A virus in *Croton lechleri*	[11]
cardenolides	inhibition of viral protein synthesis, viral cellular release, and dispersion	herpes simplex virus type 1 in *Digitalis lanata*	[12]
latex extracts	inhibition of viral multiplication	herpes simplex virus type 1 and echovirus type 11 in *Ficus carica*	[13,14]
calanolides	inhibition of viral reverse transcriptase	*Calophyllum teysmanii,* a human immunodeficiency virus	[15]

calreticulin	binding to the virus to inhibit spread or to the virus helper protein	*tobacco mosaic virus* in *Nicotiana tabacum*	[16]
PRO-VIRAL INTERACTIONS			
Component	**Interaction**	**Model**	**Reference**
latex particles	potential increase in latex fluidity, increasing viral dispersal	papaya meleira virus in *Carica papaya* L.	[17]
Hsp70 proteins	assembly and activation of viral proteins, reduction in plants in response to infection	tomato bushy stunt virus in *Nicotiana benthamiana* and *Carica papaya L.*	[18,19]
Euphorbia tirucalli latex	activation of the Epstein-Barr virus lytic cell cycle	Epstein-Barr virus in *Euphorbia tirucalli*	[20]
terpenoids	activation of the HIV-1 virus	human immunodeficiency virus type 1 in *Euphorbia officinarum*	[21]

2. Protein-Based Mechanisms

2.1. Proteases and Their Inhibitors

C. papaya and *Ficus carica* L. (common fig.) latex contain papain and ficin, respectively, which belong to the group of cysteine proteases [22]. The titer of proteases in species of the C1A papain-protease group is very high in leaf tissue and the latex of the fruit, and their participation in the metabolic pathways of different processes such as senescence, abscission, programmed cell death, and fruit ripening has been demonstrated [22]. The extraction and purification of papain from *C. papaya* leaves and fruits have been used in the food industry in winemaking [23], as a meat tenderizer [24], and in the pharmaceutical industry for the production of virus, fungal, and bacterial inhibitor drugs. Also, the use of latex proteases in medicine has been important in providing treatments for degenerative diseases due to their enzymatic action [22].

The latex exuded by *C. papaya*, a product of the laticifers that are damaged during the attack of herbivorous insects, has a toxic effect on some caterpillars, such as the cabbage moth (*Mamestra brassicae*) [25]. However, this effect disappears if the latex is washed or a cysteine protease inhibitor is added [26], such as the digestive substances of the monarch caterpillar (*Danaus plexippus*), which effectively destroys the toxicity of the latex [27]. A few prominent plant families that produce a larger amount of latex are the Caricaceae, Moraceae, and Apocynaceae, which also contain cysteine proteases in their latex, while serine proteases [28] have been found in latex samples from the Moraceae, Euphorbiaceae, Apocynaceae, and Convolvulaceae [29]. Several proteases known to be activated in laticifers are involved in the hypersensitivity response in plants to virus infection. However, the direct effect of cysteine proteases on viruses is poorly understood [30,31].

When infected by viruses, plants develop a local hypersensitive response (HR) that usually involves programmed cell death (PCD) [31]. Apoptosis, a form of animal PCD, involves cysteine proteases of the caspase group that targets specific protein substrates [32], and in plants, the activity is affected by metacaspases and papain-like Cys proteases that are induced during HR [33]; however, these activities are inhibited when caspase inhibitors are used, but protease inhibitors that do not inhibit caspases, e.g., E-64 and AEBSF, can also block PCD [30,34]. This suggests that additional proteases, such as papain-like cysteine proteases (PLCPs), can also be effectors or regulators of plant PCD [34]. In fact, cathepsin B, a papain-like cysteine protease, was shown to be required for disease resistance-causing HR in plants [35]. Against this hypothesis, the infection of *C. papaya* laticifers by papaya meleira virus (PMeV) and papaya meleira virus 2 (PMeV2) [36] leads to the accumulation of hydrogen peroxide (H_2O_2) [17], generating a reduction in the levels and activity of cysteine protease [30]. However, it could be argued that these are, by definition, susceptible plants, and a reduction in protease levels may be associated with their susceptibility [27,35].

There are many examples of cysteine and serine protease inhibitors in plant latex. However, a discernible function of these inhibitors seems to involve the inhibition of insect herbivory and microbial activity, including certain viruses [25]. Thus, a role in anti-viral protection is becoming apparent. Many viruses themselves encode proteases whose proteolytic enzymatic activity could act against host defenses [37]. For this reason, several protease inhibitor drugs, including azithromycin, were tested in the last

coronavirus pandemic, demonstrating a clear example of their increasing use in clinical medicine as antiviral agents [38]. Expression of the rice cysteine protease inhibitor gene oryzacystatin I in transgenic tobacco showed enhanced resistance to two potyviruses, *tobacco etch virus* and *potato virus Y*, which use cysteine proteases for protein processing [8]. Specific protease inhibitors are also expressed naturally in response to viral infections. Tomato plants infected with *tomato spotted wilt virus* express an unusual protease inhibitor with some features of cysteine and serine inhibitors, although the effect on the virus is unclear [9].

2.2. Loss-of-Susceptibility to Potyviruses (LSP1) Protein

A proteomic survey of lettuce latex [39] found a putative LSP1 protein, a member

of the eukaryotic translation initiation factor 4E (eIF4E) family of proteins, which are involved in translation initiation. The LSP1 protein is an isoform called eIF(iso)4E, and mutation of this gene is one of the factors associated with resistance to potyvirus infection in *Arabidopsis thaliana* [10]. This protein is a cap-binding protein that binds to the 5’ cap structure of nuclear-encoded mRNAs. The translation factor eIF(iso)4E can interact with the potyviral genome-linked protein of some potyviruses, apparently in a pro-viral manner, as Arabidopsis mutants lacking eIF(iso)4E have immunity or near immunity to *turnip mosaic potyvirus* and *tobacco etch potyvirus* [40]. The LSP1 protein is thought to play a role in the translation of mRNAs that are important for the establishment of potyvirus infection [10]. The identification of eIF(iso)4E in lettuce latex in association with proteins from the *lettuce mosaic virus*, a potyvirus, implies its potential involvement in susceptibility within this particular species [39].

2.3. Ubiquitin-Proteasome Degradation

Various proteins associated with the ubiquitin proteasome-mediated protein degradation system have been detected in lettuce latex, including a putative CAND1 (Cullin Associated and Neddylation Dissociated 1), an unmodified CUL1-interacting protein [41], More Axillary Branch 2 (MAX2) (a type of ubiquitin-protein ligase), and the 20S proteasome beta subunit G1 [42]. Similarly, expression of ubiquitin–proteasome

pathway genes has been detected in latex from the rubber tree (*Hevea brasiliensis*) and fig fruit (*Ficus carica* L.) [43,44].

Upregulation of genes associated with the ubiquitin–proteasome system in response to virus infection and propagation has been reported by a number of studies, for example in Arabidopsis following *plum pox virus* infection [45] or tobacco (*Nicotiana tabacum*) infected with *tomato mosaic virus* or *tobacco mosaic virus* [46]. Conversely, silencing of the E3 ubiquitin ligase NbSGT1, which operates as a co-chaperone during viral infection, and plant immunity generated by R genes in *Nicotiana benthamiana* compromise resistance to *tobacco mosaic virus* [47] and *potato virus X* [48,49], though not *cauliflower mosaic virus* [48].

Proteomic analysis of *C. papaya* leaf samples showed that both 20S catalytic and RPT5a subunits were upregulated following PMeV infection [18]. Those proteins were found later to occur in the *C. papaya* latex proteome, although their abundances did not change significantly during infection [30]. However, the ubiquitin–proteasome system (UPS) may function in a dual role during virus infection by impairing or facilitating viral replication or transport. This is part of the general resource redistribution within the plant body due to infection or a direct down-regulation effect on viral proteins or proteins essential for virus replication, as has been reported for various virus movement proteins. There may even be an effect on the virus's RNA or DNA integrity, as the sunflower proteasome has been shown to act as a nuclease on tobacco mosaic virus in vitro [49].

As with most aspects of plant-pathogen relationships, there are pathogen responses to plant defenses. Lettuce mosaic virus infection leads to an inhibition of proteasome RNase activity in pea plants, apparently by binding the viral protein helper component-proteinase (HcPro) to multiple sites on the 20S proteasome core [50,51].

2.4. Heat Shock Protein 70 (Hsp70) Isoforms

The plant Hsp70 protein chaperone system has been previously shown to be co-opted by infecting viruses. Hsp70 family proteins have been associated with the assembly of viral replicases, stimulation of viral RNA-dependent RNA polymerase (RdRp) activity, intracellular transport of replication proteins, and generally the assembly and activity

of viral proteins [19]. Infected plants typically express elevated Hsp70 levels [52], while silencing or inhibition of Hsp70 reduces *tomato bushy stunt virus* [53] accumulation. Contrarily, infection of *C. papaya* by PMeV and PMeV2 downregulates Hsp70 proteins in leaf tissue [18]. Several Hsp70 isoforms have been found in the *C. papaya* latex proteome. Although not statistically significant, almost all of them were in lower abundance in the infected latex [30]. This suggests that *C. papaya* Hsp70s are also important for PMeV/PMeV2 replication, and lowering the abundance of those proteins may limit viral replication.

3. Oxidative Responses

Production of reactive oxygen species (ROS) such as H_2O_2 and nitric oxide (NO) is a well-established signaling mechanism and is also involved in response to viral infection [54]. *C. papaya* laticifers infected with PMeV and PMeV2 show considerable increases in H_2O_2, phosphorus, potassium, and water levels that cause the spontaneous exudation of latex in fruits, perhaps due to an osmotic alteration [17,30]. In addition, a reduction in calcium together with an increase in hydrogen peroxide might affect signaling in the regulation of laticifer stress. In accordance with this, several isoforms of peroxidase have been isolated from *C. papaya* latex [30]. Similarly, the presence of antioxidant enzymes involved in plant defense has been demonstrated in the latex of lettuce (*Lactuca sativa*) and yellow bellflower (*Thevetia peruviana*) [55]. They include methionine sulfoxide reductase, which can rescue enzymatic activity after damage to methionine residues by ROS such as hydroxyl radicals and superoxide ions [56]. Three enzymes involved in thioredoxin reduction and oxidation, and thus cellular redox balance, were also isolated: ferredoxin oxidoreductase, ferredoxin thioredoxin reductase, and mitochondrial NADP adrenodoxin-like ferredoxin reductase [56]. Antioxidant production is one area that illustrates the disparate evolution of lactifers, as shown by a survey of three plant species from Brazil [27,57]. Latex from *Cryptostegia grandiflora* (Apocynaceae) and *Plumeria rubra* (also Apocynaceae) exhibited strong ascorbate peroxidase and superoxide dismutase activities [58], while activity in *Euphorbia tirucalli* (Euphorbiaceae) latex was undetectable [59]. In contrast, only *C. grandiflora* exhibited measureable catalase activity [60].

4. Secondary Metabolites

4.1. Phenolics and Polyphenols

Phenolic compounds are secondary metabolites involved with biotic and abiotic stresses in plants [61]. The chemical complexity of these compounds ranges from simple phenolic acids to complex tannins and lignins. Polyphenols have structural diversity because of the number of their phenol rings and other linked elements. They are present in several parts of plants (leaves, fruits, roots, etc.), including latex [61].

Hevea brasiliensis latex has quercetin and rutin, both polyphenols with relevant antiviral activity against rabies virus, poliovirus, syncytial virus, HSV-2, *respiratory syncytial virus*, dengue virus, influenza virus, and coronavirus [62]. The antiviral mechanism of these compounds is related to inhibition of viral polymerase and binding of viral nucleic acid [63].

SP-303/Provir, an oligomeric proanthocyanidin polyphenol isolated from the latex of *Croton lechleri*, has been shown to have an antiviral action against a number of human viruses, including *respiratory syncytial virus*, influenza A virus, parainfluenza virus, and *herpes virus types 1* and 2. SP-303 seems to bind directly to the viral envelope, inhibiting viral attachment [11].

Some plants contain large amounts of phenolics in their latex; for instance, the sweet potato, *Ipomoea batatas*, contains the hexadecyl, octadecyl, and eicosyl esters of p-coumaric acid in up to 10% of fresh root latex in one variety [64]. However, tests of p-coumaric acid with *herpes simplex virus type 1* [65] and type 2 [65] or *human cytomegalovirus* [66] found no antiviral effect, although there was a degree of antiviral activity against one of the adenoviruses tested [65].

4.2. Terpenoids

Terpenoids are one of the most prevalent secondary metabolites in plant latex [67] and consist of isoprene subunits. They present promising antimicrobial activity against bacteria, fungi, protozoa, and viruses. The latex of lettuce is especially rich in sesquiterpene lactones, including lactucin, lactucopicrin, and lettucenin A [68], which

have been shown to deter insect feeding as well as have an antifungal action, inhibiting the growth of *Cladosporium herbarum* [68]. Although there have been suggestions that latex terpenoids contribute to the resistance of Lactuca species to multiple viruses, a comparison of sesquiterpene lactone titers and resistance levels suggests that this is not the case [68].

4.3. Cardenolides

Cardenolides, a type of steroid, are found in the latex of various Apocynaceae plants, including milkweeds (*Asclepias* spp.) and oleander (*Nerium oleander*), as well as the latex of a Moraceae species, *Antiaris toxicaria*, apparently arising from convergent evolution [69]. A cardenolide derivative, glucoevatromonoside, isolated from a Brazilian cultivar of the Woolly foxglove (*Digitalis lanata*), has been shown to inhibit replication of *herpes simplex virus types* 1 and 2 [12]. It apparently inhibits viral protein synthesis as well as reduces cellular release and spread of virus, likely as an indirect result of virus-caused inhibition of Na+K+ATPase activity. It thus leads to cellular depletion of K+ and lowers the activation of several viral K+ dependent enzymes.

4.4. Alkaloids

Alkaloids are a group of compounds found in plants, animals, and fungi derived from amino acids [1]. In plants, alkaloids are found as non-volatile and non-odorous compounds in different tissues, including laticifers. One of the most known alkaloids is opium, obtained from the latex of *P. somniferum* and commonly used in medicine and psychedelic drugs [1]. In addition to the classical anti-herbivorous activity [67], some alkaloids affect virus replication. For example, ChM-P2 from *Chelidonium majus* latex prevented infection with HIV-1 in vitro and in vivo. Moreover, patients infected with SARS-CoV-2 treated with *C. majus* latex showed clinical improvement after three days of treatment [1]. The mechanisms involved in the antiviral activity of alkaloids include DNA and RNA synthesis inhibition and viral replication blockage [70]. Several studies showed that alkaloids interact with cell membrane receptors, indirectly disturbing the cell-virus interaction. This might have an effect on several viruses, as enveloped and non-encased viruses rely on the cell membrane as the main site for cell cycle

completion. Alkaloids may have other targets; for instance, oliverine suppresses HSV-1 DNA synthesis, lycorine inhibits dengue virus and zika virus RNA polymerases, emetine inhibits HIV-1 reverse transcriptase, and tomatidine interferes with the production of CHIKV viral particles [70]. Therefore, plant laticifers are important sites for bioactive compound production and storage [67].

5. Trials of the Biological Activity of Latex Constituents

5.1. Antiviral Activity

A number of trials have found fractions or compounds from latex to have an effect on viruses. As early as 1974, it was found that latex from three *Jatropha* sp. (Euphorbiaceae) inhibited infection with the *tobacco mosaic virus*. Other studies have been influenced by traditional medicine; for example, latex from the fig *Ficus carica* has been used to treat diseases supposedly caused by viruses. Therefore, it was tested for antiviral activity against *herpes simplex type* 1 (HSV-1), *echovirus type* 11 (ECV-11), and an adenovirus [13]. Hexane and hexane-ethyl acetate fractions were found to inhibit virus multiplication at levels below cell toxicity. Studies are often based on members of the Euphorbiaceae used in traditional medicine. It was found that a methanol extract of latex from *Codiaeum variegatum* is active against the influenza virus A/PR/8/34, while a triterpene extract of *Euphorbia tirucalli* latex is active against rhinoviruses [14].

The hunt for compounds with anti-HIV activity has also uncovered antiviral compounds from plant latex. A survey of trees in Malaysia yielded the dipyranocoumarin calanolide B, isolated from the latex of *Calophyllum teysmanii*, which went through preclinical trials [15]. Calanolide A and B act by inhibiting HIV-1 type 1 reverse transcriptase and have since been found in the bark and leaves of various Calophyllum species [15].

A study with *Chelidonium majus* L. (Papaveraceae) latex showed that compounds of latex, such as alkaloids and proteins, decreased HPV infection and inhibited oncogene viral expression [71]. *C. majus* has been used in Asian and European natural treatments of condylomas, which are visible HPV symptoms [71].

A glucoside lignin ((+)-pinoresinol 4-O-[6″-O-vanilloyl]-β-d-glucopyranoside) was isolated from the latex of *Calotropis gigantea* (Asclepiadaceae) and showed antiviral activity against influenza (H1N1) through suppression of viral replication [72]. Other compounds (6′-O-vanilloyltachioside and 6′-O-vanilloylisotachioside) were found but did not present anti-influenza activity [72].

A virus-cell-based assay with *chikungunya virus* (CHIKV) subjected to Euphorbia latex showed more potent antiviral activity than other extracts tested. 13-O-isobutyryl-12-deoxyphorbol-20-acetate and ingenol-3-mebutate were found in the latex of Euphorbia species (*E. peplus* and *E. segetalis* ssp. *pinea*) and can be related to inhibition of the CHIKV [73].

5.2. Proviral Activity

Perhaps surprisingly, there have been a number of studies showing activation rather than suppression of viral activity. An example comes from endemic Burkitt's Lymphoma (eBL), one of the commonest childhood cancers in sub-Saharan Africa, which is closely associated with the *Epstein–Barr virus* (EBV). Comparison of the distribution of the spurge *Euphorbia tirucalli* with that of eBL [74] and the discovery that methanol extracts of *E. tirucalli* tissues enhanced EBV-mediated cell transformation [75] led to suggestions that this plant may be involved as a cofactor in eBL, though it was initially unclear how. Like many of the Euphorbiaceae, *E. tirucalli* exudes a milky white latex that can be used as glue and is commonly played with by children. It was found that this latex applied to cell lines activated the EBV lytic cell cycle, implicating *E. tirucalli* latex as a possible environmental co-factor in eBL [20]. One suggestion is that 4-deoxyphorbol esters in the latex act in conjunction with EBV to alter c-MYC expression and cause chromosome aberrations [76].

Virus reactivation can be clinically useful. A major obstacle to HIV-1 eradication with antiviral mixtures (Highly Active Antiretroviral Therapy, or HAART) is the presence of latent HIV-1 cell reservoirs, which typically reactivate when HAART treatment is interrupted [77]. Thus, concurrent virus activation and HAART treatment are desirable options. Various plant products have proven useful in this regard, such as prostratin [78], and studies suggest that terpenoids isolated from the latex of *Euphorbia lactea* and *E. laurifolia* [79] or ingol diterpenes from the latex of *E. officinarum* [21] can also

cause HIV-1 activation. The compound from *E. lacteal* likely acts via the PKC pathway [79].

6. Papaya Meleira Virus Complex: Two Viruses Infecting Laticifers

Although a proteomic analysis of lettuce latex found evidence of proteins from *lettuce mosaic virus, mirafiori lettuce big-vein virus*, *lettuce big-vein virus*, *lettuce infectious yellows virus*, *lettuce ring necrosis virus*, and *lettuce necrotic yellows virus* [39], it is remarkable that, so far, only five viruses, PMeV, PMeV2, a Mexican variant of PMeV (PMeVMx), *papaya virus Q* (PpVQ), and papaya sticky fruit associated virus (PSFaV), have been isolated from latex samples [80]. This suggests that viruses have their transmission to plants impaired by the physical and chemical barrier formed by latex coagulation upon insect feeding on plant tissues [69]. Alternatively, latex is either a hostile environment for viruses or this plant fluid has been neglected in the plant virus field.

PMeV is an 8.7 kbp double-stranded RNA (dsRNA) virus (Fusagraviridae) [81] that, on co-infection with PMeV2, a 4.5 kbp single-stranded RNA (ssRNA) (umbra-like virus) [36], causes papaya sticky disease (PSD) [36,82]. Sequence similarity and phylogenetic analysis that included PMeV2, PMeV-Mx, and PpVQ suggest that these viruses may be different isolates from the same umbravirus [36]. Thus, PSD symptoms are caused by the combined infection of PMeV and PMeV2 (the PMeV complex) [80].

There are two fundamental questions to be answered: how can the PMeV complex colonize *C. papaya* laticifers, and what is its dispersion strategy within the plant and in the environment. The first is as yet unclear, but there has been progress with the second, and changes within the latex following infection have been determined [17,30].

The latex from diseased plants coagulates much less readily than that from healthy plants, and latex particles have a different morphology and a reduced titre. Viral particles bind tightly to the latex solid phase, which appears to change its morphology. The model of latex coagulation from the rubber tree results from the binding of surface proteins on the latex particles, specifically *Hevea* latex lectin-like protein (HLL) to glycosilated N-Acetyl-D-glucosamin (GluNAC) receptors, causing particle aggregation [83]. Thus, changes in surface morphology in *C. papaya* latex may disrupt coagulation,

resulting in a more liquid latex. There are also biochemical changes within the latex. Binding between HLL and GluNAC in rubber trees is calcium-dependent [83]. The latex from sticky diseased *C. papaya* contains about half the amount of calcium in the latex of healthy plants [17], although there is an increased concentration of insoluble calcium oxalate.

C. papaya latex infected by the PMeV complex also contains about half the sugar concentration of latex from asymptomatic plants. Reduction of plasmodesmata diameter in plants has been observed in response to viral infection, presumably as a means to reduce viral mobility, and this can restrict the transport of sugars from photosynthetic sugars. *C. papaya* latex consists primarily of proteins and polyisoprene molecules, and a reduction in carbohydrates would be expected to reduce the biosynthesis of both, similar to the effect on isoprene synthesis in Hevea [84]. PMeV complex-infected *C. papaya* latex also shows an increased level of potassium, possibly due to the inward rectifying K^+ channel in the laticifers. This would be expected to increase water intake and thus could account for the spontaneous bursting of laticifers and exudation of latex seen in infected tissues. Taken together, changes in the latex particles' shape, interference with their binding, and an increased water content could account for the main symptoms of PSD.

But how does the PMeV complex deal with the hostile latex environment? There does appear to be a plant defense response; for example, alkaloids were elevated in infected latex [17], and these have been shown to be effective against viruses in other circumstances. Infected laticifers have an elevated level of H_2O_2 production, which is a known signaling mechanism for viral infection, and it has been suggested that the same is occurring here, especially as production is localized adjacent to phloem cells, which have been correlated with systemic transport and the stress response [17]. Further downstream in the resistance gene regulatory pathway, the Ran gene [85] and Ran/TC4 are upregulated during PMeV complex infection [18]. Further indication of a defense response comes from the upregulation of calreticulin [18], a calcium-binding protein. Calreticulin expression has been reported as a response to viral infection in other plant species and is an essential component of the plant Ca^{2+} signaling system, which is known to activate resistance pathways [86]. In addition, calreticulin has been shown to bind directly to the *tobacco mosaic virus*, inhibiting its spread through the tobacco host [16], and in *C. papaya*, it binds to the helper component proteinase of the

papaya ringspot virus (PRSV) [16]. Similarly, the downregulation of *C. papaya* translation initiation factor, Hsp70, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [18] may be a plant response to limit viral replication.

Although these responses of *C. papaya* to PMeV complex infection may account for the absence of viral infection outside of laticifers, the success of the virus in colonizing laticifers is still unexplained. As we have seen, plant latex is a highly hostile environment, with high concentrations of proteases and other anti-pathogenic compounds. An equivalent is perhaps the insect midgut, again rich in protease enzymes, and an example from there gives an idea of the capacity of viruses to survive harsh environments [87]. *Cytoplasmic polyhedrosis virus* (CPV) is a dsRNA virus enclosed in a capsid shell made up of 120 molecules of inner capsid shell protein. Virions of CPV are resistant to chemical treatments such as high pH and SDS and to enzyme disruption by trypsin, chymotrypsin, ribonuclease A, deoxyribonuclease, and phospholipase [88]. It is also capable of endogenous mRNA transcription within an intact virus particle using viral-encoded enzymes [87]. Although PMeV complex particles have been isolated and their genome and structural proteins have been studied [81], how *C. papaya* latex components influence PMeV/PMeV2 replication and effects on the plant's laticifers remains largely unknown.

The PMeV complex is able to colonize *C. papaya* laticifers, is able to move within the plant, and is able to infect other plants. There has been little study of cytoplasmic streaming or long-distance transport within laticifers (reviewed in [89]), but transport from shoots to roots via laticifers in cassava has been proposed [90]. Such movement would provide a transport mechanism for the PMeV complex and any other laticifer viruses in the plant. Additionally, of course, rupture of a laticifer causes movement of latex within the laticifer, and this would also transport the virus, both within the laticifer and outside, to the plant body. It has been proposed that the characteristic symptom of PMeV complex infection, spontaneous exudation of watery, sticky latex, is a strategy of the virus to aid dispersion [18].

7. Plant Genetic Editing of Laticifer's Expressed Genes

Genetic editing of plants, mostly by CRISPR (clustered regularly interspaced short palindromic repeats), has been used to alter plant genes involved in disease control,

stress, and the functional analysis of proteins [91]. As such, some genes typically expressed in laticifers have been edited (Table 2) to understand laticifers' biology and to control latex composition.

Table 2. Genes typically expressed in laticifers are edited using CRISPR.

Gene	Function	Plant Host	Reference
rapid alkalinisation factor like 1	Influences root phenotype and biomass, and inulin and natural rubber yield	*Taraxacum koksaghyz*	[92]
laticifer-specific cis-prenyltransferase 3	Involved in high-quality rubber production by laticifers	*Lactuca sativa*	[93]
1-fructosyltransferase	Encodes a key enzyme in inulin biosynthesis	*Taraxacum koksaghyz*	[94]
germacrene A synthase	Involved with the degradation of sesquiterpene lactones	*Cichorium intybus L.*	[95]
rubber elongation factor and small rubber particle Protein	Belong to the stress-related protein superfamily involved in rubber biosynthesis and storage	*Taraxacum koksaghyz*	[96]
kauniolide synthase	Disruption of sesquiterpene lactone biosynthesis in laticifers	*Cichorium intybus var. sativum*	[97]
Ribonucleoprotein	Belong to a family that controls plant flowering time	*Hevea brasiliensis*	[98]

The transcriptome analysis of latex mRNA from different plant species, e.g., *Hevea brasiliensis* Muell., *Ficus carica* L., *Taraxacum kok-saghyz*, *Papaver somniferum*, and *Euphorbia tirucalli*, has revealed laticifer active promoter regions. In parallel, a *H. brasiliensis* U6 mutant promoter induced by ribonucleoproteins has been identified and characterized. The mutation frequencies ranged from 3.74% to 20.11% among the five

targeted sites [99]. Five genes of the HbFT family involved with flowering control were edited by CRISPR/Cas9. Thus, their results point to flowerless plants with increased latex production and a reduced energy loss phenotype [98].

Taraxacum koksaghyz is known as the rubber dandelion and produces high-quality, high-molecular-weight rubber particles. Several studies [92,94] have used CRISPR/Cas9 based methods to understand rubber biosynthesis and to accelerate *T. koksaghyz* domestication to be used as a rubber-producing crop. By inducing mutagenesis at the fructan 1-fructosyltransferase (1-FFT) encoding gene, mutation rates ranging from 39.4% to 88.9% were obtained. The edited plants showed shorter flowering times than wild-type plants and flowered at the sixth week [94,96]. The 1-FFT enzyme is involved in the biosynthesis of inulin, a storage carbohydrate. Similarly, overexpression of fructan-1-exohydrolase, which degrades inulin, leads to an increase in *T. koksaghyz* natural rubber synthesis [96].

The role of the *T. koksaghyz* rapid alkalinization factor-like 1 gene (TkRALFL1) in inulin and rubber production has also been evaluated using CRISPR-based gene editing [92]. Thus, heterozygous and homozygous TkRALFL1 knockout plants showed a slight increase in inulin and rubber content compared to control plants [92]. In the same species, the PEP16 gene promoter from *H. brasiliensis* (HbPEP16), which has cis-acting elements responsive to several plant hormones and is involved with rubber biosynthesis, was tested. In lettuce, CRISPR/Cas9 targeting the laticifer-specific cis-prenyltransferase 3 was used to generate rubber production deficient mutants. The edited plants showed a considerable decrease in rubber production. They were further genetically modified through the introduction of the guayule and goldenrod genes, which are involved with rubber production and rubber polymers' properties. The latex from those plants showed rubber composed of longer polymers than those of control plants [93].

A gene editing study was also carried out in chicory (*Cichorium intybus*) to inactivate the kauniolide synthase genes (Cikls1, Cikls2, and Cikls3), classified in the CYP71 family of cytochrome P450 enzymes, and analyze the resulting effects on the production of sesquiterpene lactones (STLs). The roots of edited plants showed an accumulation of costunolide and its conjugates. Although pivotal roots of edited *C. intybus* lines have shown increased free costunolide content, the major STLs and STLs-derived oxalates were completely absent in some lines compared to controls

[97]. The same species had the germacrene synthase gene (cigas) edited. As cigas are involved with STL biosynthesis, the edited plants showed a strong reduction in STL levels and an accumulation of phenols and squalene [95]. The authors highlighted the potential use of those genetic lines for improved inulin extraction.

Although there are no publications reporting expressed genes in laticifers whose products interfere with virus infection as gene editing targets, the field is expected to grow in the next few years as the protocols for genetic editing of the above-cited species have been tested and are available for further studies.

8. Conclusions

Laticifers have independently evolved in various plant species, and numerous studies indicate their specialization as plant defensive structures. Latex exudation serves to seal wounds, acting as a deterrent and potentially lethal agent for herbivorous pests. Increasing evidence suggests that latex may also play a role in plant responses to viral infections, given the observed antiviral properties of many latex components and the relative absence of virus colonization in laticifers, albeit not entirely devoid. This area of research is expanding, particularly with the advent of gene-editing tools, offering novel insights into both plant biology and medicine.

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CAPÍTULO 3

MODULACIÓN DEL GEN SALICILATO O-METILTRANSFERASA DE *CARICA PAPAYA* L. MEDIANTE CRISPR CON MIRAS A DESARROLLAR PLANTAS TOLERANTES AL COMPLEJO DEL VIRUS CAUSANTE DE LA ENFERMEDAD DE LA MELEIRA EN PAPAYA.

INTRODUCCIÓN

La búsqueda de estrategias innovadoras para controlar las enfermedades de las plantas ha impulsado la investigación en biotecnología agrícola, especialmente en el contexto de la ingeniería genética (Ouyang *et al.,* 2017). En el caso específico de *Carica papaya* L, la presencia del complejo PMeV (*papaya meleira virus*) representa un desafío significativo para la producción de papaya, ya que esta enfermedad puede causar graves daños a las plantaciones (Quito-Avila *et al.,* 2023; Antunes *et al.,* 2020).

Ante este escenario, el enfoque de modificación genética ha surgido como una herramienta prometedora para conferir resistencia a muchas enfermedades. La edición del genoma de la papaya para inducir resistencia puede realizarse utilizando la tecnología CRISPR-Cas9. Una posible estrategia es modificar los genes implicados en la respuesta inmune de la planta para aumentar la resistencia a los patógenos (Mushtaq *et al.,* 2021). Por ejemplo, la edición de genes que codifican proteínas implicadas en el reconocimiento de patógenos y la activación de la respuesta inmunitaria puede aumentar la resistencia de la planta a la infección (Rato *et al.,* 2021).

Otra estrategia consiste en mejorar las defensas frente a patógenos editando genes implicados en la síntesis de moléculas implicadas en la defensa de las plantas, como alcaloides y terpenos (Ali *et al.,* 2019; Ninkuu *et al.,* 2021). Sin embargo, cabe señalar que la edición de genes en plantas es un proceso complejo y requiere un gran conocimiento en biología molecular, genética de la planta y del virus. Además, antes de modificar genéticamente las plantas deben evaluarse cuidadosamente los posibles impactos medioambientales y de seguridad (Mushtaq *et al.,* 2021).

Las técnicas contemporáneas de fitomejoramiento han experimentado avances notables en los últimos años, entre los que destaca la eficacia de métodos como CRISPR/Cas9, que complementan las técnicas tradicionales vigentes durante décadas (Borrelli *et al.,* 2018). Este enfoque innovador ha demostrado su capacidad para aumentar la resistencia a las enfermedades en las plantas y facilitar el desarrollo de nuevas variedades de cultivos. También existe un uso creciente de la mutagénesis en plantas de diversos cultivos como estrategia para reforzar su resistencia (Borrelli *et al.,* 2018).

Con el objetivo de identificar gen(es) de interés de *C. papaya* para su edición mediante CRISPR para la tolerancia al complejo del virus de la meleira en papaya, se realizó una revisión detallada de las bases de datos elaboradas por el grupo del Laboratorio de Biotecnología Aplicada al Agronegocio (LBAA) y de los datos disponibles en la literatura. Una vez identificado un gen objetivo, se ensayaron protocolos de edición de *C. papaya* mediante CRISPR. Se seleccionó el gen salicilato O-metiltransferasa que disminuye los niveles de ácido salicílico en la planta, ante la presencia de un agente patológico, para crear una construcción de ARNg que, mediante la edición CRISPR, confiera tolerancia a las plantas de papaya en presencia de la enfermedad de la meleira e incluso pueda desarrollar resistencia con el tiempo.

METODOLOGÍA.

SELECCIÓN DEL GEN CANDIDATO PARA LA CLONACIÓN.

El proceso de selección se llevó a cabo mediante la lectura de los trabajos de tesis y disertaciones (Rodrigues, 2006; Rodrigues, 2010; Abreu, 2011; Abreu, 2014; Soares, 2016; Madroñero, 2018; Da Silva, 2021; Maurastoni, 2021) realizados en el LBAA de la Universidad Federal de Espírito Santo (UFES). Se tuvo en cuenta los años 2006 a 2021 que estudiaron sobre la interacción de la planta de *Carica papaya* y el complejo de virus PMeV. Además, se analizó la modulación de algunos genes en la transcriptoma y la proteómica, centrándose en la etapa de posfloración en donde hay mayor presencia de sintomas en la planta en presencia del virus, demostrado en los trabajos del grupo. También se realizó la lectura de artículos externos sobre genes de defensa de otras especies de planta contra otros tipos de virus que sean relevantes

para este trabajo usando el buscador web Google Académico, entre los años 2005 y 2018 (APENDICE B) para tener más opciones de genes candidatos que puedan poseer homología con la planta de papaya.

BIOINFORMÁTICA PARA EL DISEÑO DE ARNg A SER USADO EN CRISPR.

La aproximación inicial al diseño del ARNg consistió en seleccionar el gen de interés, que se obtuvo de acuerdo con las lecturas hechas con anterioridad. Es importante que la secuencia del gen esté disponible en las principales bases de datos de análisis genéticos en Internet. La secuencia del gen elegido (SAMT), también llamado evm.model.supercontig_6.164 que tiene 1197 nucleótidos con 364 aminoácidos, fue obtenido en la base de datos Phytozome genome ID:113 (Goodstein *et al.,* 2012) y con el locus XM_022052428 en NCBI, donde se muestran sus CDS y los sitios de las secuencias de los ARNg, en donde se realizará su codificación (APENDICE A).
A partir de la secuencia, se utilizaron todos los parámetros básicos que ofrece el programa on line llamado CRISPR-P (Lei *et al.,* 2014) para el diseño del ARNg. También, se utilizó el programa CRISPRdirect (Naito *et al.,* 2015) con los parámetros por defecto para la comparación de las secuencias obtenidas y eliminar las que tenían formación de estructuras secundarias.

VECTOR DE TRANSFORMACIÓN *E. COLI* DE PKSE401.

El pKSE401 es un vector de expresión vegetal (Addgene #62202) derivado del vector comercial pCambia, creada mediante la tecnología de clonación Gateway ™ para el andamiaje del ARNg y uso en la edición del genoma de la planta basada en CRISPR/Cas, posee resistencia a kanamicina (Kan) y espectinomicina (SpR), con 16438 pares de bases (pb) en total y la parte del andamiaje del ARNg tiene 76 pb (Xing, *et. al,* 2014). En el proceso de inserción del ARNg es impulsado por el promotor U6-26p, el gen de la espectomicina es digerido por la enzima BsaI y el ARNg diana se inserta en este sitio.

INSERCIÓN DE LOS OLIGONUCLEÓTIDOS EN EL VECTOR.

Se utilizó el método de golden gate assembly para acoplar la secuencia de ARNg en el vector pKSE401. Se realizó una mezcla de ligación con 2 µL de los cebadores a una concentración de 100ng/µL, 2 µL de pKSE401, 1,5 µL de tampón de ligación de ADN T4 de New England Biolabs (NEB), 1,5 µL de BSA, 1,5 µL de la enzima de restricción BsaI-HF®v2 de NEB, 1 µL de ligación de ADN T4 y 6 µL de agua ultrapura, protocolo modificado de Xing et al., 2014.

OBTENCIÓN DE CÉLULAS ELECTROCOMPETENTES EN *ESCHERICHIA COLI*.

Se inoculó una alícuota de 3 mL de *E. coli* cepa DH5α (4.0E8 células/mL en fase logarítmica) pre cultivada en 300 mL de medio líquido Luria-Bertani (LB) protocolo de Sambrook y Russel 2001. Se dejó crecer a 37 °C con agitación a 150 rpm durante 2 horas con una densidad óptica (DO) de 0,5 y longitud de onda de 600 nm. A continuación, se vertieron en tubos falcón de 50 mL, se colocaron en hielo y se centrifugaron durante 15 minutos a -4 °C a 3.000 rpm.

Luego fue desechado el sobrenadante y se resuspendió cuidadosamente en 25 mL de agua destilada autoclavada helada, repitiendo este proceso tres veces, y finalmente se resuspendió en 10 mL de glicerol al 10% autoclavado y helado distribuyéndose en alícuotas de 40 µL en microtubos, protocolo modificado de Sambrook y Russel, 2001.

ELECTROPORACIÓN DE *ESCHERICHIA COLI*.

El clonaje de la mezcla de ligación se llevó a cabo con 5 µL de plásmido bien mezclado con 40 µL de células electrocompetentes de *E. coli* en un microtubo y luego se colocó la mezcla en una cubeta de electroporación y mediante choque eléctrico por el equipo electroporador de marca eppendorf eporator® a un voltaje de 1.700V con un tiempo constante de 5 ms. Inmediatamente después de la electroporación, la mezcla se colocó en 1 mL de medio SOC con su sigla en inglés (super optimal broth with catabolite repression) protocolo de Sambrook y Russel 2001, en tubos de ensayo esterilizados con anterioridad. Luego fue colocado en agitación durante una hora a 150 rpm a 37 °C.

Para comprobar la inserción de los oligonucleótidos en el vector, se inoculó en placas de medio Luria-Bertani sólido con 50 µg/ml de antibiótico (kanamicina) a crecer durante 24 horas a 37 °C, como muestras la figura 4 el diseño consistió como control positivo (sin antibiótico y sin plásmido), control negativo (con antibiótico y sin plásmido) y los plásmidos transformados con antibiótico, tanto el del vector pKSE 401 como los construidos pSAMC1, pSAMC2, pSAMC3, pSAMC4 y pSAMC5.

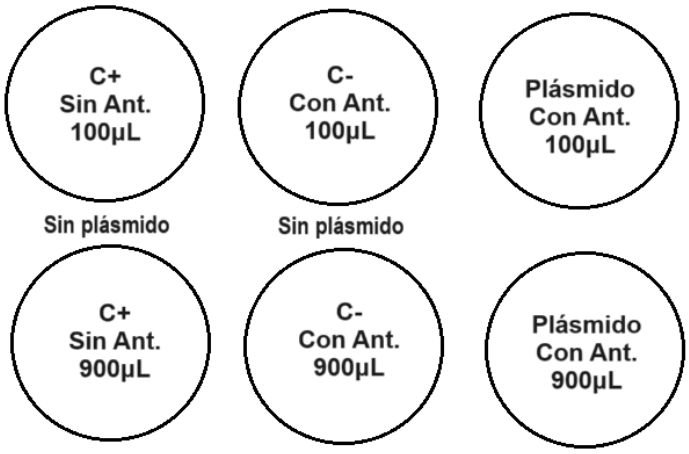

Figura 4. Diseño para la validación de la inserción del plásmido en la bacteria *E. Coli.*

EXTRACCIÓN DE ADN PLASMÍDICO

Fue colocado con anterioridad el inoculo de *E. coli* en medio LB líquido por 24 h a 37 °C. El ADN plasmídial se purificó utilizando el kit ChargeSwitch™ Pro Plasmid Miniprep de Thermofisher® y se cuantificó utilizando el nanodrop.

VERIFICACIÓN DE LA INSERCIÓN DEL ARNg EN EL PLÁSMIDO MEDIANTE PCR Y ELECTROFORESIS.

La PCR se llevó a cabo utilizando el kit Platinum™ Taq DNA Polymerase High Fidelity de Thermofisher® según la descripción del fabricante, en un termociclador (marca Applied Biosystems Veritiy) y el gel de agarosa al 1% en TAE conteniendo 0,001% de bromuro de etidio, la electroforesis se realizó durante 30 minutos a 90V.

RESULTADOS Y DISCUSIÓN.

SELECCIÓN DE ARNg PARA CRISPR

Se seleccionaron 27 secuencias que contuvieran 20 nucleótidos, entre un 40% y un 60% de guanina y citosina (GC) junto a tres nucleótidos específicos, que varían en función de la nucleasa utilizada, en este caso NGG, denominados en su sigla en inglés protospacer adyacente motive (PAM), donde N puede ser cualquier nucleótido. Las secuencias elegidas se enviaron a sintetizar en cebadores de unión a la compañía Macrogen®.

Los parámetros utilizados para eliminar las secuencias (APENDICE C) implicaban la necesidad de que la secuencia comenzara con una guanina en la posición 5' como la primera base de su transcripto, ya que el promotor U6 ARN polimerasa III es usado para expresar el ARNg y proporcionaría una fijación en la secuencia molde, también no debían presentar cinco nucleótidos repetidos de adenina (A), guanina (G), citocina (C) y de uracilo (U), ni en 3’ dos timinas (T) repetidas que provocan la terminación de la polimerasa III, además para evitar la formación de estructuras secundarias en presencia de las temperaturas melting superior a 0 ^{o}C (APENDICE C) (Bernardi y Takita, 2017).

Asimismo, es de suma importancia que la secuencia tenga 20 nucleótidos posicionado adyacente al PAM, los nucleótidos que usa la endonucleasa Cas9 (NGG), un contenido de GC cerca al 50%, donde tiene una tolerancia de 40% al 60%, para evitar que la secuencia se ligue a otra región (off-targets) y pueda causar ruido en la interpretación de los análisis posteriores (Morgante *et al.,* 2020). También, el elevado número de A en las posiciones 9-16 en la secuencia ARNg puede aumentar la afinidad de Cas9 en la secuencia molde y así generar un buen inicio en la fase de lectura, pero un número elevado de G en la misma posición puede generar un Cas9 menos funcional y presentar poca afinidad en la secuencia molde, causando una alteración de lectura en la edición o deleción de nucleótidos en la secuencia de estudio. Tampoco se recomienda una C en la posición 20 de la secuencia, ni una G en las posiciones 16 y 18 de la secuencia ARNg, para un alto apareamiento de las secuencias y un mejor corte de las enzimas de restricción (Moraes-Almeida *et al.,* 2022).

GEN SAMT ESCOGIDO PARA SER EDITADO POR CRISPR/CAS9.

Después de la revisión de varios trabajos internos y externos sobre los genes que están implicados en los mecanismos de defensa de las plantas, en especial en *Carica papaya* se escogió el gen SAMT también llamado BSMT1 en otras especies de plantas (Chen *et al.,* 2003), es una enzima crucial que cataliza la metilación del ácido salicílico (AS) para producir metil salicilato, un éster metílico del ácido salicílico. Esta enzima desempeña un papel fundamental en la regulación de los niveles de AS en las plantas, además su metilación puede regular la actividad biológica y la movilidad dentro de la planta, lo que influye en la señalización en respuesta al estrés y la defensa de la planta contra patógenos (Dempsey *et al.,* 2011), debido a que el AS se acumula en el floema y es importante en la activación de la resistencia sistémica adquirida (SAR) (Liu *et al.,* 2011; Huang *et al.,* 2020).

La vía metabólica del AS en la planta es un poco compleja y comienza desde el plastidio con el corismato dilucidado en dos vías, la primera inicia desde la vía del isocorismato (IC) y la segunda vía desde la fenilalanina amoníaco-liasa (PAL) (Dempsey *et al.,* 2011), en este estudio se centra en la primera vía mostrada en la figura 5.

En el momento de la infección primaria local es aumentado los niveles de AS, luego es biosintetizado por el gen SAMT convirtiéndolo y catalizándolo en MeSA facilitada por una BA/AS carboxil metiltransferasa (Huang *et al.,* 2020) y se ha descrito en cultivos de tomate (Tieman *et al.,* 2010), arroz (Koo *et al.,* 2007), soja (Lin *et al.,* 2013) y arabipdosis (Chen *et al.,* 2003); esta MeSA inmediatamente es translocado y transportado por la membrana a todas las células de la planta para inducir productos volátiles en respuestas de defensa en partes sanas de la planta infectada o en plantas cercanas que no han sido atacadas (Chen *et al.,* 2003; Zubieta *et al.,* 2003). En otros estudios demostraron que la producción de MeSA no es esencial para el desarrollo de SAR (Attaran *et al.,* 2009; Liu *et al.,* 2011).

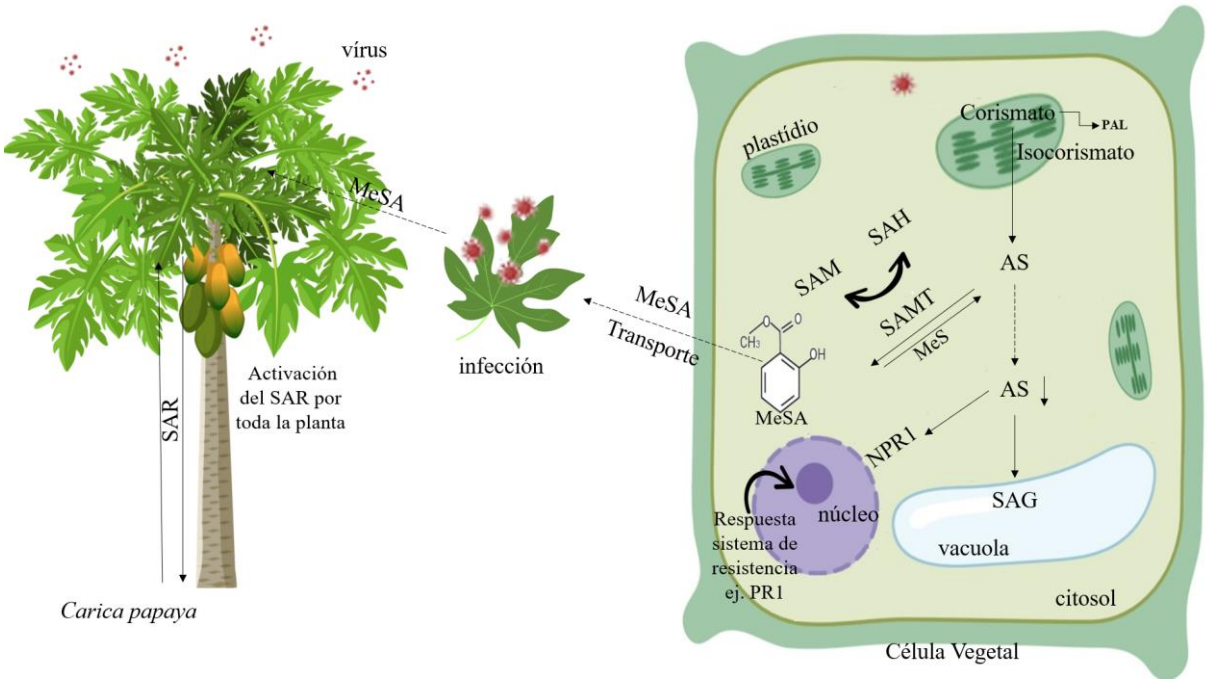

Figura 5. La metilación del ácido salicílico dentro de la célula vegetal. (AS) ácido salicílico, (MeSA) Salicilato de metila, (MeS) Metilesterasa, (NPR1) gene relacionado con la patogénesis, (SAR) Resistencia Adquirida Sistémica, (SAM) S-adenosilmetionina, (SAH) S-adenosilhomocisteína, SA 2-O-β-D- glucósido (SAG).

No obstante, se obtiene niveles significativamente reducidos de SA, lo que afecta la producción en bajo nivel de los NPR1 que se transportan al núcleo y activan los transcriptos PR1(genes asociados a la resistencia local). Al mismo tiempo, una parte de MeSA que permanece en el citosol es desmetilada por un metil esterasa (MES) (Dempsey *et al.,* 2011) para producir niveles bajos de SA; sin embargo, los niveles crecientes de AS desactivan el MES y el AS circundante es convertido por la enzima AS glucosil transferasa (ASGT) inducible por patógenos (Rangel *et al.,* 2010) y almacenado como SA O-β-glucósido (SAG) en la vacuola.

Lo que se puede entrever, es que el metabolismo de MeSA no es muy claro en algunas plantas, ya que en plantas de tabaco los autores propusieron que era una señal móvil para el SAR (Park *et al.,* 2007) pero en otros estudios demostraron que no era necesario para el SAR en arabipdosis (Attaran *et al.,* 2009; Huang *et al.,* 2020). Por otra parte, en otra investigación analizaron cómo estas enzimas han divergido a lo

largo del tiempo en diferentes especies de *Nicotiana*, lo que puede tener implicaciones en la evolución y la adaptación de estas plantas (Hippauf *et al.,* 2010).

El papel de SAMT en la papaya ha sido poco estudiado. Sin embargo, Madroñero *et al.,* 2018 observaron una regulación negativa del ácido salicílico mediada por genes como el NPR1 durante la etapa de prefloración. Estos genes están asociados con la acumulación y la regulación reducida de las respuestas de AS. También observaron que los niveles de transcripción que codifican la enzima BSMT1, que convierte AS en MeSA, aumentan después de la fructificación de la planta, lo que sugiere que la regulación de AS está involucrada en el desarrollo sintomático de la enfermedad de la meleira en papaya, siendo un gen promisorio para ser silenciado ayudando a comprender la implicación del SAR en control de la enfermedad (Madroñero *et al.,* 2018).

DISEÑO DE ARNg DEL GEN SAMT E INSERCIÓN EN EL VECTOR PKSE401.

Como resultado del programa CRISPR-P se obtuvieron 27 secuencias de ARN guía candidatas y con ayuda de CRISPRdirect se evaluó los posibles errores que se podrían presentar en su expresión para así garantizar su complementariedad y especificidad con el molde de interés, igualmente, las secuencias fueron evaluadas teniendo en cuenta criterios eliminatorios (APENDICE C).

En el APENDICE C se encuentra una lista de secuencias preseleccionadas y las mejores puntuaciones de (0.1145 a 0.7739) otorgadas a cada secuencia que cumplían los requisitos exigidos para el diseño del mejor ARNg y preferencialmente estar localizados en la región del exón cerca al codón de iniciación, ya que las mutaciones causadas por ese sistema (indels) normalmente alteran la fase de lectura (Bernardi y Takita, 2017). La tabla 3 muestra las 5 secuencias seleccionadas para la síntesis y se cambiaron las cuatro primeras bases a ATTC en dirección de la secuencia 5’ a 3’ forward (F) y en la secuencia complementar 3’ a 5’ se cambiaron a CAAA reverse (R), llamándose SAMCP del gen SAMT de Carica papaya (Xing *et al.,* 2014).

En cierta medida, cuando se insertó el ARN guía en el vector pKSE 401 figura 6b para el sistema CRISPR/Cas9, la región U6-26p se empleó como promotor para la expresión del ARNg figura 6a. Esta región es crucial, ya que es reconocida por la ARN

polimerasa III (Pol III), la polimerización mediada por este tipo de polimerasa evita las modificaciones post-transcripcionales y se encarga de impulsar la transcripción del ARNg; Una vez que el ARNg se insertó bajo el control del promotor U6-26p, la Pol III transcribirá el ARNg, lo que permitirá que este guíe la proteína Cas9 hacia el sitio específico del genoma donde se desea realizar la edición (Xing *et al.,* 2014).

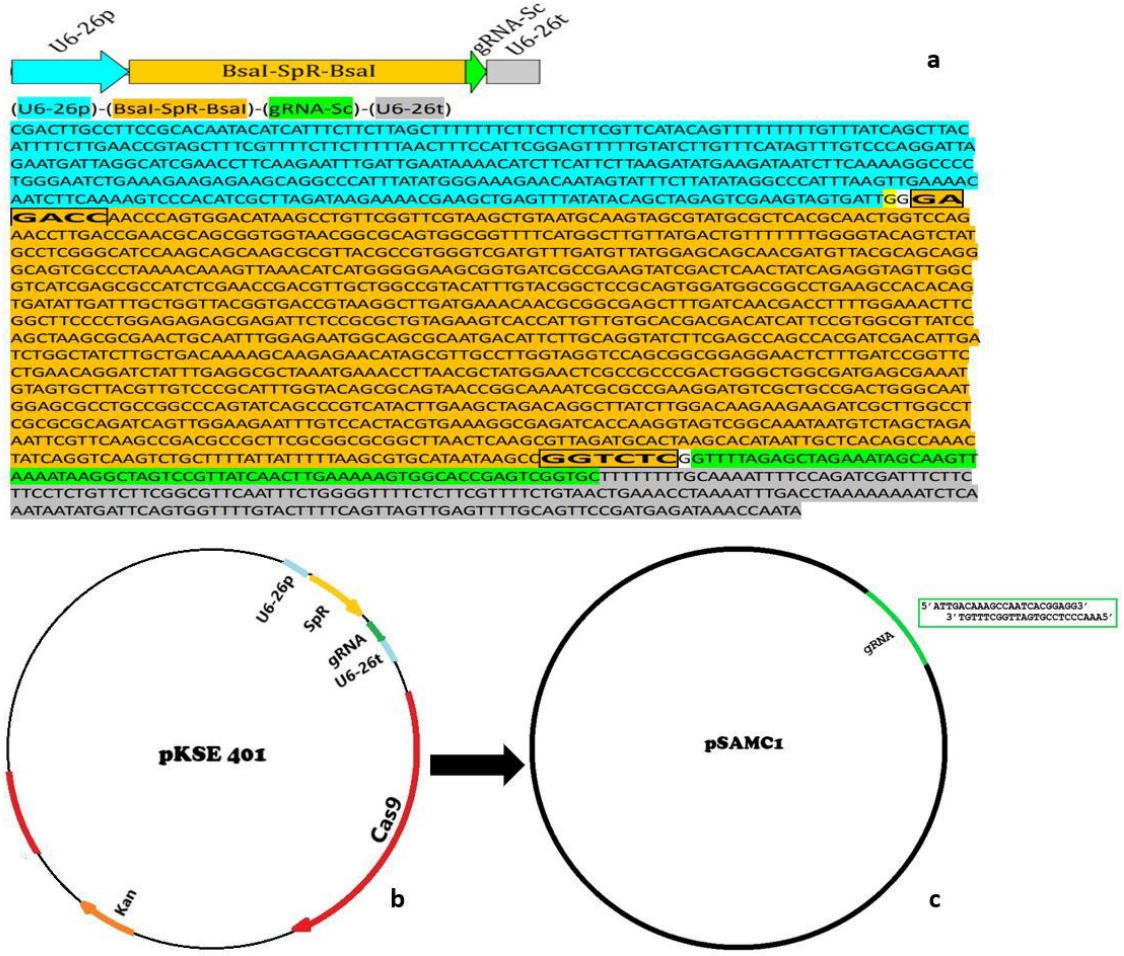

Figura 6. Esquema de la modificación del vector pKSE401. (a) Secuencia de expresión del ARNg dirigido por U6-26p, SpR gen de resistencia a la espectinomicina. Las letras agrandadas y en recuadro indican los sitios BsaI (Xing *et al.,* 2014). (b) El vector pKSE 401 con sus partes a modificar. (c) Nuevo plásmido sintetizado con el ARNg inserto de SAMC1, llamado pSAMC1.

No obstante, en la figura 6a muestra la síntesis del vector donde la enzima de restricción BsaI es utilizada para cortar los fragmentos de ADN a su vez perdiéndose esta región y uniéndolos mediante una ligasa T4, en la construcción y transfección del

ARN guía en el vector de expresión como lo muestra la figura 6c siendo eficiente en la unión específica de los fragmentos y con una duración de 5 horas usando el método golden gate assembly (Xing *et al.,* 2014).

Tabla 3. Lista de oligonucleótidos de ARNg sintetizadas para CRISPR/Cas9.

Pares de oligonucleótidos	Secuencias	Temperatura melting
SAMCP1	F 5’ ATTGACAAAGCCAATCACGGAGG 3’ R 3’ TGTTTCGGTTAGTGCCTCCCAAA 5’	63 ^{o}C
SAMCP2	F 5’ ATTGAACGACATGGTCTTAGAGG 3’ R 3’ TTGCTGTACCAGAATCTCCCAAA 5’	60 ^{o}C
SAMCP3	F 5’ ATTGAATTGCATGAGGTCTGTGG 3’ R 3’ TTAACGTACTCCAGACACCCAAA 5’	60 ^{o}C
SAMCP4	F 5’ ATTGATCCTTCCAGCAAAGAAGG 3’ R 3’ TAGGAAGGTCGTTTCTTCCCAAA 5’	61 ^{o}C
SAMCP5	F 5’ ATTGGACGGTGGATATAACGTGG 3’ R 3’ CTGCCACCTATATTGCACCCAAA 5’	61 ^{o}C

ELECTROPORACIÓN, EXTRACCIÓN DE ADN Y VERIFICACIÓN DE LA TRANSFORMACIÓN GENÉTICA EN *ESCHERICHIA COLI*.

A manera de resultado se observó crecimiento de las colonias de *E. coli* transfectadas bien definidas, en medio selectivo más antibiótico de kanamicina, con una forma redondeada de las colonias aisladas, de borde liso, color lechoso y de textura cremosa como muestra la figura 7, también se observó que tuvo mayor crecimiento el vector pKSE 401 figura 7a, ya que era un plásmido usado los últimos años, permaneciendo sus colonias activas y fuertes, igualmente tuvo un buen crecimiento pSAMC2 figura 7c, mientras que los transfectados pSAMC1, pSAMC3, pSAMC4 y pSAMC5 mostradas en la figura 7b, 7d, 7e y 7f respectivamente presentaron un crecimiento pobre teniéndose que repetir de nuevo su clonaje, debido a que las bacterias que han adquirido con éxito el ADN exógeno pueden sobrevivir en presencia del antibiótico, mientras que las que no lo obtuvieron mueren (Phue *et al.,* 2008).

Conjuntamente, los factores que afectan la eficiencia de la transfección de ADN mediante electroporación en *E. coli*, incluyen la etapa de crecimiento de las células receptoras, uso de células altamente electrocompetentes, la concentración de ADN,

la temperatura durante el proceso y los aditivos utilizados durante la preparación de la muestra (Taketo, 1988). Sin embargo, la aplicación de un campo eléctrico con alta intensidad a las células bacterianas de *E. coli* en presencia de ADN exógeno, lo que provoca la formación de poros en la membrana celular, permitiendo la entrada de la secuencia de ADN de interés (Taketo, 1988). Por tal motivo, es importante tener en cuenta que la transformación en sí misma puede ser un proceso estresante para las células de *E. coli*, lo que puede afectar su capacidad de crecimiento posterior (Durfee *et al.,* 2008).

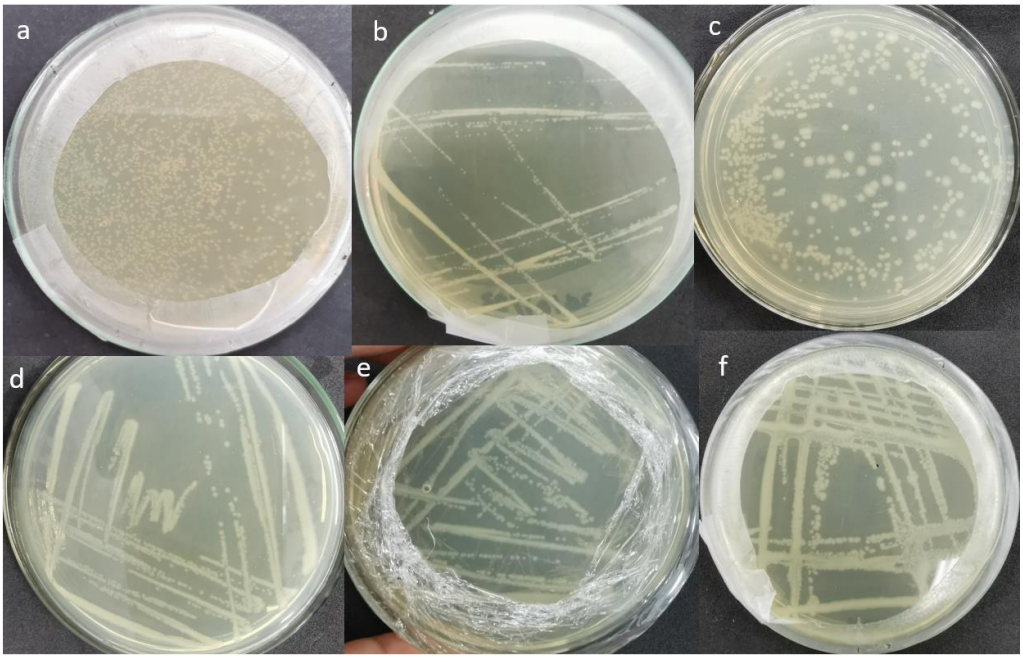

Figura 7. Crecimiento de los plásmidos en medio de cultura LB con kanamicina. (a) vector pKSE 401, (b) pSAMC1, (c) pSAMC2, (d) pSAMC3, (e) pSAMC4 y (f) pSAMC5

Después de la extracción de ADN plasmídial, se cuantificó en el NanoDrop la cantidad de ADN absoluto obtenido de las muestras siendo de 34.740 ng/µL (pSAMC1), 42.220 ng/µL (pSAMC2), 40.540 ng/µL (pSAMC3), 29.970 ng/µL (pSAMC4) y 40.150 ng/µL (pSAMC5), posteriormente se realizó la verificación de la transfección mediante una reacción de PCR de 25 µL, utilizándose 2 ng del plásmido de ARNg original como ADN molde, amplificando las bandas de interés y siendo visualizadas en el gel de

electroforesis, donde se observa el fragmento del vector pKSE401 y las bandas de los transformantes de ARNg mostrado en la figura 8.

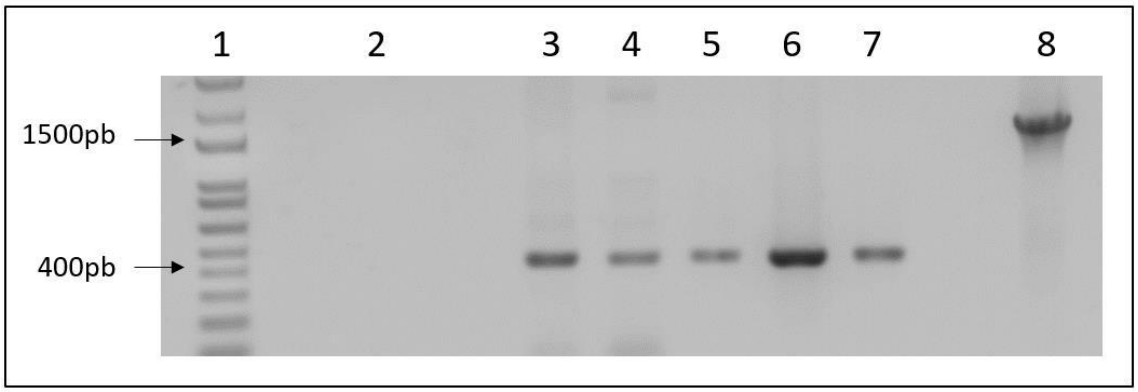

Figura 8. Gel de electroforesis confirmando la transfección de los vectores de transformación. (1) Marcador 1Kb plus, (2) Control negativo, agua ultrapura con mix, (3) pSAMC1, (4) pSAMC2, (5) pSAMC3, (6) pSAMC4, (7) pSAMC5, (8) Control positivo pKSE401.

Este resultado del perfil electroforético confirma que el carril 8, perteneciente al vector pKSE401, amplificó la región de la espectinomicina (SPR) que sirvió como control positivo. Este carril muestra un fragmento íntegro de aproximadamente 1500 pb que no fue digerido por la enzima de restricción BsaI. En contraste, los carriles del 3 al 7 de la figura 8 exhibieron la banda de interés del plásmido de *E. coli*, el cual fue clonado con ayuda del método golden gate assembly y la electroporación, la banda tiene el tamaño esperado de 400pb, indicando la integración exitosa del ADN exógeno en los plásmidos transfectados, disminuyendo la región digerida en donde se insertó la secuencia de interés como es mostrado en la figura 6a. También, el carril 2 mostró el control negativo, el cual evidenció que no se presentaron contaminantes en la ejecución del PCR.

Por lo tanto, se demostró la utilidad de las técnicas moleculares, como el método golden gate y la transformación por medio de choque eléctrico en *E. coli* para una clonación eficiente y de alto rendimiento. En consecuencia, es crucial optimizar las condiciones de transformación del cultivo para maximizar el crecimiento de las células transformadas y así minimizar cualquier efecto negativo en su viabilidad y capacidad

de crecimiento. A partir de este momento, los cultivos de *E. coli* pueden realizarse a mayor escala, con la finalidad de obtener plásmidos en cantidad suficiente para los experimentos posteriores de transformación de *C. papaya*.

CONCLUSIÓN.

Este estudio ha sentado las bases para la aplicación exitosa de la tecnología CRISPR en la papaya. En este trabajo se seleccionó el gen SAMT como gen de interés para la edición de *C. papaya*. Los resultados logrados muestran el éxito en la obtención de 5 secuencias de ARN guía, que se subclonaron en el vector y se propagaron en *E. coli*. Cada uno de los plásmidos modificados se obtuvo en cantidades suficientes para permitir los ensayos de transformación de células de *C. papaya*. Estos hallazgos representan un avance significativo en la búsqueda de estrategias para conferir resistencia a la enfermedad de la meleira en papaya causada por el complejo PMeV.

CONCLUSIONES GENERALES.

Teniendo en cuenta todos los aspectos analizados, se llega a la conclusión de que la regulación de las plantas editadas con CRISPR en América Latina es un tema complejo y en constante evolución. Mientras que algunos países han establecido pautas y marcos claros para el uso de esta tecnología en la agricultura, otros todavía están en proceso de desarrollar sus sistemas regulatorios. A pesar de estos desafíos, los beneficios potenciales de la tecnología CRISPR en la agricultura son significativos, incluyendo el aumento de los rendimientos de los cultivos, una mayor resistencia a las enfermedades y plagas, reduciendo el uso de productos químicos nocivos.

Se destacó la importancia del látex como un mecanismo de defensa de las plantas contra enfermedades y se ha demostrado que el látex puede influir en la transmisión de virus y tener actividad antiviral. Además, se han identificado compuestos específicos en el látex que tienen potencial aplicación en la agricultura y la medicina. En general, se subraya la necesidad de seguir investigando la dinámica entre las plantas con látex y los virus para comprender mejor esta interacción compleja y aprovechar su potencial en aplicaciones prácticas.

Se resaltó la importancia de la búsqueda de genes promisorios que confieran resistencia a enfermedades virales, centrándose en el gen SAMT y su potencial en el control de la enfermedad de la meleira en la papaya para ser usado posteriormente en la tecnología CRISPR, la cual es una herramienta valiosa para mejorar la resistencia de la papaya a esta enfermedad.

PERSPECTIVAS FUTURAS

Como dirección de investigación futura, sería muy interesante utilizar los plásmidos de transfección de *E. coli* para la clonación en *Agrobacterium tumefaciens* y realizar la agroinfiltración en callos de *Carica papaya* para analizar la edición del gen SAMT y futuro desarrollo de la planta.

El control de la enfermedad de la meleira en papaya requiere ingeniería continua de ARN guía para silenciar o sobreexpresar genes que confieran resistencia a plantas de papaya, por tal motivo los datos de la transcriptoma y proteómica de las investigaciones anteriores realizados en el LBAA, son de gran ayuda para la búsqueda de otros genes candidatos.

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APENDICES

APENDICE A. Esquema de los CDS del gen salicilato O-metiltransferasa de color azul claro y oscuro, incluyendo las secuencias del ARNg de color amarillo con sus respectivos PAM de color rojo, donde se realizará su edición.

ATGGATGTAGTTAAAGTGCTGCACATGAAGGGAGGAAGTGGACAAACCAGTTATGCTCTCAACTCTTTGCTTCAGC
AAAAAGTAATATCGATGACAAAGCCAATCACGGAGGAAGCCATAACCAAGCTCTACTGCAACACTTTCCCTGCAAA
GCTAGCCATAGCTGACTTGGGTTGCTCATCTGGACCGAACACCTTGCTTGTGGTTACCCAACTGATCATGACAGTT
GACAAGCTTCGACGGAAATTGAGCCAGGAGTCGCCGGAGTATGTCATCTTTCTTAACGATCTCCCAGGAAATGACT
TCAACAACATTTTCCGGTCATTACTTCCAAACTTCCAACAGAAACTGACTACTGAGATTGGAGCTGGCACCAGGCC
TTGTTTCTTGTCCGCTGTTCCGGGTTCTTTCTACGGCAGACTTTTCCCGGAAAATAGCCTGCACTTTGTTCACTCT
TCTTATAGTCTTCAATGGTTATCTCAGGTACCAGAGGGGTTGGAGAATAATAAAGGTAATATATACATGGCAAGTA
GTAGCCCTAAGAACGTGATGAGAGCATATTATGAACAATTTCAAAAGGACTTCGGCTATTTTTTGAAGTGTCGATC
AGAGGAGTTGGTGGCTGGAGGCCGTATGGTTTTAACATTTTTGGGAAGAAGCAGCCAAGATCCTTCCAGCAAAGAA
GGTTGTTACATTTGGGAGCTTTTGGCCACGGCTCTAAACGACATGGTCTTAGAGCGAATAATAGAAGAAGAGAAGC
TGGGCTCCTTTAACATCCCACAATACACTCCATCTCCAGAAGAACTGAAAACTGAGGTTATAAAAGAAAGGTCCTT
CACCGTTGACCGCCTTGAAGTTACAAGAGTCCATTGGGATGCTTATCACGGCGAATTAAGTCCGTTCGATGCAGTT
ATGGACGGTGGATATAACGTGCCAAATTGCATGAGGTCTGTGCCTGAGCCGCTGCTCGTCAGCCATTTTGGTGAAG
AAATCATTGAGGAGGTTTTTTACAGGTACCGGAAAATCCTTGCCGATCGCATGTCCAAGGAGGAAACCCAGTTTGT
CAATGTTATTATCTCGGTCGTTAAAGCTTGA

APENDICE B. Lectura de artículos externos del LBAA sobre genes de defensa.

Mecanismos	Organismo	Descripción	Título	Referencia
NRG1 Proteínas de unión a nucleótidos con repeticiones ricas en leucina (NBS-LRR).	*Nicotiana benthamiana*	Resistencia contra el *virus del mosaico del tabaco* mediante el reconocimiento de la proteína p50. El gen (NRG1), un componente de resistencia.	NRG1, a CC-NB-LRR Protein, together with N, a TIR-NB-LRR Protein, Mediates Resistance against *Tobacco Mosaic Virus*	Peart *et al,* 2005
Subunidad RPN9 de la proteasoma 26S.	*Nicotiana benthamiana*	Inhibición del transporte sistémico viral tras el silenciamiento de RPN9	Down-regulation of the 26S proteasome subunit RPN9 inhibits viral systemic transport and alters plant vascular development	Jin *et al.,* 2006
Carboxil metiltransferasa de ácido salicílico (AtBSMT1)	*Arabidopsis thaliana*	Arabidopsis que sobreexpresa AtBSMT1 con poca inducción del gen PR-1 de defensa.	Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediated pathogen resistance in *Arabidopsis thaliana*.	Koo *et al.,* 2007
miR396 miR398	*A. thaliana, G. max, O. sativa, S. bicolor, V. vinifera y Z. mays*	Un enfoque bioinformático para comprobar si los miARN vegetales de seis especies podrían tener actividad antivírica.	Plant microRNAs and their role in defense against viruses: a bioinformatics approach	Pérez-Quintero *et al.,* 2010
HSP70 HSP90 Calreticulina	*A. thaliana y Glycine max.*	Hsp70 y Hsp90 actúan en la formación de complejos de replicación unidos a la membrana y la calreticulina para promover el transporte intercelular del virus.	Cellular chaperones and folding enzymes are vital contributors to membrane bound replication and movement complexes during plant RNA virus infection	Verchot, 2012
Argonauta18	*Oryza sativa*	Proteína que forma parte del complejo de silenciamiento del ARN en las plantas, y juega un papel crucial en la defensa antiviral.	Viral-inducible Argonaute18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA	Wu *et al.,* 2015
RDR1	*Solanum tuberosum*	Arabidopsis thaliana tiene seis genes RDR; los RDR 1, 2 y 6 desempeñan un papel en el silenciamiento antiviral del ARN.	RNA-dependent RNA polymerase 1 in potato (Solanum tuberosum) and its relationship to other plant RNA-dependent RNA polymerases	Hunter *et al.*, 2016
Factores de iniciación de la traducción (eIF4E)	*Phaseolus vulgaris* L.	interfieren con la replicación viral, lo que confiere resistencia a ciertos virus.	Genetic resistance against viruses in *Phaseolus vulgaris* L. State of the art and future prospects	Meziadi *et al.,* 2017
EXPA4	*Nicotiana benthamiana*	Las expansinas son proteínas implicadas en la modificación de la pared celular y en el crecimiento de las plantas.	Tobacco alpha-expansin EXPA4 plays a role in *Nicotiana benthamiana* defence against *Tobacco mosaic virus*	Chen *et al.,* 2018

APENDICE C. Secuencias de ARNg seleccionadas del gen SAMT.

	Selección del mejor ARNg																
	Secuencia de ARNg				Longitud de la secuencia			Composición de nucleótidos					Posición específica de los nucleótidos				
Secuencias propuestas	La secuencia a partir de 5' G para evitar errores	Repetición de base (AAAAA, GGGGG, CCCCC, UUUUU)	Repetición de timina (TTY ou TTYY em 3')	En posición 3' GCC	Las secuencias cortas de 17 a 18 nucleótidos son más específicas pero menos eficaces	Las secuencias largas de más de 20 nucleótidos son menos eficaces.	Secuencia de 20 nucleótidos	El elevado número de A en las posiciones 9 - 16 puede aumentar la afinidad de Cas9.	Un número elevado de G en 9 - 16 puede dar lugar a una Cas9 menos funcional.	40 a 60% de GC se considera óptimo	G en las posiciones 4 - 5 mayor actividad	GC en los 6 nucleótidos próximos a la secuencia PAM.	Las posiciones 19 y 20 son preferibles a G o A	No se recomienda una C en la posición 20	En las posiciones 16 y 18, es preferible C, especialmente	No se recomienda G en las posiciones 16 y 18.	Puntuación
GAAATTGAGCCAGGAGTCGCCGG	✔	✔	✔	✔	✔	✔	✔	✘	✘	55%	✘	✔	✔ 19	✘	✔	✘ 16	
GAAGTGTCGATCAGAGGAGTTGG	✔	✔	✔	✔	✔	✔	✔	✘	✘	50%	✔ 4	✘	✔ 19	✔	✘	✘ 16	
TCAGAGGAGTTGGTGGCTGGAGG	✔	✔	✔	✔	✔	✔	✔	✘	✘	60%	✔ 4	✔	✔	✔	✘	✘ 16	
AAAGCTAGCCATAGCTGACTTGG	✔	✔	✔	✔	✔	✔	✔	✘	✔	45%	✔ 4	✘	✘	✔	✘	✔	
TGGACCGAACACCTTGCTTGTGG	✔	✔	✘	✔	✔	✔	✔	✔ 9	✘ 16	55%	✘	✔	✔ 20	✔	✘	✘ 16	
GATGACAAAGCCAATCACGGAGG	✔	✔	✔	✔	✔	✔	✔	✔ 9	✔	50%	✔ 4	✘	✔	✔	✔	✔	0.7739
CGATCAGAGGAGTTGGTGGCTGG	✔	✔	✘	✔	✔	✔	✔	✘	✘	60%	✘	✔	✔ 19	✘	✘	✘	
GCTGTTCCGGGTTCTTTCTACGG	✔	✔	✘	✔	✔	✔	✔	✘	✘ 9	50%	✔ 4	✘	✔ 20	✔	✔	✔	
TGACAGTTGACAAGCTTCGACGG	✔	✔	✘	✔	✔	✔	✔	✘	✘ 9	45%	✘	✘	✔ 19	✔	✔	✔	
GCTTCGACGGAAATTGAGCCAGG	✔	✔	✘	✘	✔	✔	✔	✘	✘ 9	55%	✘	✔	✘	✘	✘	✘	
CTAAACGACATGGTCTTAGAGGG	✔	✔	✘	✔	✔	✔	✔	✘	✔	40%	✘	✘	✔ 19	✔	✘	✔	
TATGGACGGTGGATATAACGTGG	✔	✔	✔	✔	✔	✔	✔	✘	✘ 9	45%	✔	✘	✔ 20	✔	✘	✔	0.4687
AAAGTGCTGCACATGAAGGGAGG	✔	✔	✔	✔	✔	✔	✔	✔ 16	✘ 9	50%	✔ 4	✘	✔	✔	✔	✘ 18	
TCCAGAAGAACTGAAAACTGAGG	✔	✔	✔	✔	✔	✔	✔	✔	✔	40%	✔ 5	✘	✔ 20	✔	✔	✔	0.3335
AAGCTAGCCATAGCTGACTTGGG	✔	✔	✘	✔	✔	✔	✔	✘	✘ 16	45%	✘	✔	✘	✔	✔	✘ 16	
TGGTGAAGAAATCATTGAGGAGG	✔	✔	✘	✔	✔	✔	✔	✔ 9	✔	40%	✔ 5	✘	✔	✔	✘	✔	
GTGTCGATCAGAGGAGTTGGTGG	✔	✔	✘	✔	✔	✔	✔	✘	✘ 16	55%	✘	✔	✔	✔	✘	✘ 16	
GGCCACGGCTCTAAACGACATGG	✔	✔	✘	✔	✔	✔	✔	✘	✔	60%	✘	✘	✔ 20	✔	✔ 16	✔	
CCTTGCCGATCGCATGTCCAAGG	✔	✔	✔	✔	✔	✔	✔	✔ 9	✘ 16	60%	✔ 5	✘	✔ 20	✔	✔ 18	✘ 16	
ATAACGTGGCAAATTGCATGAGG	✔	✔	✘	✔	✔	✔	✔	✘	✘	40%	✘	✔	✔ 20	✔	✘	✘ 16	
AGGAGGTTTTTTACAGGTACCGG	✘	✔	✔	✔	✔	✔	✔	✘	✘ 16	40%	✔ 5	✘	✘	✘	✘	✘ 16	
TCTAAACGACATGGTCTTAGAGG	✔	✔	✘	✔	✔	✔	✔	✔ 9	✔	40%	✘	✘	✔ 20	✔	✔ 16	✔	0.2597
CAAGATCCTTCCAGCAAAGAAGG	✔	✔	✔	✔	✔	✔	✔	✔ 16	✔	45%	✔ 4	✘	✔ 20	✔	✘	✔	0.1152
GGCAAATTGCATGAGGTCTGTGG	✔	✔	✔	✔	✔	✔	✔	✘	✘	50%	✘	✘	✔ 20	✔	✔ 18	✘ 16	0.1145
CTGAGATTGGAGCTGGCACCAGG	✔	✔	✔	✔	✔	✔	✔	✘	✘	60%	✔ 5	✔	✘	✘	✘	✘ 16	
TGTTTCTTGTCCGCTGTTCCGGG	✔	✔	✘	✔	✔	✔	✔	✘	✘	50%	✘	✘	✘	✘	✘	✘ 16	
CTGCACATGAAGGGAGGAAGTGG	✔	✔	✔	✔	✔	✔	✔	✘	✘	55%	✘	✘	✔ 20	✔	✘	✘ 16	