DOCINADE

Doctorado en Ciencias Naturales para el Desarrollo

Énfasis en Sistemas de Producción Agrícola (SPA)

Tesis de Doctorado

Inducción de variabilidad genética en una variedad comercial de arroz (*Oryza sativa*) mediante el uso de radiación gama a partir de la selección *in vitro* de mutantes tolerantes a ariloxifenoxipropionatos para su uso en el fitomejoramiento del cultivo

ALEJANDRO HERNANDEZ SOTO

Dra Ana Abdelnour Esquivel
Director de Tesis

Dra Marta Valdez Melara
Asesor de Tesis

Dr. Fabián Echeverría Beirute
Asesor de Tesis

Cartago, Costa Rica, enero 2023

MIEMBROS DEL TRIBUNAL EXAMINADOR

TEODOLITO GUILLEN GIRON (FIRMA)

Firmado digitalmente por TEODOLITO GUILLEN GIRON (FIRMA)
Fecha: 2023.01.26 17:42:24 -06'00'

Dr. Teodolito Guillén Girón

Dirección de Posgrados TEC

ANA MARIA ABDELNOUR ESQUIVEL (FIRMA)

Firmado digitalmente por ANA MARIA ABDELNOUR ESQUIVEL (FIRMA)
Fecha: 2023.02.01 19:06:00 -06'00'

Dra Ana Abdelnour Esquivel

Director de tesis

Marta Valdez Melara

Firmado digitalmente por Marta Valdez Melara
Fecha: 2023.01.26 14:55:20 -06'00'

Dra Marta Valdez Melara

Asesora de Tesis

FABIAN ECHEVERRIA BEIRUTE (FIRMA)

Firmado digitalmente por FABIAN ECHEVERRIA BEIRUTE (FIRMA)
Fecha: 2023.01.26 09:44:43 -06'00'

Dr. Fabián Echeverría Beirute

Asesor de tesis

Firmado por GIOVANNI SAENZ ARCE (FIRMA)
PERSONA FISICA, CPF-01-1153-0569.
Fecha declarada: 07/02/2023 11:50 PM

Dr. Giovanni Sáenz Arce

Coordinador General DOCINADE

Tabla de contenidos

Índice de figuras

Índice de tablas

Declaración de autenticidad

Yo Alejandro Hernández Soto, estudiante del Doctorado en Ciencias Naturales para el Desarrollo, declaro que la Tesis Doctoral que presento para su exposición y defensa titulada "*Inducción de variabilidad genética en una variedad comercial de arroz (Oryza sativa) mediante el uso de radiación gama a partir de la selección in vitro de mutantes tolerantes a ariloxifenoxipropionatos para su uso en el fitomejoramiento del cultivo*" y comité asesor de tesis son la Dra Ana Abdelnour Esquivel (directora de tesis), Dra Marta Valdez Melara (asesor) y el Dr. Fabián Echeverría Beirute (asesor), es original y que todas las fuentes utilizadas para su realización han sido debidamente citadas en el mismo. Este material no lo he presentado, en forma parcial o total, como una tesis en esta u otra institución.

Cartago, Costa Rica, 25 de enero de 2023.

ALEJANDRO HERNANDEZ SOTO (FIRMA)
PERSONA FISICA, CPF-01-1100-0510.
Fecha declarada: 13/02/2023 01:56:05 PM
Lugar: San Jose, Costa Rica Contacto: alhernandez@itcr.ac.cr

Firma

Alejandro Hernández Soto

Agradecimientos

Jason Pérez por toda su colaboración en el desarrollo del trabajo en laboratorio, dedicación, colaboración y gestión oportuna que hicieron posible la obtención de resultados de laboratorio que culminaron en artículos científicos.

Al Doctor Andrés Gatica por permitir colaborar en múltiples proyectos y facilitar el acceso a infraestructura, aporte de contenidos técnicos en la redacción de artículos y apoyo para la pasantía.

Dedicatoria

A mi esposa Elena Ortiz, a mis hijos Elena Hernández Ortiz y José Gabriel Hernández Ortiz a quienes motivaron e hicieron posible este trabajo.

A mis padres por su confianza, apoyo.

A Dios por darme salud y bendición para culminar esta etapa.

Resumen

El principio de mejoramiento genético basado en mutaciones consiste en generar cambios hereditarios en el ADN mediante agentes externos. La mutagénesis inducida mediante radiación gamma ofrece una alternativa para desarrollar líneas de arroz resistentes al estrés biótico y abiótico al acelerar el proceso de mutación espontánea y aumentar el conjunto de variantes alélicas disponibles para la mejora genética. En el presente trabajo se exploran las bases técnicas para lograr la mutagénesis *in vitro* mediante radiación gamma (60Co) con el rasgo de tolerancia al ariloxifenoxipropionato Fluazifop-P-Butil como modelo para crear variabilidad en una variedad local de arroz Lazarroz FL. El primer parámetro estandarizado que se logró fue la radiosensibilidad de los callos embriogénicos. La dosis letal 30 se calculó a partir de 1680 callos con exposición de gradiente de 0-120 Gy en 81,8 Gy (77,507-86,403). El segundo parámetro fue la sensibilidad a los ariloxifenoxipropionatos como agente de selección. La dosis letal media fue de 6,93 mg / L (0,425 mg / L - 15,743 mg / L, R_2 = 0,402, 1000n) para callos y la dosis letal media de 3.771mg / L (R_2 = 1, 290n) para plantas *in vitro*. La secuenciación del gen diana *ACCasa2* y las secuencias de control *mat*K, *rbc*L con el pre-identificador NCBI MZ558337, MZ558334, MZ558335, demostraron que la variedad comercial utilizada tiene ausencia de mutaciones. El sistema se ejecutó con 60Gy en callo embriogénico, regeneración, preselección a 5mg/L y selección final a 5mg/L de Fluazifop-P-Butil. Lo anterior resultó en un único mutante putativo tolerante a partir de 8000 vitroplantas regeneradas. La línea tolerante presentó una tasa de mutación aproximada de 1/1000pb en el gen blanco *ACC2* (T2222I/T2222M) y en los controles *mat*K, *rbc*L. Es posible que la mutación en el gen blanco *ACC2* esté vinculada a la tolerancia, sin embargo, no se puede descartar otros mecanismos no evaluados. El trabajo exploró el uso de semilla como tejido de partida para la radiación *in vitro*, lo que permitió una exposición de 50-350Gy, y un incremento en el número de mutantes putativos de 31. Un método alterno para generar mutaciones con mayor precisión es mediante CRISPR-Cas9, el proyecto exploró la técnica en levaduras de un gen homólogo de arroz como modelo para proveer tolerancia a salinidad. Se logró una mutación en el gen *NTH2*, *nth2 1271_1272delTA,* lo que incrementó la tolerancia de la levadura al estrés salino con capacidad de crecimiento a 0.8M NaCl a una tasa de 0.2179 ± 0.0061 h^{-1} en comparación con la silvestre de 0.1580 ± 0.0009 h^{-1}.

Palabras claves

Mutagénesis; Cobalto-60; cultivo *in vitro*; mejoramiento genético; CRISPR-Cas9; levadura.

Abstract

The mutation breeding principle is to generate heritable changes in the DNA by external agents. Induced mutagenesis by gamma rays offers a promising alternative for developing rice varieties resistant to biotic and abiotic stresses. It could accelerate the spontaneous mutation process and increase the pool of allelic variants available for genetic improvement. Here we present the technical bases to achieve *in vitro* Gamma radiation (60Co) mutagenesis with Fluazifop P-Butyl tolerant trait as a model to create rice variability in a local variety used by farmers, Lazarroz FL. The first standardized parameter achieved was the radiosensitivity of embryogenic calli. The dose was 81.8Gy (77.507-86.403) for LD_{30}, obtained from 1680 callus tested on a 0-120Gy gradient. The second parameter was the sensibility to the aryloxyphenoxypropionates as a selection agent. The dose was 6.93mg/L (0.425 mg/L- 15.743 mg/L, R_2= 0.402, 1000 callus) for callus LD50, and 3.771mg/L (R_2= 1, 290n) LD50 for Vitro plants. Target gene sequencing ACCasa2, and control sequences *mat*K, *rbc*L summited to the NCBI id MZ558337, MZ558334, MZ558335, demonstrated that the commercial variety used had an absence of mutations. The mutation system was done at 60Gy on embryogenic calli, regeneration, preselection on 5mg/L and final selection on 10mg/L Fluazyfop P-Butyl resulting in 1/8000 putative tolerant *vitro plant*. The tolerant line had a mutation rate of approximately 1/1000bp in the control genes *mat*K, *rbc*L as well as the target gene *ACC2* (T2222I/T2222M). The mutation may be linked to the tolerance; however, it could also be the result of other non-evaluated detoxification mechanism. The *in vitro* system was improved by using seeds as a starting point for irradiation, allowing an exposition window of 50-350Gy and 31 putative mutants. An alternative method to generate precise mutations is CRISPR-CAS9. The project explored the technique in a yeast gene homologous to rice as a model to provide salt tolerance. We achieved a mutation on gene *NTH2*, *nth2 1271_1272delTA,* resulting in increased salt tolerance of the yeast capable of better growing under 0.8M NaCl at a rate of 0.2179 ± 0.0061 h−1 versus wild type 0.1580 ± 0.0009 h−1.

Keywords

Mutagenesis; Cobalt-60; plant tissue culture; plant breeding; CRISPR-Cas9; yeast.

1. Introducción

El arroz (*Oryza sativa*) es uno de los cereales de mayor consumo mundial, responsable del 20% de las calorías de la dieta de al menos 3500 millones de personas particularmente de Africa, Asia y América (Gutaker *et al.*, 2020). En Costa Rica se consume 406,599 toneladas métricas de arroz, que se abastecen en un 35% con producción nacional de cantones de alta vulnerabilidad económica, particularmente la región Chorotega, Brunca y Pacífico Central. Los agricultores de Costa Rica tienen una preferencia por cuatro cultivares, Lazarroz FL con un 45%, Senumisa 20FL (18%), Palmar18 (11%) y Puita INTA (11%) (CONARROZ, 2021; Morales *et al.*, 2018; Orozco & Carrodeguas, 2022). Los programas de fitomejoramiento de arroz en Costa Rica deben considerar estos materiales de partida para añadir variabilidad en caracteres que mejoren el cultivo.

Las mutaciones son útiles para el mejoramiento de cultivos como el arroz, sin embargo, la tasa de mutaciones espontáneas es baja, lo que impide el avance de programas de fitomejoramiento (Oladosu *et al.*, 2016). Las mutaciones con métodos físicos, químicos o biológicos permiten desarrollar variabilidad mediante cambios en el material genético (Nakagawa & Kato, 2017). La radiación gamma en semillas es capaz de generar mutaciones con una dosis de 250Gy tales como sustituciones únicas (57), deleciones (17.7), inserciones (5.9) (F. Li *et al.*, 2019). Un método alternativo que disminuye el trabajo y selección en campo es la inducción de mutaciones *in vitro* a partir de callo, suspensiones celulares y embriones inmaduros, para lo cual se requiere la optimización a nivel de laboratorio según el material de partida (Viana *et al.*, 2019).

Las mutaciones en arroz permiten el desarrollo de características de interés agronómico y de importancia para la sostenibilidad del cultivo, algunas de las cuales se describen a continuación. El incremento en el número de granos está asociado a mutaciones en el gen *Os01g0197700* (*Gn1a*, *OsCKX2*), el tamaño del grano al gen *Os03g0407400* (*GS3*); mutaciones en el gen *Os08g0509600* (*IPA1*) están relacionadas con un mayor número de granos y alta frecuencia; mientras que la floración en día corto o largo en el gen *Os06g0275000* (*Hd1/ SE1*) (Fan & Li, 2019; M. Li *et al.*, 2016; Shen *et al.*, 2017; Song *et al.*, 2022; Tanaka *et al.*, 2020; Yang *et al.*, 2019). El rendimiento puede aumentar de 41.3 a un 68.3% mediante la sobre expresión del gen DREB1C(Wei *et al.*, 2022). El aroma está relacionado a mutaciones en el gen *Os08g0424500* (Shen *et al.*, 2017). El genotipo de planta erecta y corta por mutaciones en el gen *Os09g0441900* (*DEP1*) (M. Li *et al.*, 2016). Existen genes relacionados a la domesticación que ocurrió por mutación en los genes como el *Os06g0133000* (*WX1*) (Chen *et al.*, 2019). Otros genes de interés

son la tolerancia a estrés salino relacionados con el gen *Os06g0183100* (A. Zhang *et al.*, 2019). Además, existe resistencia a bacterias relacionada con los genes *Os11t0508600-01* y *Os08g0535200* (H. Li *et al.*, 2020; Xu *et al.*, 2020). En el caso de resistencia a moléculas químicas comúnmente llamados herbicidas, se conocen mutaciones relacionadas con trifluralina en el gen *Os11g14220* (L. Liu *et al.*, 2021), quizalofop *Os05g22940.1* (*ACC2*) (de Andrade *et al.*, 2018; X. Liu *et al.*, 2020; Xu *et al.*, 2020), así como las moléculas químicas que actúan sobre las enzimas Acetolactato sintasa en el gen *Os02g0510200* (Bzour *et al.*, 2018; Q. Lin *et al.*, 2020; Sagare *et al.*, 2020; Shimatani *et al.*, 2017; Xu *et al.*, 2020) y sobre la enzima *EPSPS* (J. Li *et al.*, 2016). Es posible lograr mutaciones inducidas mediante radiación gamma en los genes descritos con anterioridad para lograr las características deseadas según se detalla a continuación (F. Li *et al.*, 2019).

Las radiaciones ionizantes producen daños directos o indirectos en el ADN. Estos daños pueden ser de dos tipos, puntuales lo que generan mutaciones en el material genético o bien afectan de manera letal al organismo (Nakagawa & Kato, 2017). Las mutaciones ocurren de forma aleatoria a lo largo del genoma y son fuente de diversidad útil para el fitomejoramiento. Una manera de optimizar el proceso de mutación es utilizando marcadores que correlacionen con estos cambios en el genoma como la resistencia a un agente químico (Murphy & Tranel, 2019; Tan & Li, 2022). El uso de un agente de selección químico permite el establecimiento y optimización de los protocolos *in vitro* de manera predecible, en este caso a partir de una variedad comercial de mayor uso por el agricultor costarricense Lazarroz FL. Los ariloxifenoxipropionatos son recomendados a nivel mundial para el control de gramíneas, este tipo de moléculas actúan a nivel de la síntesis de ácidos grasos y la resistencia está dada por mutaciones puntuales en el gen *ACC2* que cataliza la caboxilación de acetil-CoA a Malonil-CoA (de Andrade *et al.*, 2018; Takano *et al.*, 2020; R. Zhang *et al.*, 2019). Lo anterior permite las bases teóricas para determinar el equilibrio entre la dosis correcta de radiación gamma, muerte celular, protocolos *in vitro* y mutaciones puntuales. La presente tesis plantea el sistema de creación de variabilidad genética *in vitro* al utilizar como agente de selección una molécula química estrechamente relacionada con mutaciones puntuales en el gen *ACC2* (de Andrade *et al.*, 2018; Murphy & Tranel, 2019; Pereira *et al.*, 2019).

El objetivo de esta investigación es establecer un modelo de inducción de variabilidad genética de Arroz (*Oryza sativa)* Lazarroz FL mediante radiación gamma usando como agente de selección los ariloxifenoxipropionatos, particularmente la molécula Fluazifop, para su uso como una fuente de genes para el mejoramiento genético. Mutaciones en el gen blanco *ACCasa*

(*ACC2*, *Os05g0295300*, *LOC_Os05g22940* B9FK36) están directamente relacionadas a la resistencia a ariloxifenoxipropionatos. Para ello, el proyecto planteó el establecimiento *in vitro* de callo embriogénicos del escutelo de semillas de arroz, la determinación de la dosis letal media (DL50) del agente de selección, así como de la dosis de la irradiación gama, para generar los procesos posteriores de irradiación y selección de células tolerantes. Finalmente, las líneas tolerantes se regeneraron, multiplicaron y fueron transferidas al invernadero para la obtención de la M2. Adicionalmente, estas plantas de la M1 y M2 con tolerancia a ariloxifenoxipropionatos se analizaron mediante secuenciación de los genes blanco *ACCasa*, mediante herramientas bioinformáticas para determinar el tipo de mutación y la heredabilidad de la resistencia.

Pregunta de investigación

El presente trabajo tiene como pregunta de investigación determinar condiciones óptimas para generar mutaciones en arroz *in vitro* de una variedad comercial (Lazarroz FL) mediante radiaciones gamma usando como modelo las mutaciones que otorgan resistencia a los ariloxifenoxipropionatos.

Marco teórico:

Mutación. El mejoramiento de cultivos se basa en la variabilidad genética de las plantas. Las mutaciones son la mayor fuente de esta variabilidad, que es posteriormente amplificada por recombinación genética de cromosomas homólogos durante la meiosis (Viana *et al.*, 2019). El término mutación fue acuñado a finales del siglo XIX por Hugo de Vries en referencia a la ocurrencia de cambios o “saltos” en características que llevan al desarrollo de variaciones y de nuevas especies, como resultado de redescubrir las leyes de la herencia mendeliana (Oladosu *et al.*, 2016). La radiación ionizante es el agente mutagénico de mayor uso en el mejoramiento genético de cultivos vegetales (F. Li *et al.*, 2019). La base conceptual de la capacidad de las radiaciones ionizantes de producir mutaciones en organismos superiores fue demostrada por Muller en 1927 en mosca de la fruta (Drosophila) (Muller, 1927) y en cultivos vegetales por Stadler en 1928 en maíz (*Zea mays*) mediante rayos X (Stadler, 1928).

La mayoría de las variedades que se utilizan en agricultura fueron producidas por mutagénesis inducida por métodos físicos, particularmente por radiación gamma cuyo origen es el cobalto radiactivo (60Co) y/o Cesio (137Cs). La preferencia de este sistema se debe a su capacidad de penetración de los tejidos vegetales. Existen otros agentes físicos que pueden ser utilizados para la mutagénesis física tales como los rayos X; los neutrones producidos por 235U; las partículas beta producidos por núcleos de Helio; partículas alfa producidos por 32P y 14C; protones

producidos por núcleos de Hidrógeno; y los iones producidos por aceleración de partículas (en inglés ion beam) (Oladosu *et al.*, 2016).

La base de datos de la FAO/IAEA registra más de 3200 variedades de cultivos liberadas en todo el mundo, incluyendo 830 registros que corresponden a arroz obtenidos por este método en su mayoría a partir de semillas (FAO/IEAE 2019[1]). En suma, en Costa Rica, existen antecedentes del uso exitoso de estas técnicas de radiaciones ionizantes, en la década de los noventa se desarrolló la variedad CAMAGO-8, que fue utilizada comercialmente por su tolerancia a *Pyricularia oryzae*. La UNA, UCR y TEC se unieron en un proyecto FEES-CONARE durante el 2016-2018 para buscar variantes de arroz tolerantes a sequía y salinidad (Abdelnour-Esquivel *et al.*, 2020).

Existen otros métodos como inducción con Etil Metano Sulfonato (EMS) con el inconveniente de que las mutaciones no se distribuyen uniformemente en el cromosoma, tiene regiones preferidas con contenidos altos de GC, elementos transponibles y regiones con niveles bajos de expresión génica y heterocromatina y tienen poca capacidad de penetración del tejido (Hu *et al.*, 2020; Yan *et al*., 2021). Este método no debe ser descartado como opción, pero en el presente trabajo no se utilizó como primera opción debido al riesgo del manejo del químico EMS en condiciones de laboratorio.

Avance en el mejoramiento de cultivos. El mejoramiento genético de cultivos se encuentra en evolución e integración de métodos no convencionales. En el mejoramiento convencional la introducción de nuevos caracteres ocurre por mutación inducida, así como por introgresión por medio de cruzas intergenéricas e interespecíficas. Lo anterior permite introducir rasgos como el incremento en rendimiento, resistencia a enfermedades y mejora de la calidad de grano (Hernández-Soto *et al*., 2021; Oladosu *et al*., 2019).

Los métodos no convencionales, tales como los Nuevos Métodos de Mejora Genética (en inglés *NBTs*, *New Breeding Techniques*) son el producto del avance en el entendimiento a nivel molecular de los cultivos mediante las ómicas, la fisiología y los métodos automatizados de caracterización fenotípica(Singer *et al*., 2021). Este entendimiento no sólo permite una mayor precisión en la mejora del cultivo, sino el abordaje de caracteres más complejos controlados por múltiples genes mediante la introducción de genes por cisgénesis, transgénesis y ARN de interferencia para un gen funcional o bien para factores de transcripción que interactúan con múltiples genes (Anders *et al*., 2021; Mohd Saad *et al*., 2022; Oladosu *et al*., 2019). Lo mismo es

[1] FAO/IAEA (2019) Mutant Variety Database. http://mvd.iaea.org/. Visitado el 12 de junio de 2019.

posible también sin la introducción de nuevo material genético mediante técnicas como la edición de genomas mediada por meganucleasas como las nucleasas de dedos de zinc (ZFN) y las nucleasas efectoras similares a factores de transcripción (TALEN), así como el sistema CRISPR-Cas9 que permiten la corrección o la introducción de mutaciones de manera precisa (Karmakar *et al*., 2022; Singer *et al*., 2021; Smyth, 2022).

La ventaja de la alta integración de las herramientas no convencionales es ahorro en tiempo (Karmakar *et al*., 2022; Smyth, 2022). El análisis de fenotipo, el conocimiento genómico de parentales silvestres, el procesamiento de datos por aprendizaje automático (en inglés machine learning) y posterior uso de ese conocimiento para generar correcciones puntuales mediante edición de genomas tiene el potencial de aumentar la precisión y reducir los tiempos de desarrollo de cultivos más resilientes y sostenibles (Mohd Saad *et al*., 2022). La presente tesis aborda métodos *in vitro* útiles para el uso de estas técnicas con métodos convencionales que son útiles para el mejoramiento no convencional y explora de manera práctica el uso de CRISPR-CAS9 con un modelo de levadura estos conceptos para la mejora de arroz.

¿Por qué utilizar la resistencia a ariloxifenoxipropionato como modelo para generar mutaciones?

Los agentes de selección permiten optimizar los procedimientos, existen mutaciones específicas relacionadas con la resistencia ariloxifenoxipropionato en el gen *ACC2* por lo que los resultados se correlacionan. Adicionalmente, la tolerancia a herbicidas es una característica que podría servir para el fitomejoramiento del cultivo. A continuación, se detallan los tres argumentos indicados con anterioridad.

1. Los procedimientos de generación de variabilidad mediante radiación gamma son posibles, pero requieren optimización. Un agente de selección como la resistencia a ariloxifenoxipropionatos permitiría esta optimización. Los procedimientos de inducción de mutaciones mediante la radiación gamma son posibles gracias a que el ITCR cuenta con un irradiador de cobalto Gamma-Cell, las facilidades en cultivo de tejidos y equipo en el Centro de Investigación en Biotecnología y la Escuela de Física. La inducción de mutación es un procedimiento común y se realiza con un irradiador de cobalto Gamma-Cell. Lo anterior es la premisa para lograr la inducción de mutaciones mediante radiación gamma y posteriormente seleccionar mediante la exposición *in vitro* a dosis correctas de radiación y herbicida como agente de selección. Las células que sobrevivan generarán líneas que adquirieron la tolerancia como resultado de la mutación.

2. Mutaciones específicas en el gen *ACC2* están relacionadas con la tolerancia a herbicidas ariloxifenoxipropionatos por lo que son un indicador para determinar si el sistema de generación de mutaciones es funcional. La enzima es la Acetil-CoA carboxilasa *ACC2* (EC 6.4.1.2) ubicado en el cromosoma 5: 14,067,726-14,079,652 en la hebra reversa (*ACC2*, *BGIOSGA018366-TA*, *A2Y2U1*). Específicamente, mutaciones en el dominio caboxiltransferasa entre los aminoácidos 1,781- 2,078 y 2,027- 2,096 relacionadas a la tolerancia de herbicidas ariloxifenoxipropionatos (Hernández-Soto *et al.*, 2021). En esta región, se conocen de mutaciones como Ile1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg, Gly-2096-Ala (Powles & Yu, 2010). La mayoría de estas mutaciones son del tipo transversiones por ejemplo Gln-1756-Glu C/G (CAG por GAG), Ile1781-Leu/Val/Ala/Thr (ATA por CTA Leu, GTA Val), y algunas pueden ser transiciones como A/G Ile1781-Val (ATA por GTA). La mutación reportada en trigo de A2004V en la accesión AIQ78380.1 por transición de C por T confirmada por Zhang (R. Zhang *et al.*, 2019), le otorga tolerancia a quizalofop a 10gramos de ia por ha−1, mientras que esta molécula en leguminosas como soya y lentejas es de 31-92g ia por Ha (Ostlie *et al.*, 2014; R. Zhang *et al.*, 2019). Mutaciones en *Oryza japonica* de la *ACCasa 2* (*LOC_Os05g22940*) correspondientes a I1781V, C2088R y W2027C proveen tolerancia a herbicidas (X. Liu *et al.*, 2020). Existe el antecedente de un arroz comercial con la mutación por transversión A/T que ocasiona un cambio de aminoácido I1781L en el gen *ACCasa* de arroz de herencia mendeliana y está disponible en el mercado estadounidense desde el 2018 (Camacho *et al.*, 2019). Otros sitios de mutación por transversión en malezas resistentes a estos herbicidas están disponibles en la accesión AJ310767.1 de la maleza resistente *Alopecurus myosuroides* (Hernández-Soto *et al.*, 2021).

El gen *ACC2* se puede caracterizar mediante secuenciación con lo que se dispondría de un método visible y cuantitativo para determinar las condiciones óptimas que resultan en los fenotipos esperados.

3. La tolerancia a moléculas químicas es una característica potencialmente útil para el fitomejoramiento. La práctica de usar suelos inundados y cultivo de arroz mediante transplante es eficiente y ampliamente usada en países asiáticos particularmente para el control de arvenses (Wang *et al.*, 2017) pero el impacto ambiental por la producción de metano (Basavalingaiah *et al.*, 2020), así como la huella hídrica es muy elevado con un promedio de 1325 m3/t (48% verde, 44% azul y 8% gris) (Lovarelli *et al.*, 2016). La práctica puede resultar en degradación química y fitotoxicidad en suelos ultisoles ricos en hierro, que se solubilizan al acidificarse por efecto del agua, lo ocasiona también mayor compactación del suelo. La degradación puede ser también a

nivel de pérdida de materia orgánica (Wang *et al*., 2017). Alternativamente el arroz se cultiva de manera directa (en secano), con la ventaja de menos uso de agua, menor producción de metano, maduración en menor tiempo, pero la desventaja de la presencia y manejo de arvenses (Sagare *et al*., 2020). Una estrategia para solventar esta problemática es el desarrollo por fitomejoramiento de variedades que muestren resistencia a moléculas químicas (herbicidas) o a condiciones que limitan la producción (Reddy *et al*. 2017). En el caso específico del arroz, uno de los mayores problemas en el control de arvenses es el arroz maleza (Oryza sativa f. spontanea) (Nadir *et al*., 2017). El arroz tolerante a herbicidas es una solución ya adoptada en el pasado con tolerancia a herbicidas imidazolinonas como resultado de las mutaciones S653N, A122T y G654E en el gen *Acetolactato Sintasa* (*ALS*) de nombre comercial Clearfield (tales como CL121, CL141 contienen G654E; CL161 y CLXL8 contienen S653N con 32% más resistencia; INTA Puitá-CL contiene A122T). Sin embargo, el gen responsable de la resistencia se cruza eventualmente con el arroz maleza y por tanto adquiere la resistencia que bajo una constante presión de selección puede llegar a niveles del 20-60% (Singh *et al.*, 2017). Una molécula alternativa es la tolerancia a herbicidas de distinto modo de acción como los ariloxifenoxipropionatos como el Fluazifop.

El Fluazifop cuyo nombre es Fluazifop-p-butyl, peso molecular de 327,25g $mole^{-1}$ es un herbicida que pertenece a los Ariloxifenoxipropionato que actúan bloqueando el dominio funcional de la enzima *Acetil-CoA carboxilasa*. La enzima *Acetil-CoA carboxilasa* (EC 6.4.1.2) es una enzima multifuncional para la síntesis de ácidos grasos primarios en cloroplastos, mitocondrias, así como en la síntesis de ácidos grasos de cadena larga en el citoplasma. El arroz convencional es susceptible al herbicida Fluazifop a concentraciones de 210g ia/Ha y 420g ia/Ha en aplicación en suelo que afectan en un 13% y 15% de daño respectivamente y proveen de poco efecto residual de arvenses al aplicarse como pre- emergente (Lancaster *et al.*, 2018). Existen mutaciones que confieren resistencia a herbicidas en trigo A2004V ariloxifenoxipropionato, específicamente a quizalofop a 10gramos de ia por ha−1, mientras que esta molécula en leguminosas como soya y lentejas es de 31-92g ia por Ha (Ostlie *et al.*, 2014; R. Zhang *et al.*, 2019). A nivel de selección por mutación, existe un reporte de dosis de selección de 1uM y 2uM por Litro (PM 327,25g mol^{-1}) es decir de 0,327mg y 0,654 mg respectivamente en callo embriogénico, así como selección en invernadero con aspersión de 150mg/L de las plantas resistentes (X. Liu *et al.*, 2020).

El presente proyecto plantea realizar la inducción por mutación en etapas *in vitro* mediante callos embriogénicos y selección mediada por ariloxifenoxipropionatos. Las vitroplantas que sobrevivan

generarían líneas que adquirieron la tolerancia potencialmente relacionada con la mutación en el gen *ACCasa2*. Los genes se procederán a caracterizar mediante secuenciación de los genes blanco de las líneas mutantes regeneradas M1 con tolerancia al herbicida.

Marco metodológico:

La investigación es cuantitativa en su diseño (McCusker & Gunaydin, 2015) y utiliza dos factores en etapas independientes. El primer factor es la radiosensibilidad de los callos de arroz para el cálculo de la dosis letal media (LD50), en dosis de 0, 20, 40, 60, 80, 100 y 120 Gy, con 5-10 repeticiones, y 20 unidades (callos) para un total de 1500 callos. La segunda es la dosis letal media del herbicida que se realiza en dosis seriadas del herbicida, mediante una primera aproximación de dosis letal media de 0,10,100mg de herbicida 10 repeticiones, y 20 unidades (callos o semillas germinadas in vitro) cada uno y posteriores ensayos de una mejor aproximación a la dosis letal media. Con esta información se generan los datos de línea base para generar mutantes tolerantes y someterlos a selección con el agente de selección con el fin de identificar las líneas resistentes. Posteriormente se analizarían a nivel genético las plantas tolerantes para entender la resistencia, se llevarían al invernadero y eventualmente a campo para un análisis de comportamiento. La metodología específica se describe en el apartado de procedimientos.

OE 1 Establecer el cultivo in vitro de callo embriogénico de una variedad comercial de arroz y la radiosensibilidad de estos a radiaciones gamma (Co-60).

Preparación de material. Semilla de arroz (Oryza sativa subsp. indica) Lazarroz FL en granza, facilitada por productores de arroz, se utilizaron como material de partida para la introducción e inducción de callo embriogénico según se detalla a continuación. En la desinfección y el establecimiento in vitro se utilizaron 6000 semillas. La palea y la lemma se eliminaron utilizando una lija No. 100 y posteriormente se utilizó una doble incubación en una solución de hipoclorito de sodio (NaOCl) al 5,25 % (v/v) con 5 µl de Tween 20 ® por cada 10 ml, en agitación en orbital a 200 rpm por 20 minutos. En la cámara de transferencia de flujo laminar, se realizó cinco lavados con agua destilada estéril, seguido de un enjuague con la solución biocida (Methylisothiazolone (2-methyl-4-isothiazolin-3-one, MIT) y Methylchloroisothiazolinone (5-chloro-2-methyl-4-isothiazolin-3-one, CIT)), reactivos Químicos Gamma, Laboratorios ARVI S.A) al 40% (v/v).

Inducción del callo embriogénico. Se colocaron 15 semillas por plato Petri con 20ml de medio de inducción de callos según lo descrito por Bai *et al.* (2014), el cual consiste de las sales minerales y vitaminas de Murashige y Skoog (MS, 1962) suplementado con 2,5 $mg.L^{-1}$ ácido 2,4-diclorofenoxiacético (2,4-D), 30 $g.L^{-1}$ sacarosa, 0,3 $g.L^{-1}$ caseína hidrolizada y 0,5 $g.L^{-1}$ prolina

(Bai *et al.*, 2014; Murashige & Skoog, 1962; Pawar *et al.*, 2015). El pH se ajustó a 5,8 y el medio se esterilizará en autoclave (presión de 1,2 ATM.cm^{-2} y 121 °C de temperatura por 25 min). Los cultivos se mantuvieron en condiciones de oscuridad durante la inducción de callo embriogénico por 20 días. La inducción se tuvo de optimizar lo que resultó en un medio MS con 2 mg L^{-1} 2,4-D, en lugar de 2.5mg L.

Regeneración de los embriones somáticos. Se evaluó la regeneración de 200 callos en medio semisólido con sales minerales MS, 30 g.L^{-1} maltosa, 0,5 mg.L^{-1} ácido α-naftalenacético (ANA), 3 mg.L^{-1} 6-bencilaminopurina (BAP), gelificado con 4 g.L^{-1} Phytagel ®, y el pH fue ajustado a 5,8 (Sudhakar *et al.*, 1998). Las condiciones de cultivo serán de luz directa con una intensidad lumínica de 72 µmol·s^{-1}·m-2, un fotoperiodo de 16h y una temperatura de 26 ± 2 °C. La metodología de regeneración se tuvo que optimizar. Se propuso el uso de kinetina y tidiazurón como fitohormonas alternativas para la regeneración en caso de ser necesario, por ejemplo (Mohd Din *et al.*, 2016) reporta el uso del medio MS con 0.5 mg.L^{-1} 6-bencilaminopurina (BAP), 1.5 mg.L^{-1} Kinetina, 0,5 mg.L^{-1} ácido α-naftalenacético (ANA), y 0,5 mg.L^{-1} tidiazurón (TDZ). Lo anterior se debe a Tidiazurón ocasiona formacion de brotes y específicamente en arroz genera un cambio de expresión genética asociado a la regeneración a concentraciones de 1mg/L (Chakrabarty *et al.*, 2010; Indoliya *et al.*, 2016). La regeneración mejoró mediante el uso de inmersión temporal en un 100%.

Determinación de la radiosensibilidad. Los callos embriogénicos se irradiaron en el sistema de irradiación tipo Ob-Servo Ignis (Institute of Isotopes Co, Ltd., Budapest, Hungary), con 24 fuentes de cobalto 60 (tipo CoS 44HH-N). La radio sensibilidad se calculó al exponer grupos de 20 callos a dosis de 0, 20, 40, 60, 80, 100 y 120 Gy. Se irradiaron en promedio 180 callos por dosis, para un total aproximado de 1500 callos evaluados. Cada ensayo se analizó en un diseño completamente aleatorio y con un análisis de regresión lineal de Probit y Logit con el paquete estadístico IBM SPSS versión 26. Los callos se colocaron en medio de multiplicación con el fin de evaluar exclusivamente la variable de radiosensibilidad.

OE 2. Determinar la concentración correcta de dosis letal, que permita el posterior ensayo de inducción y selección *in vitro* de líneas de arroz con tolerancia al herbicida.

Determinar la sensibilidad a los herbicidas de callos embriogénicos en regeneración. Callos embriogénicos no irradiados se cultivaron en el medio de multiplicación de callo según lo descrito por Bai *et al.* (2014) con dosis de 0, 10 y 100mg de herbicida ariloxifenoxipropionato por Litro, para un total de 2000callos por herbicida. Los cultivos se mantuvieron con un fotoperiodo de 16h

luz con intensidad de 72 µmol·s^{-1}·m^{-2} y una temperatura de 26 ± 2 °C. Después de seis semanas de cultivo, se evaluó el porcentaje de callos que sobrevivieron y regeneraron plantas. Cada ensayo se analizará en un diseño completamente aleatorio y con un análisis de regresión lineal de Probit y Logit con el paquete estadístico IBM SPSS versión 26. Una vez determinado una aproximación de la dosis letal media, se realizarán ajustes en las dosis para repetir el ensayo con las dosis que se aproximen a la dosis calculada.

Alternativamente se realizó un análisis de toxicidad en *plántulas* germinadas *in vitro*. Esto debido al acceso limitado al laboratorio por la pandemia. El análisis se realizará en iguales condiciones, esto es, con dosis de 0, 10 y 100mg de herbicida ariloxifenoxipropionato por Litro. El número de individuos disminuiría a 40 plántulas por dosis de herbicida en medio de regeneración semisólido con sales minerales MS, 30 g.L^{-1} maltosa, 0,5 mg.L^{-1} ácido α-naftalenacético (ANA), 3 mg.L^{-1} 6-bencilaminopurina (BAP), gelificado con 4 g.L^{-1} Phytagel ®, con pH fue ajustado a 5,7(Sudhakar *et al.*, 1998). Después de seis semanas de cultivo, se evaluará el número de plantas que sobrevivan. Cada ensayo se analizará en un diseño completamente aleatorio y con un análisis de regresión lineal de Probit y Logit con el paquete estadístico IBM SPSS versión 26. Una vez determinado una aproximación de la dosis letal media, se realizarán ajustes en las dosis para repetir el ensayo con las dosis que se aproximen a la dosis calculada.

<u>Identificar líneas promisorias de arroz con tolerancia a los herbicidas.</u>

Los callos embriogénicos de arroz (2000 callos) de 21 días de inducción en el medio optimizado, se sometieron a la dosis letal media calculada para la radiación gamma (60Gy) y se regeneraron en medio de regeneración mencionado con anterioridad por un periodo de 2 meses (Sudhakar *et al*., 1998). Las plántulas regeneradas se sometieron a la dosis letal del agente de selección (5mg/L de Fusilade) en el medio de regeneración con un fotoperiodo de 16h luz e intensidad lumínica de 72 µmol·s^{-1}·m-2 y una temperatura de 26 ± 2 °C. Después 8 semanas se evaluó la sobrevivencia y las plantas sólo se seleccionaron las tolerantes (100 plantas). Posteriormente se incrementó a la dosis del agente de selección (10mg/L de Fusilade) para identificar potenciales líneas resistentes. La planta resultante se mantuvo en multiplicación en los medios de cultivo respectivos.

OE 3. Verificar los cambios genéticos asociados a la resistencia de al menos una de las líneas promisorias de arroz con tolerancia a herbicida.

La determinación de la mutación presente en líneas tolerantes a los herbicidas se basó en el estudio del gen reportado en la literatura como responsables de la tolerancia, ACCasa. Las plantas con tolerancia serán cultivadas hasta llegar a invernadero donde serán analizadas mediante secuenciación del gen ACCasa. La extracción de ADN se realizará a partir de hojas de vitroplantas resistentes al herbicida, mediante el Kit NucleoSpin™ Tissue (Macherey-Nagel), según las condiciones del fabricante. El ADN se visualizó en un gel de agarosa al 1,5% con buffer TAE 1X. La corrida se realizó durante 45 min a 70 V en una cámara de electroforesis horizontal. La secuenciación se realizará mediante el método de sanger. El diseño molecular de para el diseño de los imprimadores se realizó con Primer3 Plus, sobre la base de las secuencias disponibles en línea ACCasa (*ACC1 Os10g0363300*, *LOC_Os10g21910 Q8S6N5*; *ACC2*, *Os05g0295300, LOC_Os05g22940, B9FK36*). Para ello se realizó un análisis bioinformático de las secuencias disponibles en las bases de datos para el diseño, amplificación y secuenciación de los genes de interés tanto en el control, como en al menos un arroz maleza. Los resultados fueron procesados y analizados por herramientas bioinformáticas para entender el tipo de mutación, su cambio a nivel de aminoácidos y su relación con la resistencia al herbicida. Una vez obtenidas las plantas en el invernadero, se analizó para verificar su tolerancia al herbicida al exponerlas la línea a 150mg/L.

2. Objetivos

2.1. Objetivo general

Establecer un sistema de inducción de variabilidad genética de arroz (*Oryza sativa*) *in vitro* mediante radiación gamma usando como agente de selección ariloxifenoxipropionatos para su uso como una fuente de genes para el mejoramiento genético del cultivo.

2.2. Objetivos específicos

1. Establecer el cultivo *in vitro* de callo embriogénico de una variedad comercial de arroz y la radiosensibilidad de estos a radiaciones gamma (Co-60).
2. Determinar la concentración correcta de dosis letal de ariloxifenoxipropionatos, que permita el posterior ensayo de inducción y selección *in vitro* de líneas de arroz con tolerancia al herbicida.
3. Verificar los cambios genéticos asociados a la resistencia de al menos una de las líneas promisorias de arroz con tolerancia a herbicida.

3. Síntesis

Los artículos científicos de la presente tesis se presentan bajo el siguiente orden.

El primer artículo (Hernández-Soto *et al.*, 2021) es una revisión de literatura donde se exploran potenciales genes blanco para las técnicas de mutación. Las mutaciones son una herramienta para crear variabilidad genética, sin embargo, las dos preguntas que motivaron esta revisión de literatura fueron las siguientes. La primera si a nivel exploratorio existían fenotipos asociados a uno o dos genes específicos. La importancia para el fitomejoramiento radica en que la mutación pueda ser seleccionada en el proceso de manera más expedita, identificarse a nivel genético y validarlo en la descendencia para efectos de la heredabilidad. Efectivamente se lograron identificar genes relacionados con rendimiento, número de granos, floración, aroma, tolerancia a estrés biótico y abiótico. La segunda pregunta era si el agente de selección, tolerancia a ariloxifenoxipropionatos era efectivamente útil para correlacionarla con mutaciones de interés en genes blanco y qué otros genes o caracteres podrían ser útiles para el mismo fin. La revisión de literatura demostró que el gen *Os05g22940.1* (*ACC2*) es un excelente candidato dado que mutaciones en el extremo carboxi terminal se relacionan directamente con tolerancia. De igual manera la revisión de literatura permitió identificar otros genes útiles que se detallan a continuación. Los genes de tolerancia a agentes químicos como la trifluralina en el gen *Os11g14220*, las moléculas químicas que actúan sobre las enzimas *Acetolactato sintasa* en el gen *Os02g0510200*, y sobre la enzima *EPSPS*. Existen además genes de tolerancia a salinidad que podrían ser útiles durante la etapa de exposición a un agente de selección.

El segundo artículo (Hernández Soto *et al.*, 2022) consolida los primeros resultados experimentales. El artículo explica la herramienta de creación de variabilidad genética mediante radiación gamma *in vitro* de arroz y presenta las conclusiones de los objetivos 1 y 2. El artículo explora la identidad genética del material comercial que se utilizó, Lazarroz FL mediante marcadores moleculares estándar *matK* y *rbcL*, presenta el establecimiento y optimización de los protocolos *in vitro* de manera predecible y precisa. La optimización de los protocolos de cultivos de tejidos, de la radiación a 60Gy y regeneración requirió de buscar formas alternativas para alcanzar los objetivos según se detalla a continuación. La línea Lazarroz FL era recalcitrante para

la regeneración y presentaba porcentajes de regeneración muy bajos (30%) o nulos. Se requirió de optimización de los protocolos y se logró identificar a la inmersión temporal como una solución sumamente eficiente. Lo anterior hizo que se validasen los protocolos de inmersión temporal con un control (CR-5272), la variedad en estudio Lazarroz FL y los materiales recalcitrantes CR-1821, CR-1113, que mejoraron su regeneración.

En el tercer artículo (Hernández-Soto *et al.*, 2022) corresponde un pre-print del segundo y tercer objetivo de la tesis y presenta la consolidación del trabajo de estandarización previamente hecho en los objetivos 1 y 2. El sistema se ejecutó con base en lo aprendido, para ello se calculó la dosis letal del agente de selección (Fluazifop-p-Butil) y se re-evaluó el tejido *in vitro* a irradiar, a saber, callo y semilla. La dosis letal media en callo fue de 6,93 mg/L (0,425 mg/L - 15,743 mg/L, R2 = 0,402, 1000n); mientras que fue 3.771mg / L (R2 = 1, 290n) en plantas *in vitro* regeneradas a partir de callo. Se seleccionó la dosis de 5 mg/L (DL100) y una segunda selección con 10 mg/L para evitar falsos positivos. Se obtuvo solamente un mutante putativo de 8000 plantas irradiadas, regeneradas y seleccionadas. El mutante putativo disponía de la mutación T2222I/T2222M en el gen blanco y de mutaciones en los genes control *matK* y *rbcL*, en una proporción aproximada de 1/1000 pares de bases. La evaluación de lo obtenido llevó a un cambio en el tipo de tejido a irradiar de callo embriogénico a semilla. Esto permitió disponer de una mayor diversidad de dosis gamma dado que la semilla fue más tolerante, dispuso además de una etapa de inducción de callo y regeneración más predecible, así como un incremento de 1 a 31 mutantes putativos que serán evaluados en el futuro.

El cuarto artículo (Hernández-Soto *et al.*, 2022) corresponde a la pasantía realizada en el Laboratorio del Dr. Gatica, Universidad de Costa Rica en el marco del Proyecto UCREA “Edición del genoma de arroz: alternativa para contribuir a la mitigación del cambio climático y una contribución al logro de la seguridad alimentaria”. El trabajo es una exploración de técnicas de mutación de mayor precisión mediante *CRISPR-Cas9* en levaduras como un modelo para arroz, en búsqueda del rasgo de tolerancia a estrés salino mediado por trehalosa. La trehalosa en un disacárido de reserva con capacidad de funcionar con un osmoprotector tanto en arroz como en levadura (Fichtner & Lunn, 2021; Rapoport *et al.*, 2019; X. Zhang *et al.*, 2020). En arroz existe un solo gen OsTRE Os10g0521000 que codifica para la enzima que degrada la trehalosa. En levadura existen tres enzimas una vacuolar *ATH1* y dos neutras *NTH1*, *NTH2* que son homólogas a la de arroz. Estas últimas dos enzimas son responsables del 75 y 25% de la actividad

enzimática respectivamente de la hidrólisis intracelular. Originalmente se planteó usar como pregunta de investigación si disminuir un 25% de la actividad enzimática mediante la disrupción del *NTH2* resultaba en tolerancia en levadura de tal manera que se tuviese al menos un acercamiento sobre el potencial de lograr algo similar en arroz. Se realizó una mutación en el gen *NTH2* que resulta en una mayor tolerancia de la levadura a condiciones salinas. El artículo se enfoca en el diseño y prueba de concepto para la levadura exclusivamente, y demuestra el potencial de la creación de variabilidad genética, en este caso mediante una técnica de mayor precisión.

Current Plant Biology 27 (2021) 100211

Contents lists available at ScienceDirect

Current Plant Biology

journal homepage: www.elsevier.com/locate/cpb

Review article

Rice breeding in the new era: Comparison of useful agronomic traits

Alejandro Hernández-Soto [a,*], Fabián Echeverría-Beirute [b], Ana Abdelnour-Esquivel [a], Marta Valdez-Melara [c], Jens Boch [d], Andres Gatica-Arias [c]

[a] Technologic Institute of Costa Rica, DOCINADE, Biology School, Biotechnology Research Center, Costa Rica, P.O. Box 159-7050, Cartago, Costa Rica
[b] Technologic Institute of Costa Rica, DOCINADE, APDO, 223-21001, Alajuela, San Carlos, Ciudad Quesada, Costa Rica
[c] University of Costa Rica, School of Biology, Plant Biotechnology Laboratory, P.O. Box 2060, San José, Costa Rica
[d] Institute of Plant Genetics, Leibniz Universität Hannover, 30419, Hannover, Germany

ARTICLE INFO

Keywords:
Mutagenesis
Domestication
CRISPR
Gamma radiation
Chemical mutagen
Biotic stress
Abiotic stress

ABSTRACT

Understanding agronomic traits at a genetic level enables the leveraging of this knowledge to produce crops that are more productive and resilient, have better quality and are adjusted for consumer preferences. In the last decade, rice has become a model to validate the function of specific genes, resulting in valuable but scattered information. Here, we aimed to identify particular genes in rice related to traits that can be targeted by different mutation techniques in the breeding of crops. We selected gain of function, misfunction, and specific mutations associated with phenotypes of agronomic interest. The review includes specific trait-related genes involved in domestication, stress, herbicide tolerance, pathogen resistance, grain number/quality/weight, plant structure, nitrogen use, and others. The information presented can be used for rice, other cereals, and orphan crops to achieve a superior and sustainable production in challenging farming conditions.

1. Introduction

Induced mutagenesis is a valuable tool to support functional genomics studies and the development of new genotypes. Rice serves as an outstanding model because of its impact on the worldwide food supply chain and the availability of genomic and agronomic resources to utilize. Rice was the first crop sequenced in 2004 [1], biotechnological techniques are available, and the genomic information is available to search for specific target mutations, such as from the Rice Genome Annotation Project and Oryza Genome which can contribute to the precise engineering of the crop [2–4]. Biological, chemical, and physical agents can induce mutagenesis. Typical methods are radiation (first used on vegetables in 1928), ethyl methanesulfonate (EMS) (which produces 2–10 mutations per Mb), and new breeding techniques to introduce specific mutations via genetic engineering [5–8]. In this review, we present rice traits that have emerged or have been validated in the last decade (2010–2021) and were derived from technological advances in genomics [9]. This paper is focused on characteristics that could be targeted by mutagenesis of rice lines and related crops to produce predictable changes in gains or losses of function. We present traits that could result from the use of different techniques; remarkably, genome editing represents an exciting opportunity to transfer the information about gene-trait relationships to other crops to improve their traits. Consequently, they represent a challenge from a regulatory point of view for countries that have established a different regulation for genome-edited plants in contrast to other mutagenesis techniques.

2. Methods

The methodology applied a search based on PubMed articles and keywords: rice, traits, stress tolerance, resistance, breeding; selection of papers with agronomic traits linked to specific genes described within 2010–2021. Finally, verification of each gene, trait, and mutation was performed using specialized web servers such as Gramene, Ensembl-Plants, Rice Diversity, FunRiceGenes, Rice Genome Annotation Project, Oryza Base, and Rice Information GateWay [10]. The search resulted in

Abbreviations: EMS, Ethyl methanesulfonate; PTR, Puddled Transplanted Rice; DSR, Direct Seeded Rice; DAS, Days After Sowing; HRAC, The Herbicide Resistance Action Committee; WSSA, Weed Science Society of America; *MoA*, Mode of Action; BLS, Bacterial Blight Streak; AC, Amylose Content; ALS, Acetolactate Synthase; NUE, nitrogen use efficiency.

* Corresponding author.
E-mail addresses: alhernandez@itcr.ac.cr (A. Hernández-Soto), fecheverria@itcr.ac.cr (F. Echeverría-Beirute), aabdelnour@itcr.ac.cr (A. Abdelnour-Esquivel), marta.valdez@ucr.ac.cr (M. Valdez-Melara), jens.boch@genetik.uni-hannover.de (J. Boch), andres.gatica@ucr.ac.cr (A. Gatica-Arias).

https://doi.org/10.1016/j.cpb.2021.100211
Received 11 May 2021; Received in revised form 15 June 2021; Accepted 17 June 2021
Available online 18 June 2021

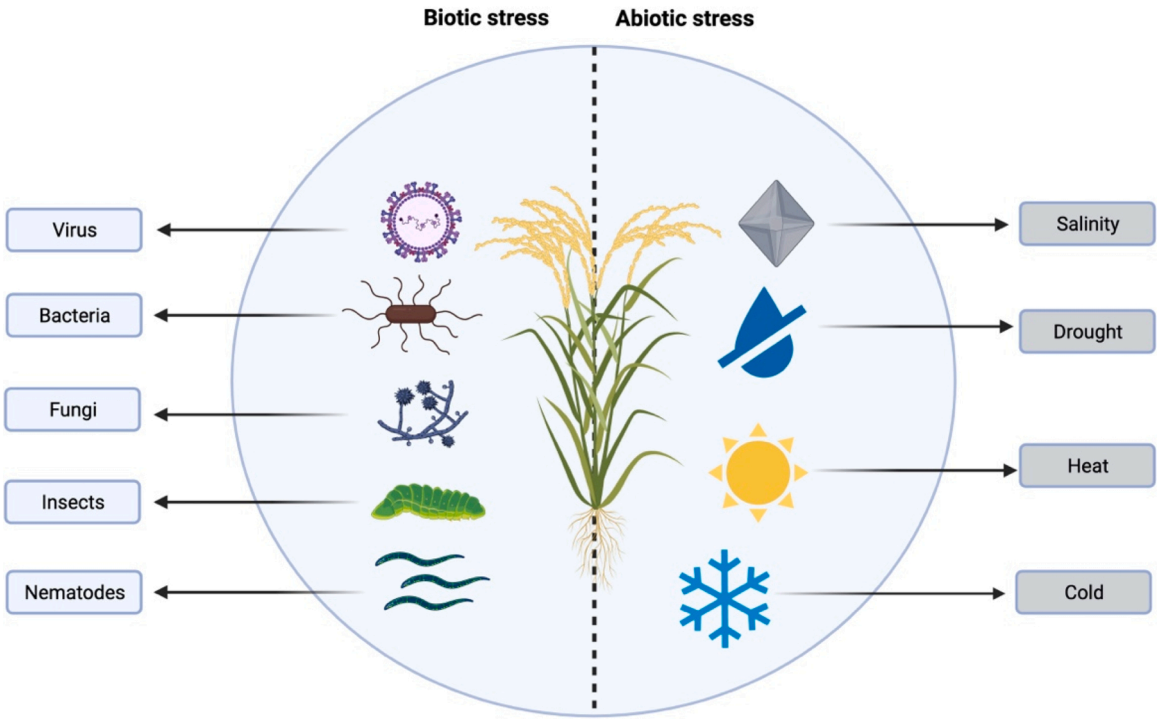

Fig. 1. Representation of biotic and abiotic stress factors that affect rice production. Created with BioRender.com.

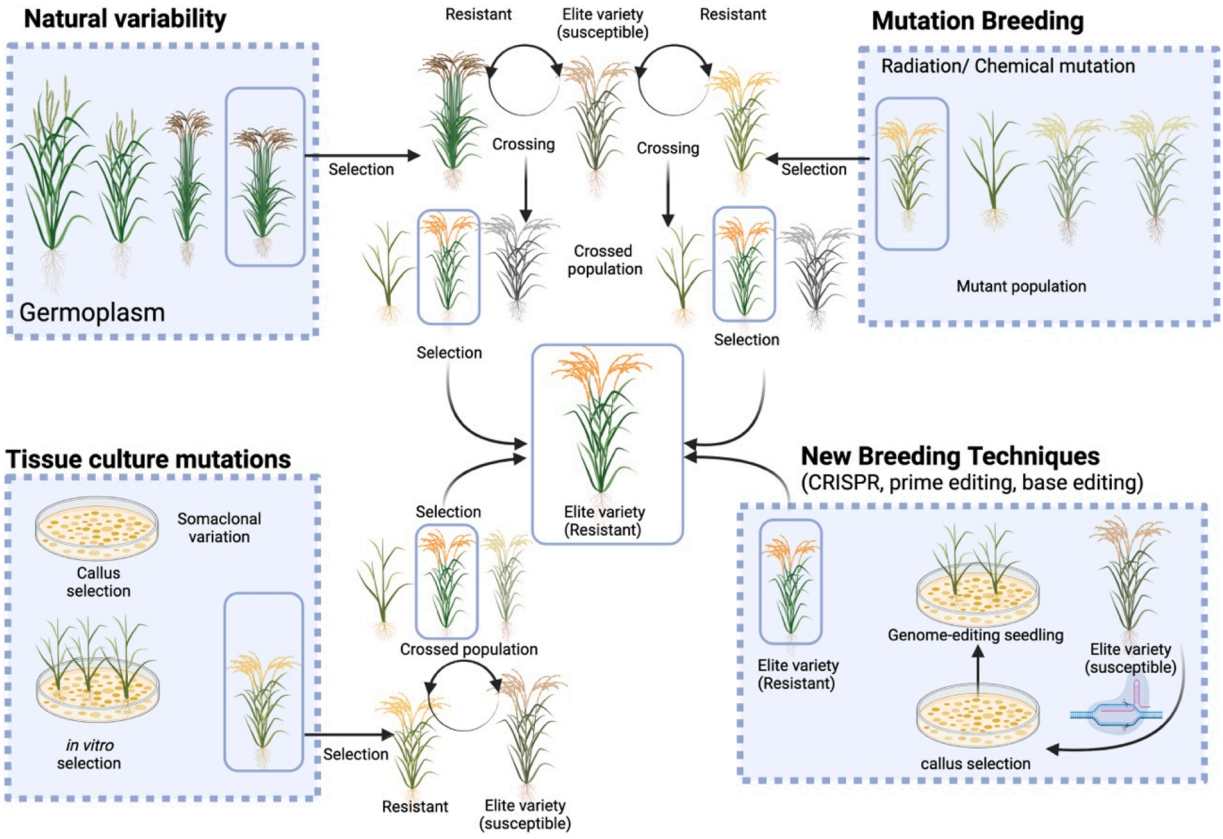

Fig. 2. Schematic representation of different systems used for breeding rice: natural variability, mutation breeding, tissue culture mutation, and new breeding techniques. Created with BioRender.com.

a selection of 117 papers out of 500.

(*continued on next column*)

(*continued*)

Webserver	Link
Webserver	Link
Gramene	https://ensembl.gramene.org/genome_browser/index.html
EnsemblPlants	http://plants.ensembl.org/index.html
Rice Diversity	http://www.ricediversity.org/data/index.cfm
FunRiceGenes	http://funricegenes.ncpgr.cn/
Rice Genome Annotation Project, Michigan State University	http://rice.plantbiology.msu.edu
Oryza Base	https://shigen.nig.ac.jp/rice/oryzabase/
Rice Information GateWay	http://rice.hzau.edu.cn/

3. Importance for breeding rice

Rice, such as many other tropical crops, is susceptible to a large set of biotic (fungi, bacteria, nematodes, insects, and viruses) and abiotic (salinity, drought, heat, and cold) stresses that cause yield and economic losses (Fig. 1). In general, biotic stress cause losses worldwide up to 35 % of the total food production [11]. As an example, losses in rice due to insects can account for over 40 %. Moreover, losses caused by the fungal pathogens *Magnaporthe grisea*, *Thanatephorus cucumeris*, and *C. miyabeanus* have been estimated worldwide at 35 %, 24 %, and 16 %, respectively [12]. On the other hand, abiotic stress represents the primary cause of crop losses worldwide, and yield losses can be as high as 50 % of crop production [128].

In this regard, the generation of rice-resistant varieties to biotic and abiotic conditions represents one of the major challenges that breeders face. For decades, breeding strategies include selection, hybridization, mutation induction using chemical and physical agents, and somaclonal variation. More recently, the availability of genome editing technologies, genome sequences, efficient tissue culture, and transformation methodologies could remarkably facilitate the breeding of rice (Fig. 2).

4. Rice breeding systems

Several methods are available for breeding rice with natural or induced mutagenesis; among them, we can mention mutation breeding, tissue culture, and new breeding techniques (CRISPR mutagenesis, base editing, and prime editing) (Fig. 2).

4.1. Mutation breeding

The mutation breeding principle is to generate heritable changes in the DNA by external agents. The changes result by exposing plant cells to physical (UV, X-ray, gamma radiation) or chemical (sodium azide and ethyl methanesulfonate) agents [13]. Induced mutagenesis offers a promising alternative for developing rice varieties resistant to biotic and abiotic stresses since it could accelerate the spontaneous mutation process and increase the pool of allelic variants available for genetic improvement [14,15,8].

4.2. Tissue culture

Totipotency, a distinguishable characteristic of plant cells, in principle allows each cell to regenerate an entire plant. This process involves the culture of plant tissue fragments or individual cells on special growth media enabling the cells to grow, divide, and differentiate into organs [16]. Among the techniques available, somaclonal variation are spontaneous changes in the DNA leading to genetic and phenotypic variations among clonally propagated plants. The somaclonal variants obtained could be detected using in vitro selection by applying selective pressure in culture conditions [17,18].

4.3. New breeding techniques

4.3.1. CRISPR/Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 (CRISPR/Cas9) system from *Streptococcus pyogenes* targets a specific genomic sequence using an engineered 20 base pair (bp) RNA guide sequence that binds to matching DNA and the Cas9 protein, upon recognition of an additional 3' localized PAM sequence 5′-NGG-3′, generates a double-strand break at a desired location in the genome. This genome editing method allows the insertion, deletion, or modification of DNA with high specificity and efficiency [5].

4.3.2. CRISPR/Cpf1 system

The nuclease Cas12a requires a small crRNA for inducing double strand breaks with efficiencies similar to those of CRISPR/Cas9. Moreover, this nuclease uses a 18–23 nt spacer for its maximum efficiency and specificity and identifies a T-rich PAM located 5' upstream of the guide and generates staggered ends with 5' overhangs [19].

4.3.3. Base editing

This system allows the conversion of nucleotides without inducing double-stranded DNA breaks or using donor templates. It is based on Cas9 nickase fusions to a nucleotide deaminase domain and has been used for changing a C–G base pairs into T-A (cytidine deaminase base editor), or A–T into G-C (adenosine deaminase base editor) [20].

4.3.4. Prime editing

This system uses a catalytically impaired Cas9 endonuclease fused to a reverse transcriptase enzyme, and a prime editing guide RNA (pegRNA). This complex is capable of identifying a target site and replace the target DNA nucleotides without double-stranded DNA breaks or using donor templates [21,22].

5. Agronomic traits of interest

5.1. Domestication genes

The *Oryza* genus is composed of species with a variety of genome structures, including six diploids (n = 12; named AA, BB, CC, ee, ff, gg) and five polyploids (n = 24, named BBCC, CCDD, HHJJ, HHKK, and KKLL) [23–26]. Only two diploid (2n = 24) species of rice have been domesticated and used for cultivation: *Oryza sativa* and African *O. glaberrima.* Rice domestication favored the selection of specific loss of function alleles. Wild relatives typically have functional versions of these genes such as *sh4*, *waxy*, *BH4*, *qSH1*, *AN1*, *brown pericarp, PROG1*, and *OsG1*. The *sh4* gene is related to reduced seed shattering (*Os04g0670900*). The *waxy* gene controls the amylose content (*Os06g0133000*). *BH4* is related to the hull color of the seeds (*Os04g0460200*). The gene *qSH1* is involved in seed shattering (*Os01g0848400*). The *AN1* gene is related to seeds, morphology, and grain shape (*Os04g0350700*). RC *Brown pericarp* is involved in the seed coat (Os07g0211500). *PROG1* is related to an erect plant structure (*Os07g0153600*). *OsLG1* is related to a closed-panicle structure (*Os04g0656500*) [27]. The importance of such genes and their domesticated alleles is critical in understanding how *de novo* domestication can be achieved from wild *Oryza* varieties and how such genes can be further used for breeding of rice and other crops.

This concept was demonstrated in polyploid *O. alta* (CCDD) by Yu et al. [28], targeting *SD1, GS3, IPA1, Ghd7, Gn1a, Wx, Bh4, TAC1, An-1* homologs, as well as African landraces of *Oryza glaberrima* by disrupting the *HTD1* (*O. sativa Os04g0550600*), *GS3* (*O. sativa Os03g0407400*), *GW2* (*O. sativa Os01g0197700*) and *GN1A* (*O. sativa Os02g0244100*) genes [29]. For plant breeding, the use of non-domesticated, more genetically diverse rice species that better adapt to stress conditions, such as African landraces *O. glaberrima*, *O. barthii*, *O. meridionalis* (AA),

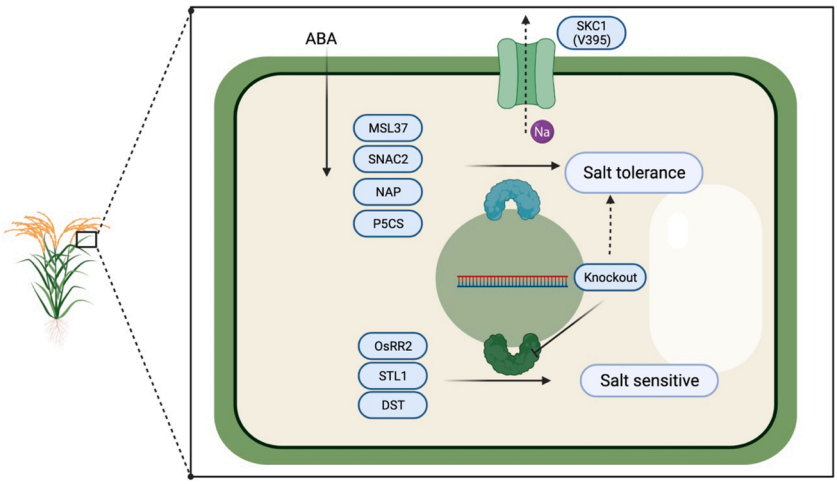

Fig. 3. Representation of salt tolerance traits mediated by three different methods: 1) overexpression, 2) knockout of specific genes, and 3) particular sodium channels. Note that the first corresponds to transcription factors that trigger adaptive responses labeled *MSL37*, *NAC2*, *NAP*, and *P5CS*. The second is a knockout of those that result in salt sensitivity: *OsRR2*, *STL1*, *DST*; and the sodium channel *SKC1* in rice. The third is the sodium channel *SKC1* containing amino acid V395. Created with BioRender.com.

Australian landraces *O. longistaminata* (AA), *O. australiensis* (EE), and Asian landraces *O. rufipogon* (AA) or *Porteresia coarct*ata (*O.coarctata*) (KKLL), harbors a valuable potential of developing more sustainable rice crops [30,31].

5.2. Stress tolerance

Rice susceptibility to salt is evidenced by a yield decrease due to delays in heading and panicle sterility especially in salt-sensitive varieties like MI48, IR29 [31–33]. In contrast, salt tolerance in varieties like Pokkali, Cheriviroppu, FL478, IR651, CSR27, FL30, Fontan, SR86, IR9884−54-3 results from ion exclusion, osmotic and tissue tolerance with multiple genes involved in the process, which confers agronomic stability of this trait [31–40]. The orchestrated stress system can be targeted for achieving salt tolerance by mutating genes encoding key transcription factors, specifically *OsRR22* (*Os06g0183100*), *STL1* (*Os04g0110600*), and the zinc finger transcription factor encoded by *DST* (*Os03g0786400*) [38,41–44]. Other transcription factors are critical in stress adaptation, which results in stress sensitivity when inactivated. This is the case for *MSL37* (*Os11g0163500*) which encodes a positive salt stress transcription factor response by regulating ion transporters, *P5CS* (*Os05g0455500*), which causes accumulation of the osmoprotectant proline, the transcription factor *SNAC2* (*Os01g0884300*), which is key in root adaptation, and *OsNAP* (*Os03g0327800*), which triggers a stress response mediated by ABA [45–49]. For details, see Fig. 3 and Table 1.

Osmoprotection by accumulating molecules such as trehalose or proline is a possible pathway leading to salt tolerance, as proven currently in plants like rice and *Arabidopsis* [59,47,60,61]. Other individual genes can confer osmoprotection, such as the Na + transporter *SKC1* (*Os01g0307500*) with a V395 L that provides salt tolerance [50]. Knocking out an independent gene, *OsEPFL9* (*Os01g0824500*), results in increased water use efficiency under stress because of the reduced stomatal count [51,52].

Other stress tolerance pathways can be modified by specific alleles, too. Low cadmium accumulation occurs after mutating the metal transporter genes *OsNramp5* (*LC196140;* japonica homologue: *Os07g0257200*) and *OsNramp1* (*LC196122*; japonica homologue: *Os07g0258400*). Plants are able to resist heat stress only when the gene *OsNTL3* (*Os01g0261200*) is functioning correctly, whereas cold tolerance can result from mutation of the *OsMYB30* (*Os02g0624300*) gene. Finally, more cuticle wax is deposited when the gene *DHS* (*Os02g0682300*) is mutated [54–58,62]

5.3. Herbicide resistance monogenic traits

Rice is usually cultivated under two agronomical systems: paddy-transplanted-rice (PTR) and direct-seeded-rice (DSR). The first is the conventional method, which requires water flooding and represents a sustainability issue because of water scarcity, methane production, and the consumption of nonrenewable energy [63]. DSR, on the other hand, represents opportunities for efficient water and nitrogen use, and a reduction of both greenhouse gas emissions and labor demand, especially in countries such as China, where 90 % of rice is currently produced under PTR [64]. However, weed management is a challenge in DSR, specifically during the first 41 days after sowing (DAS). This includes complication by weedy rice (*O. sativa f. spontanea)*, which is a variety of rice that is morphologically similar to cultivated rice, but grows as a weed. It produces far fewer grains than cultivated rice, and can result in rice yield losses of up to 50 % [25]. Weedy rice usually has increased seed longevity, seed shattering and stress tolerance which makes it difficult to control [65]. The use of chemical control represents a tool to manage weeds including weedy rice, but poses additional challenges.

The Herbicide Resistance Action Committee (HRAC) and the Weed Science Society of America (WSSA) classify herbicides into 34 groups and one unknown group based on their "mode of action" (*MoA*) at the biochemical level [66–68]. The discovery of a new mode of action has been rare in the last 30 years. A good example is leptospermone, and its analogous inhibitors that act as hydroxyphenylpyruvate inhibitors of dioxygenase (HPPD) [69]. Different modes of herbicide use, such as rotations, delay the emergence of herbicide-resistant weeds. However, weeds are evolving to resist multiple *MoA* types of herbicides. For example, *Chloris radiata* is found in Colombian rice fields with dual resistance to glyphosate (mode of action 9) and the acetolactate synthase (ALS) inhibitor imazomox (mode of action 2) [70]. Weedy rice infestation in the USA resulted in 5.7 million tons of harvest lost and $457 million in environmental costs between 2002–2014 [71]. To control weedy rice, herbicide tolerance was introduced into cultivated rice 20 years ago based on an *acetohydroxy acid synthase AHAS/ALS* (*Os02g0510200*) gene mutation, providing tolerance to the mode of action 2 [72]. Currently, rice herbicide tolerant varieties are used in the USA (700,000 Ha), Brazil (600,000 Ha), Uruguay (70,000 Ha), Argentina (32,000 Ha), Malaysia (95,000 Ha), and Italy (60,000 Ha), as well as in many Central America countries, such as Costa Rica, Honduras, Panamá, and the Dominican Republic [73]. The incorrect use of this variety allowed introgression and outcrossing of the resistance into weedy rice, which means that weed herbicide control requires stricter farming practices, such as herbicide rotation [74]. Alternatives such as aryloxphenoxy propionate-resistant rice (mode of action 1), which is the result of mutations in the *ACCase2* (*Os5g0295300*) gene, already exist and will allow for herbicide rotation [75,76].

According to the literature, at least five target genes have the

Table 1
Rice genes and mutations involved in stress tolerance or sensitivity traits.

Gene	Position	Protein	Obtained mutation	Method	Trait details	Reference
OsRR22 *Os06g0183100* *STL1*	Chr 6	Q5SML5	Knockout	CRISPR/Cas9	Two-component response regulator ORR22. Salt tolerance 0.75 % NaCl.	[38]
Os04g0110600 *Salt tolerance Level 1, Stress repressive zinc finger protein 4*	Chr 4	Q7XXF2	SNP	None	hap1 tolerance 0.9 % salt, the gene is the homolog of Arabidopsis salt tolerance gene SRP1 (Stress associated RNA-binding protein 1, AT2G17975). Knock-out mutation in the srp1 allele reduced sensitivity to ABA and salt stress.	[44]
MSL37 *Os11g0163500* *OsGTgamma-2, OsGTγ-2*	Chr 11	Q53PP7	Natural variability-Knockout	Spontaneous mutation-CRISPR/Cas9	Knock-out results in salt sensitivity. The transcription factor is a positive salt stress regulator, and binds to promoters of OsHKT2; 1, OsNHX1 and OsHKT1.	[42]
Os03g0786400 OsDST, DLN102, OsDLN102, Negative regulation of response to salt stress	Chr 3	Q10CE2	Knockdown	Mutant/ CRISPR/ Cas9	Knockdown improved the tolerance to stress, as also observed in the dst mutant. C2H2 zinc finger transcription factor, drought and salt tolerance, stomatal aperture control	[41,43]
P5C *Os05g0455500*	Chr 5	O04226	Natural: cultivar LPT123 is salt-susceptible versus salt-tolerant line LPT123-TC171	None	The enzyme increases the proline accumulation and salt resistance mediated by ABA application.	[48]
SKC1 *Os01g0307500* *OsHKT1;5,* *OsHKT8*	Chr 1	Q0JNB6	Wild relatives	None	Variant V395 (is salt tolerant), while L395 is sensitive.	[50]
Os10g0521000 *Based on Z.mays GRMZM2G162690 and A. thaliana AT4G24040*	Chr 10	Q9FWC1	Substitutio S163T	CRISPR/Cas9	Mutation of domain WDS to replicate *Selaginella moellendoffii* WDT. The enzyme may be less efficient in allowing the accumulation of trehalose.	[47]
OsEPFL9 *Os01g0824500* Epidermal Patterning Factor Like-9	Chr 1	Q5JN76	Knockout	CRISPR/Cpf1	Increased water use efficiency under stress because of reduced stomatal count	[51,52]
DHS *Os02g0682300* Drought hypersensitive	Chr 2	Q6EU38	Knockout-Overexpression	CRISPR/Cas9-gene transfer	Knockout results in more cuticular wax. Overexpression (DHS OE) plantlets grew more slowly. The enzyme is a ubiquitin that degrades ROC4 that positively regulates cuticular wax biosynthesis	[53]
RCS1 *Os12g0625000* O-acetylserine (thiol) lyase, Cysteine synthase. *arsenite tolerant 1*	Chr 12	Q9XEA6	S189N	EMS	Tolerates 20 μM As (III). The mutation increases As tolerance/decreased accumulation in the grain/increase Se accumulation in the grain.	[141]
OsNramp5 *LC196140 (indica rice)* *Os07g0257200 (japonica rice)* *Manganese and Cadmium transporter, Mn and Cd uptake,*	Chr 7	B8B4U0 (*indica rice*) Q8H4H5 I7GYG6 (*japonica rice*)	Knockout	CRISPR/Cas9	Low Cd accumulation	[54,55, 56]
OsNramp1 *LC196122 (indica rice)* *Os07g0258400* Metal transporter *(japonica rice)*	Chr 7	A2YK11 (*indica rice*) Q0D7E4 (*japonica rice*)	Knockout	CRISPR/Cas9	Low Cd accumulation. It works as a plasma membrane-localized transporter/uptake for Mn and Cd; it is complementary to OsNRAMP5 in the uptake of Mn and Cd.	[56]
OsNTL3 *Os01g0261200* *Thermotolerance*	Chr 1	Q7GCL7	Natural variability-	None	OsNTL3 is required for heat stress tolerance in rice. Loss-of-function mutation of OsNTL3 confers heat sensitivity. It regulates the expression of genes involved in ER protein folding.	[57]
OsMYB30 *Os02g0624300,* Cold tolerance gene	Chr 2	Q6K1S6	Knockout	CRISPR/Cas9	The protein OsMYB30 is a nuclear protein that acts as a negative regulator of cold tolerance. Mutant shows increased cold tolerance.	[58]

potential to develop herbicide-resistant rice varieties with a different mode of action. Two of those have already been described above: *ACCase2* on aryloxphenoxy propionates (*MoA-1*) and *AHAS/ALS* on ALS (*MoA-2*). For *ACCase2*, mutations such as I1781 L, S1866 F, I1879 V, A1884 P, W2027C, W2125S, D2176 G, and C2186R/P1927 F/G2201A/W2125C in exon 32 provide herbicide tolerance at a different level. *AHAS/ALS* alleles cause ALS (*MoA-2*) resistance when carrying the following mutations: A96 V/A122 T/P171 H/P171S/P197S/C287 T and W548 L/W574 L/S627I/S653I/S653 N/G654E. *OsTubA2* (*Os11g0247300*) provides tolerance to dinitroanilines (*MoA-3*) with a mutation in the fourth exon, M268 T. *HPPD* (*Os02g0280700*), provides tolerance to triketones with a natural insertion (GGAACCAAAAGAATTAGAGACGATATCA) in the fourth exon. Finally, the double mutation known as "TIPS" (T102I + P106S) in the *OsEPSPS* (*Os02g0510200*) gene provides tolerance to *MoA-9* (glyphosate). For details, see Fig. 4 and Table 2.

Weeds that are tolerant to the inhibition of photosynthesis at PSII by herbicides can also provide insights to generate herbicide tolerant crops. The S264 G mutation in *psbA* increases tolerance more than 50-fold to triazine in herbicide-tolerant radish (*MoA-5*). However, it can also compromise fitness because of less efficient photosynthesis [77]. Other mutations, such as Val219Ile, Asn266Thr, Phe255Ile, and Ala251Val, can also provide tolerance [68]. It is important to note that the *psbA* mutation Val-219-Ile provides tolerance to the amide propanil *MoA-5* on

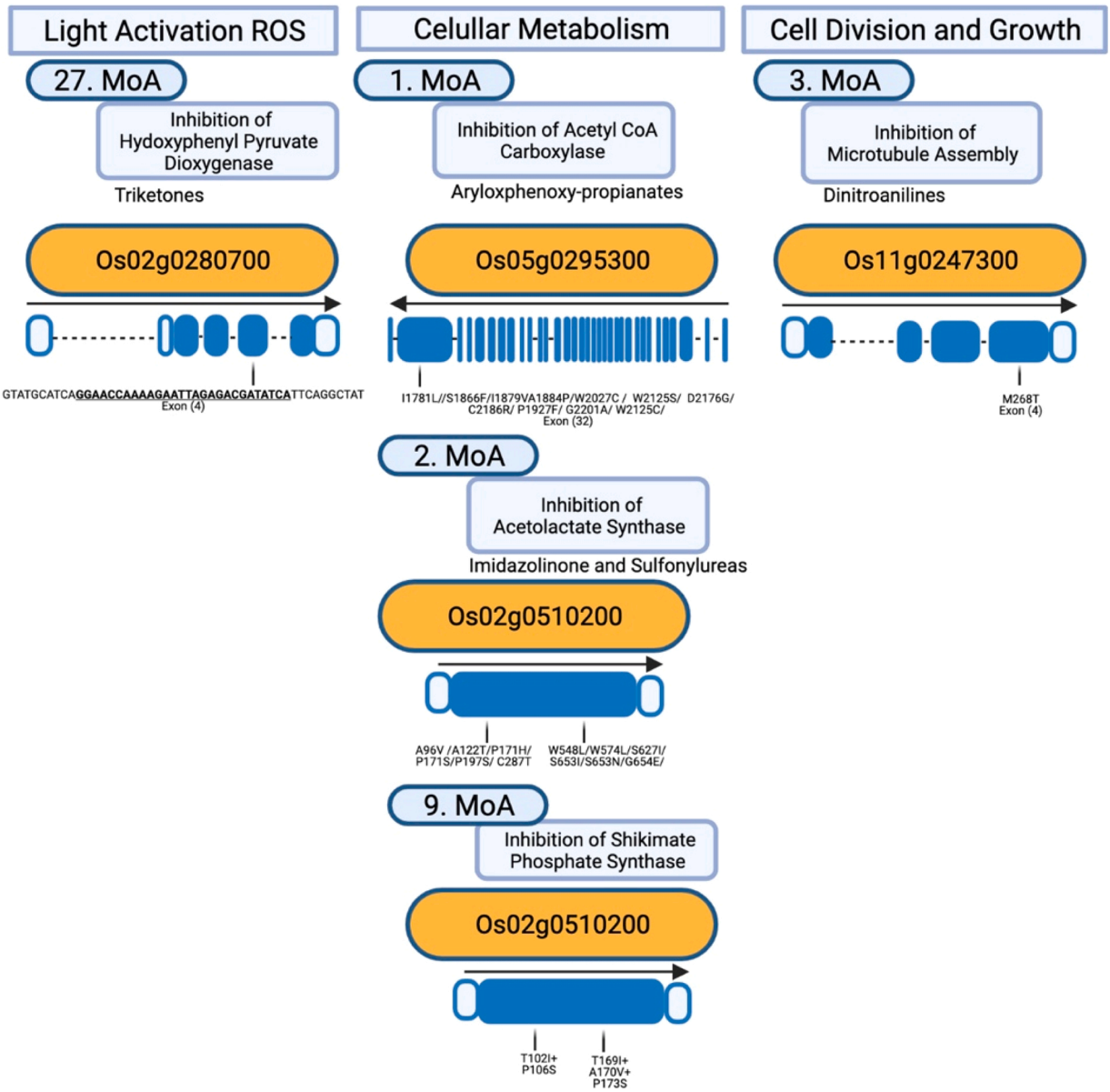

Fig. 4. Representation of five rice genes and the corresponding mutation that results in herbicide tolerance. The genes are shown organized by their Mode of Action (MoA). Note the name of the gene in orange circles, the exons in blue filled boxes and the corresponding untranslated exon regions in the blue empty boxes. Created with BioRender.com (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Cyperus difformis [79]. Propanil is widely used in rice cultivation because the crop is naturally capable of degrading the molecule by a putative enzyme located in the mitochondria, and an additional pathway could increase its tolerance [80,81]. The described mutations could also result in herbicide tolerance in rice when targeting the homologous gene AAS46167, encoding protein P0C434, to address an additional *MoA*.

Rice is also known to be resistant to Bentazon (*MoA-6*), as it is degraded by cytochrome P450 *CYP81A6* [82]. Additionally, the P450 gene *CYP72A31* is responsible for conferring tolerance to bispyribac sodium (BS) in *Oryza sativa* cv. *indica*, while its absence in japonica rice varieties results in BS-sensitivity [83,84].

5.4. Bacteria, fungi and virus resistance

Rice breeding of pathogen resistance is possible by mutation of specific promoter regions of the *Sweet 14,11,13* genes (*Os11g0508600*, *Os08g0535200*, *Os12g0476200*), respectively, since they are required for infection by bacterial *Xanthomonas oryzae* pv. *oryzae* pathogens causing bacterial leaf blight (BLB) [85–87]. The pathogen emerges by breaking the resistance of varieties planted in approximately 80 % of the total crop cultivation area carrying the resistance gene *Xa4* on chromosome 11 introduced in the 60 s [88]. Some *Xanthomonas oryzae* pathovars can also infect wild grasses and could become an emergent pathogen that is difficult to control [89].

A gene to target for fungal resistance is the transcription factor *IPA1* (*Os08g0509600)*; higher expression levels of IPA1 result in increased yield and immunity when tested against the fungal pathogen *Magnaporthe oryzae*. Resistance relies on time- and pathogen-specific phosphorylation and activation of the transcription factor at Ser163. Subsequently, phosphorylated IPA1 activates the WRKY45 promoter and following basal resistant gene expression within 48 h after infection, while the nonphosphorylated IPA1 protein binds to the DEP1 promoter related to yield (Jing [53]). A different way to achieve *M. oryzae* resistance is by mutation of *OsERF922 ethylene response factor 922* (*Os01g0752500*) [90]. Another important trait is tungro spherical virus

Table 2
Rice genes and mutations in herbicide resistance traits.

Gene (*)	Position	Protein	Obtained mutation	Method	Trait details	References
OsTubA2 *Os11g0247300*	Chr 11	Q53M51	M268T	CRISPR/Cas9-Base editor	*In vitro* trifluralin 4 mg/, pendimethaline 6.6 mg/L	[140]
ACCase2			W2027C	Seeds-Gamma Rays 280Gy	quizalofop-p-ethyl =75 g/ha; haloxyfop-p-methyl =62.35 g/ha	[76]
			I1879V W2125S	CRISPR/Cas9-Base editor	haloxyfop-R-methyl, 1 and 2 μM *in vitro*.	[142,42]
	Chr 5	B9FK36	I1781L	Tissue culture mutation	quizalofop-p-ethyl = 235 g ai ha-1	[75]
Os05g0295300			D2176G G2201A	CRISPR-Prime Editing	Herbicide resistance	[139]
			C2186R	CRISPR-Base editor	Herbicide resistance	[134,42]
			P1927 F, W2125C, S1866 F and A1884 P	CRISPR-Base editor	Herbicide resistance 34 g/Ha. High tolerance P1927 F, W2125C versus low tolerance S1866 F and A1884P	[133,42]
psbA *AAS46167* (Photosystem II protein D1, psbA)	Chloroplast	P0C434	S264G	Wild radish, Spontaneous mutation-	Atrazine > 50-fold (4000(187 g a.i. ha-1 atrazine), (S) Bromoxynil	[77]
HPPD *Os02g0280700* Inhibitor Sensitive 1	Chr 2	Fe(II)/2-oxoglutarate–dependent oxygenase	28-bp deletion allele (his1).	wild Nipponbare lacked deletion (HIS1)	b-Triketone herbicides, HIS1detoxifies b-triketone herbicides by hydroxylation.	[135]
AHAS, ALS			W548L P171S	CRISPR-Prime Editing	Herbicide tolerance	[22,139]
Os02g0510200			A96V (C287 T)	CRISPR/Cas9 Base editor	Imazamox (quantity not reported)	[137]
			G654E	Chemical mutation	Clearfield 121 Clearfield 141 IRGA422	[73,71]
	Chr 2		S653N	Chemical mutation	Named CL161 and CLXL8 increased herbicide tolerance	[73]
		Q6K2E8	W548 L or P171S	Recombinant protein	Herbicide tolerance	[131]
Acetohydroxy acid synthase			W548 E549	CRISPR-Prime Editing	Herbicide tolerance	[139]
			A122T	Sodium Azide	IMINTA1, IMINTA4	[136]
			W548L S627I	CRISPR	Herbicide tolerance	[138]
	Arabidopsis		P171 H/W548 L	Recombinant	100 mM IQ	[131]
	Modeling		W574 L P197S S653I		100 mM CS/BM/IQ/IP/PS 100 mM BM 100 mM IP	
OsEPSPS	Chr 6	A0A0N7KLH2	T169I A170 V P173S	CRISPR-Prime Editing	NA	[132]
Os06g0133900			T102I + P106S	CRISPR	*In vitro* resistance 1 mg l–1 glyphosate, 400x dilution Greenhouse.	[78]

(S)= Susceptible, genomic.
(*) Additional information at The Rice Annotation Project (RAP). [85–87].
CS, chlorsulfuron; BM, bensulfuron-methyl; IQ, imazaquin; IP, imazapyr; PM, pyriminobac; PS, pyrithiobac-sodium; BS, bispyribac- sodium.

Table 3
Rice genes and mutations with pathogen-resistant traits.

Gene	Position	Protein	Obtained Mutation	Method	Trait details	Reference
Os11g0508600 *Sweet 14*	Chr 11	Q2R3P9	promoter edited	TALEN / CRISPR-Cas9	*Xanthomonas oryzae* pv. *oryzae* resistance, probably by avoiding sugar access for the pathogen growth	[86,87]
Os08g0535200 *Sweet11*	Chr 8	Q6YZF3	promoter edited	CRISPR-Cas9	*Xanthomonas oryzae* pv. *oryzae* resistance, probably by avoiding sugar access for the pathogen growth	[86,87]
Os12g0476200 *Sweet13*	Chr 12	Q2QR07	promoter edited	CRISPR-Cas9	*Xanthomonas oryzae* pv. *oryzae* resistance, probably by avoiding sugar access for the pathogen growth	[86,87]
Os07g0555200 *translation initiation factor 4 gamma gene (eIF4G)*	Chr 7	B9FXV5	Knockout and mutations on SVLFPNLAGKS	CRISPR-Cas9	Resistance to rice tungro spherical virus (RTSV)	[91]
Os01g0752500, ethylene response factor 922 OsERF922, LOC_Os01g54890.1	Chr 1	Q5JMX7	Knockout	CRISPR	*Magnaporthe oryzae*, Blast resistance	[90]

resistance which results by mutation of gene eIF4G (Os07g0555200) coding a translational factor that is key in the initiation of the virus mRNA [91]. For details, see Table 3.

5.5. Grain number, quality, weight and plant structure

Rice quality traits are essential to achieve a better yield, consumer preference, and growth efficiency. The genes involved in grain number

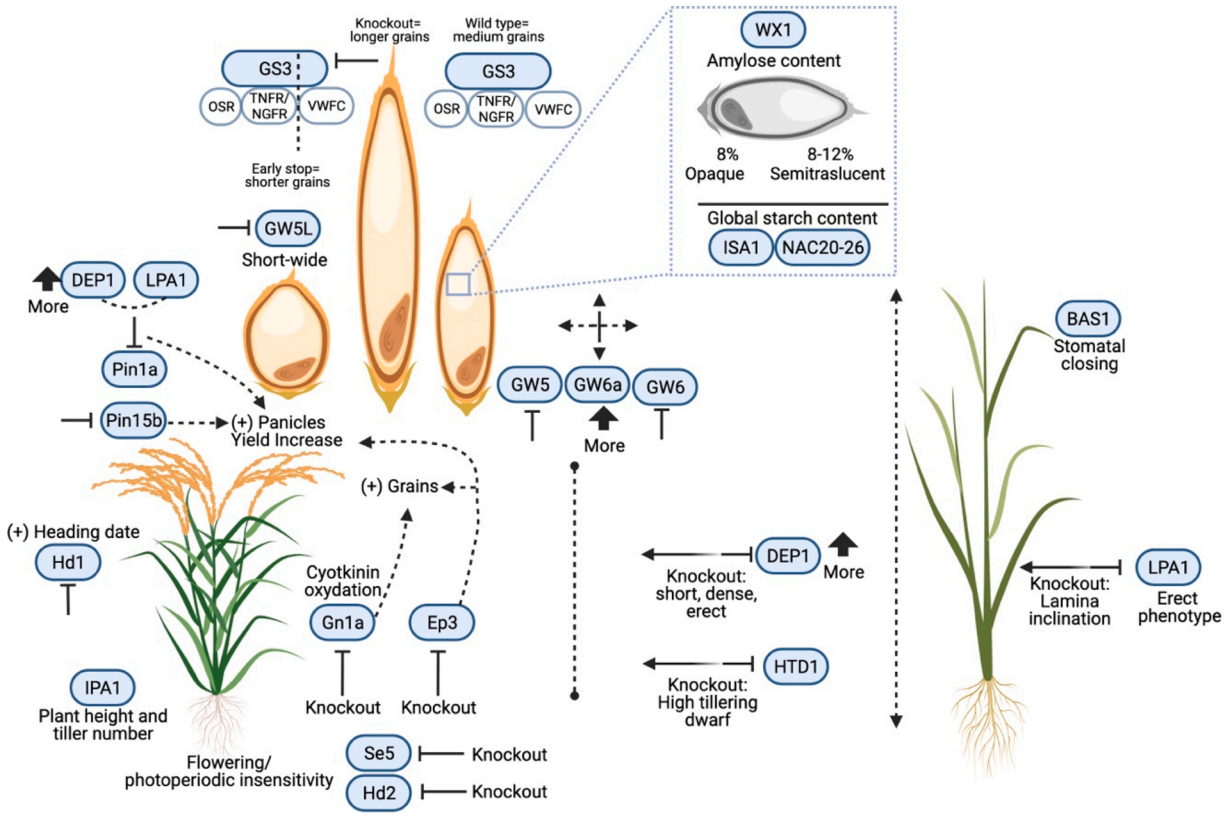

Fig. 5. Representation of traits such as grain number, quality, weight and plant structure and gene relationships in rice. Note that heading and flowering are positively influenced by *Se5, Hd2,* and *Hd1* knockout; structure by *DEP1, HTD1, IPA1, LPA1, Pin1a,* and *Pin15b*; grain size by *Gn1a,* and *Ep3*; grain size by *GS3, GW6a, GW5,* and *GW5L*; and grain starch by *ISA1, NAC20-26,* and *WX1*. Created with BioRender.com.

and size, plant density, structure, panicles, and flowering have complex interactions. However, recent findings and key mutations now provide insight into their regulatory mechanisms and greater predictability in achieving the desired phenotype (for details, see Fig. 5 and Table 4).

5.5.1. Grain size

The *GS3 Grain Size3* gene (*Os03g0407400*) is responsible for negatively controlling the grain length. Its mutation can result in better or worse weight and size that correlates with the composition of its domains: organ size regulation (OSR), a transmembrane necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR), and a von Willebrand factor type C (VWFC) (Meiru [78,94–96,58]). The wild type allele contains all of the domains and results in medium grains [95]. Loss of function results in long-grain varieties; for example, Minghui 63 has a stop mutation C165A at the second exon, resulting in a loss of function and a long-grain phenotype (Meiru [78,58]). In contrast, a mutation or deletion in the fifth exon creates a truncated protein with no VWFC domain and a short seed phenotype [95,114]. Grain size, in general, is controlled by several additional genes: higher expression of *GW6a* (*Os06g0650300*), and knockout of *GW5* (*Os05g0187500*), *GW6* (*Os06g0623700*), and *GW5L* (*Os01g0190500*) results in increased grain size [104–107,103].

5.5.2. Grain number

Malfunction of the gene *Os01g0197700* (*GN1a*) produces an increment of grain per panicle number and flowering because of a lower degradation of cytokines produced by the corresponding cytokinin oxidation enzyme [92,78,94]. Another gene that correlates with increased production and downregulates cytokine level regulation is *EP3 Erect Panicle 3* (*Os02g0260200*) [94,115].

5.5.3. Grain starch

Grain starch quality is an essential trait, which depends on the relative content of amylose and protein. The global starch content relies on the gene *ISA1* (*Os08g0520900*) and the protein content relies on NAC20−26 (*Os01g0104500*, *Os01g0393100*) [101,102]. The waxy gene *WX1* (*Os06g0133000*) controls the grain amylose content (AC). Mutations in this gene correlate with a phenotype that ranges from opaque (8%), semitranslucent (8–12 %), and transparent (12 % or more) grains [97–100,39].

5.5.4. Flowering

Flowering and photoperiodic insensitivity results from overexpression of *OsMeCP (Os12g0620400* [110] or by knocking out several genes. For example, *Se5*, *Hd2* and *Hd1* [4,94,111,112]. Another critical regulator of heading date and grain weight seems to be *HGW*, but its homozygous null mutant is embryonic lethal [113].

5.5.5. Structure

Farmers prefer smaller plants with many panicles and fewer tillering traits. Knockout of the *DEP1* (*Os09g0441900*) gene, as well as the loss of function of the *HTD1* (*Os04g0550600*) gene introgressed from landraces produces short, dense, erect panicles [29,78,108].

The transcription factor *IPA1 Ideal Plant Architecture1* (*Os08g0509600*), is related to fungal resistance and yield as mention previously, and its specific mutations between bases 854 and 876 can increase the production of the transcription factor protein because they interrupt transcript cleavage due to the micro RNA OsmiR156. For example, C874A in the third exon (leucine to isoleucine) generates a rice plant with a reduced tiller number, increased lodging resistance, and an enhanced grain yield [78.53].

The number of panicles and consequently the yield can be increased by mutating the genes *Pin1A* and *Pin15b* or indirectly blocking their

Table 4
Rice genes and mutations involved in grain quality, quantity, weight, and plant structural traits.

Gene	Position	Protein	Obtained mutation	Method	Trait details	Reference
OsDEP1 *Os09g0441900*	Chr 2	Q67UU9	Mutation, promoter	Spontaneous mutation -CRISPR /Cas9	More expression, yield increase 15 %. The interaction between DEP1 and LPA1 suppresses *PIN1a* expression, leading to an increase in planting density. The panicle number per plant was the main contributor to the increase in grains per rice plant in the DEP1 mutants.	[92,93, 116]
Gn1a *Os01g0197700* *OsCKX2*	Chr 1	Q4ADV8	Knockout	CRISPR/Cas9	Catalyzes the oxidation of cytokinin, enhanced the grain yield by increasing the grain number per panicle. Twice flowering relative to the wild type.	[78,92,94]
GS3 *Os03g0407400*	Chr 3	C6L686	Knockout	CRISPR/Cas9 -Spontaneous mutation	δ subunit of G protein. Regulator of grain size and organ size. Produces a longer grain length. Knockout and deletions produce short seeds, such as 320 bp and 13 bp deletions in the fifth exon of GS3 that occurred in a japonica-like ancestor. The 4 bp and 1 + 3 bp deletions occurred in an indica-like ancestor. Farmers and early breeders imposed artificial selection favoring short seeds	[94,95,96] b
IPA1 *Os08g0509600* Transcription factor Ideal Plant Architecture 1	Chr 8	Q7EXZ2	Knockout	CRISPR/Cas9	Squamosa promoter-binding-like protein 14. Specific mutations between bases 854 to 876 result in more protein and produce less tillering, more grains and a higher frequency of seed set. It reduces unproductive tillers and increases the number of grains per panicle, while higher IPA1 levels enhance immunity.	[78,62]
WX1 *Os06g0133000* granule-bound *starch synthase I GBSSI, OsGBSS1, waxy*	Chr 6	Q0DEV5	Knockout, mutations	CRISPR/Cas9 P124 F, R125W T178I, T178S, R158H, Y191H, R158H, G159A, D161 N, G159 K, G159A, G159E, V160 F, S415 P	Modulate the synthesis of amylose in the endosperm. Amylose contents change the appearance of the rice endosperm >12 % results in transparent endosperm/ semitranslucent (8–12 %)/or opaque (<8%). Favorable rice palatability usually requires low to intermediate AC (10–20 %). The null wax results in an absence of amylose, resulting in starch granules with 100% amylopectin production, referred to as waxy or glutenous starch. S415 P changes phosphorylation, resulting in moderate enzyme activity and a content of amylose.	[39,97,98, 99,100]
ISA1 *Os08g0520900* isoamylase 1	Chr 8	D0TZF0	Knockout	CRISPR/Cas9	Decreased endosperm contents of total starch, amylose and amylopectin. Increased soluble sugar content and starch gel consistency.	[101]
OsNAC20 *Os01g0104500* *OsNAC26* *Os01g0393100*	Chr 1	Q9FTY0 (*OsNAC20*) Q5VNK1 *(OsNAC26)*	Knockout	CRISPR/Cas9	Double knockout osnac20/26 displayed a floury grain caused by decreased starch and storage protein content. Both proteins transactivate the expression of SSI, Pul, GluA1, GluB4/5, α-globulin and 16 kD prolamin and indirectly influence DPE1 expression to regulate starch and storage protein synthesis.	[102]
GW5 *Os05g0187500* Grain Size on Chromosome 5, qSW5/GW5, GSE5	Chr 5	Q75KY5 A0A1D8GZC0	Knockout	Spontaneous mutation	*GW5* could function as a key regulator to coordinate the performance of the other grain size genes. *gw5* contributes to an increased grain width and weight. Positive regulator of brassinosteroid signaling.	[103]
GW5L *Os01g0190500* GW5L homologue of GW5	Chr 1	B8ADP5	Knockout	Spontaneous mutation	Knockout results in shorter and wider grains. Overexpression could confer salt stress resistance through an association with calmodulin protein OsCaM1−1.	[104]
GW6a *Os06g0650300* OsglHAT1, Grain weight on chromosome 6	Chr 6	Q67UR2	Over expression	Spontaneous mutation	Histone H4 acetyltransferase, regulation of grain weight, yield, and plant biomass. Elevated OsglHAT1 expression enhances the grain weight and yield. Increases global acetylation levels of histone H4.	[105,106]
GW6 *Os06g0623700* *TOTAL GRAIN WEIGHT6, total grain weight6,*	Chr 6	Q69U01	Loss of function	Spontaneous mutation	Loss of function of the Kasalath allele enhances the grain weight through pleiotropic effects on source organs and leads to significant yield increases. Encodes a protein with indole-3-acetic acid (IAA)-glucose hydrolase activity.	[107]
OsPIN5b	Chr 8	Q6ZIB5	Knockout	CRISPR	Increased panicle length in the mutant.	[58]

(*continued on next page*)

Table 4 (*continued*)

Gene	Position	Protein	Obtained mutation	Method	Trait details	Reference
Os08g0529000 *a panicle length gene* *Hd1/ SE1* *Os06g0275000*	Chr 6	Q9FDX8	Knockout	CRISPR-Cas9/ Spontaneous mutation	Zinc finger protein, Heading date. Under long day conditions suppresses HD3A/FT expression, causing the suppression of flowering.	[94,4]
HTD1 *Os04g0550600* High-Tillering Dwarf 1	Chr 4	Q7XU29	Loss of function	Spontaneous mutation/ CRISPR	Landraces contain HTD1, while domesticated rice have *htd1*. The defect in HTD1 is responsible for both high-tillering and dwarf phenotypes in the htd1 mutant. Auxin induces HTD1 expression. The protein negatively regulates the outgrowth of axillary buds and is related to strigolactones biosynthesis	[108,29]
LPA1 *Os03g0237250* Loose Plant Architecture1	Chr 3	L7PBL4	Overexpression/ Knockout	Spontaneous mutation	Plant architecture. Related to lamina inclination by suppressing auxin signaling. LPA1 is an active transcriptional repressor. Negatively controls the tiller and lamina joint angle in an expression level-dependent manner. LPA1 overexpressors contain higher levels of IAA, increases planting density and resistance to sheath blight disease via activation of PIN-FORMED 1a. Exaggerated lamina angles observed in knockout mutants (lpa1). lpa1 mutants might exhibit less efficient auxin flux.	[109]
OsMeCP *Os12g0620400* methyl-CpG binding domain protein, Methyl-CpG binding domain containing protein	Chr 12	Q0ILV0	Overexpression/ RNAi/ CRISPR Knockout	CRISPR/Cas9 knockout, Gene transfer overexpression and RNAi	Overexpression of OsMBD707 results in larger tiller angles and reduced photoperiod sensitivity.	[110]
Hd2 *Os07g0695100* Heading date 2	Chr 7	Q0D3B6	2−8bp deletion in Hd2	Hap_3 and Hap_6 mutants	Early flowering/low photosensitivity. Plants can be planted at any time of year	[111]
Ep3 *Os02g0260200* ERECT PANICLE 3,	Chr 2	G3CKN6	Mutation (knockout, recesive)	60Co Irradiated japonica cultivar Zhonghua 11, CRISPR/Cas9 knockout	Increased panicle size. Mutants modulate cytokinin level in plant tissues by down regulating cytokinin oxidase/dehydrogenase	[60,94]
Se5 *Os06g0603000* *Photosensitivity*5	Chr 6	Q69XJ4	Gamma rays	*s*73 mutant	Identified in a gamma-irradiated Bahia collection, displays early flowering and photoperiodic insensitivity due to a null mutation.	[112]
HGW *Os06g0160400 heading and grain weight, heading date- and grain weight-related protein*	Chr 6	B6TN35	Natural	Spontaneous mutation	Is a key regulator of heading date and grain weight. Encodes a protein with a UBA domain. Homozygous null mutant is embryonic lethal.	[113]

expression. The indirect mechanism results in higher expression of *DEP1* and *LPA1*, which interact to suppress *PIN1a* expression [93,92,116]. *LPA1* is also important in the erect phenotype, and its mutation results in lamina inclination [109,117].

5.6. Other traits

Other rice traits provide value for breeding and for satisfying consumer preferences, such as nitrogen use, fragrance, oleic acid content, and color. Regarding nitrogen provision, there is a better efficiency with a higher expression of the nitrate transporter *OsNPF6.1* and the two transcription factors *OsNAC42* and *OsNLP4* [118,119]. Mutation of the *FAD2* gene results in an oleic acid increment [120,121]. Furthermore, a mutation in the *Osor* (*Os02g0651300*) gene results in potential orange-colored rice [122], and the fragrance can be increased or decreased by modulating the *BADH2* gene, which prevents the formation of the aromatic compound 2AP (2-acetyl-1-pyrroline) [94]. For details, check Table 5.

6. Regulatory approaches

The traits presented in this article can result from the application of conventional or new breeding techniques, such as genome editing. It is important to note that the advance in sequencing technologies allow for a detection of mutations; however, it is unfeasible to identify the specific technique or natural cause that resulted in a mutation like a single nucleotide polymorphism or a few nucleotide variations [123,129,130]. Trying to create a legal system that differentiates between genome editing and other mutagenesis approaches or natural variations represents a challenge, given that detection is not achievable under realistic circumstances [123]. It is a challenge to regulate a product that cannot be practically distinguished once in the market, but that falls under a norm that requests such a differentiation. Such a legal norm is currently applied in Europe. A supreme court resolution on case C-528/16 enforced that the genetically modified organism (GMO) norm (Directive 2001/18/EC) is applied on genome-edited plants [124]. A recent study of the European Commission delivered to the Council of the European Union in April 2021 has collected opinions from different stakeholders and concluded that "*similar products with similar risk profiles can be obtained with conventional breeding techniques, certain genome editing*

Table 5
Rice genes and mutations in traits such as oleic acid, color, fragrancy, and nitrogen use.

Gene	Position	Protein	Obtained Mutation	Method	Trait details	Reference
FAD2 *Os02g0716500* fatty acid desaturase 2	Chr 2	Q6ZGW6	Knockout	CRISPR/Cas9- RNAi	Increased oleic acid (twice) and decreased linoleic acid content.	[120, 121]
Osor *Os02g0651300*	Chr 2	Q6H3Y3	Knockout	CRISPR/Cas9	β-carotene accumulation resulting in orange-colored calli.	[122]
BADH2 *Os08g0424500*	Chr 8	A0A0P0XG36	Knockout	CRISPR/Cas9	Betaine aldehyde dehydrogenase 2, prevents the formation of 2-acetyl-1-pyrroline (2AP), which gives fragrant rice its aromatic properties. Change in fragrance.	[94]
OsNPF6.1 *Os01g0103100* Nitrate transporter	Chr 1	Q9FTZ3	HapB, 160 Gly to Asp and two additional CACG motifs at the promoter -0.5Kb and -1kb	Natural, validation with CRISPR/CAS9 Knockout-Gene transfer	Nitrate transporter OsNPF6.1 is more efficient and has increased expression.	[118]
OsNAC42 *Os09g0493700* NUE (nitrogen use efficiency)-related transcription factor	Chr 9	Q0J0L8	Natural- Knockout	Natural, validation with CRISPR/CAS9 Knockout-lost-of- function SNP mutation (Pro51 changed to Leu, P51 L)	Transcription factor OsNAC42 related to the expression of the nitrate transporter OsNPF6.1. Loss of function decreased expression of nitrate transporter OsNPF6.1	[118]
OsNLP4 *Os09g0549450* transcriptional factor, Promotion of nitrogen use efficiency (NUE)	Chr 9	A0A0P0XQL5	Natural	Natural, HapB distributed in South China, India and South-East Asia 131 T (UTR), 181 T (UTR), 614A, 842 T, 2889C, 4662 T (UTR), 4674 T(UTR), 4888C (UTR)	The gene is upregulated by nitrogen starvation. OsNLP4 binds to the NRE motif and promotes the expression of OsNiR that encodes a critical nitrite reductase in nitrogen assimilation.	[119]

techniques and cisgenesis. It may not be justified to apply different levels of regulatory oversight to similar products with similar levels of risk" [125]. An adjustment of the GMO norms should be endorsed to correspond with such a conclusion.

The legal status of a genome editing product depends on norms established at a country level based on a discriminate process to determine whether the final product is a Living Modified Organism (LMO) or not. For countries like Argentina, Australia, Colombia, Brazil, and the United States, a variety is equivalent to conventional in the absence of a foreign DNA [126,127]. For details, see Table 6.

The legal frameworks dealing with genome editing plants currently are country-specific. Still, there is some common background in the international definitions of a Living Modified Organism (LMO) given in the Cartagena Protocol on Biosafety as "*any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology*". The keyword in defining such differentiation is "*novel combination of genetic material*," which is usually explained in legal terms as the presence of foreign DNA, as described previously.

There is an international Central America norm RT 65.06.01:18 approved by Resolution 60−2019 that provides a legally binding definition on Article 4.6 for "novel combination of genetic material," currently applied in Honduras and Guatemala. The definition states in simple words, that a new combination of genetic material means a stable insertion of DNA that could not be obtained by conventional breeding or available in nature. The procedures and information requested in both countries are aligned with the international definition and are available in decree CD-008-SENASA-2019 for Honduras and 271-MAGA chapter VI for Guatemala. This legal antecedent provides a background for comparative laws within countries with norms still in discussion. The latter is interesting because the Supreme Court of Guatemala endorsed the international standard in Case Resolution 6767−2019. For details, see Table 6.

7. Conclusion

Induced mutations targeting specific genes associated with known phenotypes, as described in this review, will allow for advances in more precise rice breeding to improve varieties that farmers are currently using. It can also result in new varieties and *de novo* domestication from wild relatives and the results can be extrapolated to other crops with homologous traits. Farmers urgently require advanced breeding to respond to the challenges of climate change, consumer demands, water scarcity, nitrogen usage, and sustainable production.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Funding

This study was financed by the Project "Producción de mutantes de arroz (*Oryza sativa*) tolerantes a herbicidas utilizando rayos gamma para contribuir con el manejo sostenible del cultivo" (1510-1022, Research Vice-Rectory of TEC, Costa Rica), and is part of the Doctoral Thesis of the first author, Doctorado en Ciencia Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia, Cartago, Costa Rica.

Authors' contributions

A.H.-S conceived the paper, designed and coordinated the inputs, analyzed the data, and wrote the manuscript; F.E.-B reviewed, discussed the content and edited the paper; A. A-E discussed the results and edited the paper; A.G.-A. wrote, reviewed, discussed the results and edited the paper; M.V.-M. discussed the results and edited the paper; J.B. reviewed, discussed the content and edited the paper. All authors read and approved the final manuscript.

Table 6
Genome Editing related norms and links.

Norm	Country	Link (Visited on May, 2021)
Court of Justice's judgment in Case C-528/16.	The EU	https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:62016CJ0528
EC study on new genomic techniques	The EU	https://ec.europa.eu/food/plant/gmo/modern_biotech/new-genomic-techniques_en
Food Hygiene Handling Procedures for Food and Additives Derived from Genome Editing Technology	Japan	https://www.mhlw.go.jp/content/000550824.pdf
RESOL-2021-21-APN-SABYDR#MAGYP	Argentine	https://www.boletinoficial.gob.ar/detalleAviso/primera/240529/20210208
Resolution 00029299	Colombia	https://www.ica.gov.co/getattachment/2d02cc52-d1c5-4123-8a5a-aea9ad2ce926/2018R29299.aspx
Secure	USA	https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/biotech-rule-revision/secure-rule/secure-about/
Resolution CTNBio-No 16	Brasil	http://ctnbio.mctic.gov.br/en/resolucoes-normativas/-/asset_publisher/OgW431Rs9dQ6/content/resolucao-normativa-nº-16-de-15-de-janeiro-de-2018
Applicability of Resolution N° 1.523/2001	Chile	http://www.sag.cl/ambitos-de-accion/aplicabilidad-de-resolucion-ndeg-15232001-en-material-de-propagacion-desarrollado-por-nuevas-tecnicas-de-fitomejoramiento
Resolution 20565-2019	Paraguay	https://conbio.mag.gov.py/media/ckfinder/files/Resolucion%20565%20de%202019.pdf
Resolution 60-2019, approving RT 65.06.01:18	Centralamerica GT-HN	https://www.sieca.int/index.php/download/resolucion-no-60-2019-aprueba-rt-65-06-0118-bioseguridad-de-organismos-vivos-para-uso-agropecuario/
271-MAGA	Guatemala	https://visar.maga.gob.gt/visar/2019/20/MANPROCT.pdf
CD-SENASA-008-2019	Honduras	http://senasa.gob.hn/images/ACD/2019/ACUERDO-CD-SENASA-008-2019%20GACETA%2035047.PDF
Supreme Court Resolution 6767-2019	Guatemala	http://138.94.255.164/Sentencias/846825.6767-2019.pdf
WTO- G/SPS/GEN/1658/Rev.3 WTO International Statement on Agricultural Applications of Precision Biotechnology WTO- G/SPS/GEN/1699	Argentina, Australia, Brazil, Canada, the Dominican Republic, Guatemala, Honduras, Paraguay, the United States of America and Uruguay.	https://docs.wto.org/dol2fe/Pages/SS/directdoc.aspx?filename=q:/G/SPS/GEN1658R3.pdf
South America Ministries of Agriculture	Argentina, Brazil, Chile, Paraguay and Uruguay	https://docs.wto.org/dol2fe/Pages/SS/directdoc.aspx?filename=q:/G/SPS/GEN1699.pdf

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

Not applicable.

References

[1] T. Matsumoto, J. Wu, H. Kanamori, Y. Katayose, M. Fujisawa, N. Namiki, H. Mizuno, K. Yamamoto, B.A. Antonio, T. Baba, K. Sakata, Y. Nagamura, H. Aoki, K. Arikawa, K. Arita, T. Bito, Y. Chiden, N. Fujitsuka, R. Fukunaka, et al., The map-based sequence of the rice genome, Nature 436 (7052) (2005) 793–800, https://doi.org/10.1038/nature03895.

[2] H. Kajiya-Kanegae, H. Ohyanagi, T. Ebata, Y. Tanizawa, A. Onogi, Y. Sawada, M. Y. Hirai, Z.X. Wang, B. Han, A. Toyoda, A. Fujiyama, H. Iwata, K. Tsuda, T. Suzuki, M. Nosaka-Takahashi, K.Ichi Nonomura, Y. Nakamura, S. Kawamoto, N. Kurata, Y. Sato, OryzaGenome2.1: database of diverse genotypes in wild *Oryza* species, Rice 14 (24) (2021), https://doi.org/10.1186/s12284-021-00468-x.

[3] Y. Kawahara, M. de la Bastide, J.P. Hamilton, H. Kanamori, W.R. Mccombie, S. Ouyang, D.C. Schwartz, T. Tanaka, J. Wu, S. Zhou, K.L. Childs, R.M. Davidson, H. Lin, L. Quesada-Ocampo, B. Vaillancourt, H. Sakai, S.S. Lee, J. Kim, H. Numa, et al., Improvement of the *Oryza sativa nipponbare* reference genome using next generation sequence and optical map data, Rice 6 (1) (2013) 3–10, https://doi.org/10.1186/1939-8433-6-4.

[4] N. Tanaka, M. Shenton, Y. Kawahara, M. Kumagai, H. Sakai, H. Kanamori, J. Yonemaru, S. Fukuoka, K. Sugimoto, M. Ishimoto, J. Wu, K. Ebana, Whole-genome sequencing of the NARO World Rice Core Collection (WRC) as the basis for diversity and association studies, Plant Cell Physiol. 61 (5) (2020) 922–932, https://doi.org/10.1093/pcp/pcaa019.

[5] F.M. Romero, A. Gatica-Arias, CRISPR/Cas9: development and application in rice breeding, Rice Sci. 26 (5) (2019) 265–281, https://doi.org/10.1016/j.rsci.2019.08.001.

[6] X. Serrat, R. Esteban, N. Guibourt, L. Moysset, S. Nogués, E. Lalanne, EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations, Plant Methods 10 (5) (2014) 1–13, https://doi.org/10.1186/1746-4811-10-5.

[7] J.D. Soriano, Mutagenic effects of gamma radiation on rice, Bot. Gaz. 123 (1) (1961) 57–63. http://www.jstor.org/stable/2473547.

[8] V.E. Viana, C. Pegoraro, C. Busanello, A. Costa de Oliveira, Mutagenesis in rice: the basis for breeding a new super plant, Front. Plant Sci. 10 (1326) (2019) 1–28, https://doi.org/10.3389/fpls.2019.01326.

[9] E. Benavente, E. Giménez, Modern approaches for the genetic improvement of rice, wheat and maize for abiotic constraints-related traits: a comparative overview, Agronomy 11 (2) (2021) 376, https://doi.org/10.3390/agronomy11020376.

[10] W. Yao, G. Li, Y. Yu, Y. Ouyang, funRiceGenes dataset for comprehensive understanding and application of rice functional genes, Gigascience 7 (1) (2018), https://doi.org/10.1093/gigascience/gix119 gix119.

[11] N.K. Bainsla, H.P. Meena, Breeding for resistance to biotic stresses., in: J.V. Yadav P, S. Kumar (Eds.), Recent Advances in Plant Stress Physiology, Daya Publishing House, 2016 https://doi.org/10.1002/9780470752708.ch22.

[12] E.C. Oerke, H.W. Dehne, Safeguarding production - losses in major crops and the role of crop protection, Crop. Prot. 23 (4) (2004) 275–285, https://doi.org/10.1016/j.cropro.2003.10.001.

[13] C. Mba, R. Afza, S. Bado, S.M. Jain, Induced mutagenesis in plants using physical and chemical agents, Plant Cell Culture: Essential Methods (March) (2010) 111–130, https://doi.org/10.1002/9780470686522.ch7.

[14] J. Gressel, A.A. Levy, Agriculture: the selector of improbable mutations, Proc. Natl. Acad. Sci. U.S.A. 103 (33) (2006) 12215–12216, https://doi.org/10.1073/pnas.0603666103.

[15] Y. Oladosu, M.Y. Rafii, N. Abdullah, G. Hussin, A. Ramli, H.A. Rahim, G. Miah, M. Usman, Principle and application of plant mutagenesis in crop improvement: a review, Biotechnol. Biotechnol. Equip. 30 (1) (2016) 1–16, https://doi.org/10.1080/13102818.2015.1087333.

[16] A. Fehér, Callus, dedifferentiation, totipotency, somatic embryogenesis: What these terms mean in the era of molecular plant biology? Front. Plant Sci. 10 (536) (2019) https://doi.org/10.3389/fpls.2019.00536.

[17] M.W. Bairu, A.O. Aremu, J. van Staden, Somaclonal variation in plants: causes and detection methods, Plant Growth Regul. 63 (2) (2011) 147–173, https://doi.org/10.1007/s10725-010-9554-x.

[18] P.J. Larkin, W.R. Scowcroft, Somaclonal variation - a novel source of variability from cell cultures for plant improvement, Theor. Appl. Genet. 60 (4) (1981) 197–214, https://doi.org/10.1007/BF02342540.

[19] P. Schindele, F. Wolter, H. Puchta, Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13, FEBS Lett. 592 (12) (2018) 1954–1967, https://doi.org/10.1002/1873-3468.13073.

[20] V. Marx, Base editing a CRISPR way, Nat. Methods 15 (10) (2018) 767–770, https://doi.org/10.1038/s41592-018-0146-4.

[21] A.V. Anzalone, P.B. Randolph, J.R. Davis, A.A. Sousa, L.W. Koblan, J.M. Levy, P. J. Chen, C. Wilson, G.A. Newby, A. Raguram, D.R. Liu, Search-and-replace genome editing without double-strand breaks or donor DNA, Nature 576 (7785) (2019) 149–157, https://doi.org/10.1038/s41586-019-1711-4.

[22] Q. Lin, Y. Zong, C. Xue, S. Wang, S. Jin, Z. Zhu, Y. Wang, A.V. Anzalone, A. Raguram, J.L. Doman, D.R. Liu, C. Gao, Prime genome editing in rice and wheat, Nat. Biotechnol. 38 (5) (2020) 582–585, https://doi.org/10.1038/s41587-020-0455-x.

[23] E. Chen, X. Huang, Z. Tian, R.A. Wing, B. Han, The genomics of *Oryza* species provides insights into rice domestication and heterosis, Annu. Rev. Plant Biol. 70 (2019) 639–665, https://doi.org/10.1146/annurev-arplant-050718-100320.

[24] K. Kim, S.C. Lee, J. Lee, Y. Yu, K. Yang, B.S. Choi, H.J. Koh, N.E. Waminal, H. Il Choi, N.H. Kim, W. Jang, H.S. Park, J. Lee, H.O. Lee, H.J. Joh, H.J. Lee, J. Y. Park, S. Perumal, M. Jayakodi, et al., Complete chloroplast and ribosomal sequences for 30 accessions elucidate evolution of *Oryza* AA genome species, Sci. Rep. 5 (15655) (2015) 1–13, https://doi.org/10.1038/srep15655.

[25] S. Nadir, H.B. Xiong, Q. Zhu, X.L. Zhang, H.Y. Xu, J. Li, W. Dongchen, D. Henry, X.Q. Guo, S. Khan, H.S. Suh, D.S. Lee, L.J. Chen, Weedy rice in sustainable rice production. A review, Agron. Sustain. Dev. 37 (46) (2017), https://doi.org/10.1007/s13593-017-0456-4.

[26] R.A. Wing, M.D. Purugganan, Q. Zhang, The rice genome revolution: from an ancient grain to Green Super Rice, Nat. Rev. Genet. 19 (8) (2018) 505–517, https://doi.org/10.1038/s41576-018-0024-z.

[27] L.F. Li, Y.L. Li, Y. Jia, A.L. Caicedo, K.M. Olsen, Signatures of adaptation in the weedy rice genome, Nat. Genet. 49 (5) (2017) 811–814, https://doi.org/10.1038/ng.3825.

[28] H. Yu, T. Lin, X. Meng, H. Du, J. Zhang, G. Liu, M. Chen, Y. Jing, L. Kou, X. Li, Q. Gao, Y. Liang, X. Liu, Z. Fan, Y. Liang, Z. Cheng, M. Chen, Z. Tian, Y. Wang, et al., A route to de novo domestication of wild allotetraploid rice, Cell 184 (5) (2021) 1156–1170, https://doi.org/10.1016/j.cell.2021.01.013, e14.

[29] E. Lacchini, E. Kiegle, M. Castellani, H. Adam, S. Jouannic, V. Gregis, M.M. Kater, CRISPR-mediated accelerated domestication of African rice landraces, PLoS One 15 (3) (2020), e0229782, https://doi.org/10.1371/journal.pone.0229782.

[30] J. Mammadov, R. Buyyarapu, S.K. Guttikonda, K. Parliament, I. Y. Abdurakhmonov, S.P. Kumpatla, Wild relatives of maize, Rice, cotton, and soybean: treasure troves for tolerance to biotic and abiotic stresses, Front. Plant Sci. 9 (886) (2018), https://doi.org/10.3389/fpls.2018.00886.

[31] I.N.B.L. Reddy, B.K. Kim, I.S. Yoon, K.H. Kim, T.R. Kwon, Salt tolerance in rice: focus on mechanisms and approaches, Rice Sci. 24 (3) (2017) 123–144, https://doi.org/10.1016/j.rsci.2016.09.004.

[32] L.J. Ferreira, V. Azevedo, J. Maroco, M.M. Oliveira, A.P. Santos, Salt tolerant and sensitive rice varieties display differential methylome flexibility under salt stress, PLoS One 10 (5) (2015) 1–19, https://doi.org/10.1371/journal.pone.0124060.

[33] P. Mishra, V. Mishra, T. Takabe, V. Rai, N.K. Singh, Elucidation of salt-tolerance metabolic pathways in contrasting rice genotypes and their segregating progenies, Plant Cell Rep. 35 (6) (2016) 1273–1286, https://doi.org/10.1007/s00299-016-1959-1.

[34] P.S.H. Sexcion, J.A. Egdane, A.M. Ismail, M.L. Dionisio-Sese, Morpho-physiological traits associated with tolerance of salinity during seedling stage in rice (*Oryza sativa* L.), Philippine Journal of Crop Science (Philippines) 34 (2) (2009) 27–37.

[35] H. Takagi, M. Tamiru, A. Abe, K. Yoshida, A. Uemura, H. Yaegashi, T. Obara, K. Oikawa, H. Utsushi, E. Kanzaki, C. Mitsuoka, S. Natsume, S. Kosugi, H. Kanzaki, H. Matsumura, N. Urasaki, S. Kamoun, R. Terauchi, MutMap accelerates breeding of a salt-tolerant rice cultivar, Nat. Biotechnol. 33 (5) (2015) 445–449, https://doi.org/10.1038/nbt.3188.

[36] M.A. Rahman, M.J. Thomson, M. Shah-E-Alam, M. de Ocampo, J. Egdane, et al., Exploring novel genetic sources of salinity tolerance in rice through molecular and physiological characterization, Ann. Bot. 117 (6) (2016) 1083–1097, https://doi.org/10.1093/aob/mcw030.

[37] R. Chen, Y. Cheng, S. Han, B. van Handel, L. Dong, et al., Whole genome sequencing and comparative transcriptome analysis of a novel seawater adapted, salt-resistant rice cultivar - *sea rice 86*, BMC Genomics 18 (1) (2017) 1–11, https://doi.org/10.1186/s12864-017-4037-3.

[38] A. Zhang, Y. Liu, F. Wang, T. Li, Z. Chen, D. Kong, J. Bi, F. Zhang, X. Luo, J. Wang, J. Tang, X. Yu, G. Liu, L. Luo, Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the *OsRR22* gene, Mol. Breed. 39 (47) (2019) 1–10, https://doi.org/10.1007/s11032-019-0954-y.

[39] C. Zhang, J. Zhu, S. Chen, X. Fan, Q. Li, Y. Lu, M. Wang, H. Yu, C. Yi, S. Tang, M. Gu, Q. Liu, *Wxlv*, the ancestral allele of rice *waxy* gene, Mol. Plant 12 (8) (2019) 1157–1166, https://doi.org/10.1016/j.molp.2019.05.011.

[40] H. Qin, Y. Li, R. Huang, Advances and challenges in the breeding of salt-tolerant rice, Int. J. Mol. Sci. 21 (21) (2020) 8385, https://doi.org/10.3390/ijms21218385.

[41] L.G. Cui, J.X. Shan, M. Shi, J.P. Gao, H.X. Lin, *DCA1* acts as a transcriptional co-activator of *DST* and contributes to drought and salt tolerance in rice, PLoS Genet. 11 (10) (2015), e1005617, https://doi.org/10.1371/journal.pgen.1005617.

[42] X. Liu, D. Wu, T. Shan, S. Xu, R. Qin, H. Li, M. Negm, D. Wu, J. Li, The trihelix transcription factor *OsGTγ-2* is involved adaption to salt stress in rice, Plant Mol. Biol. 103 (4–5) (2020) 545–560, https://doi.org/10.1007/s11103-020-01010-1.

[43] V.V. Santosh Kumar, R.K. Verma, S.K. Yadav, P. Yadav, A. Watts, M.V. Rao, V. Chinnusamy, CRISPR-Cas9 mediated genome editing of drought and salt tolerance (*OsDST*) gene in *indica* mega rice cultivar MTU1010, Physiol. Mol. Biol. Plants 26 (6) (2020) 1099–1110, https://doi.org/10.1007/s12298-020-00819-w.

[44] J. Yuan, X. Wang, Y. Zhao, N.U. Khan, Z. Zhao, Y. Zhang, X. Wen, F. Tang, F. Wang, Z. Li, Genetic basis and identification of candidate genes for salt tolerance in rice by GWAS, Sci. Rep. 10 (9958) (2020) 1–9, https://doi.org/10.1038/s41598-020-66604-7.

[45] X. Chen, Y. Wang, B. Lv, J. Li, L. Luo, S. Lu, X. Zhang, H. Ma, F. Ming, The *NAC* family transcription factor *OsNAP* confers abiotic stress response through the *ABA* pathway, Plant Cell Physiol. 55 (3) (2014) 604–619, https://doi.org/10.1093/pcp/pct204.

[46] D.K. Lee, P.J. Chung, J.S. Jeong, G. Jang, S.W. Bang, H. Jung, Y.S. Kim, S.H. Ha, Ydo Choi, J.K. Kim, The rice OsNAC6 transcription factor orchestrates multiple molecular mechanisms involving root structural adaptions and nicotianamine biosynthesis for drought tolerance, Plant Biotechnol. J. 15 (6) (2017) 754–764, https://doi.org/10.1111/pbi.12673.

[47] L. Nuñez-Muñoz, B. Vargas-Hernández, J. Hinojosa-Moya, R. Ruiz-Medrano, B. Xoconostle-Cázares, Plant drought tolerance provided through genome editing of the *trehalase* gene, Plant Signal. Behav. 16 (4) (2021), https://doi.org/10.1080/15592324.2021.1877005 e1877005-3.

[48] S. Sripinyowanich, P. Klomsakul, B. Boonburapong, T. Bangyeekhun, T. Asami, H. Gu, T. Buaboocha, S. Chadchawan, Exogenous ABA induces salt tolerance in *indica* rice (*Oryza sativa* L.): the role of *OsP5CS1* and *OsP5CR* gene expression during salt stress, Environ. Exp. Bot. 86 (2013) 94–105, https://doi.org/10.1016/j.envexpbot.2010.01.009.

[49] H. Takasaki, K. Maruyama, S. Kidokoro, Y. Ito, Y. Fujita, K. Shinozaki, K. Yamaguchi-Shinozaki, K. Nakashima, The abiotic stress-responsive NAC-type transcription factor *OsNAC5* regulates stress-inducible genes and stress tolerance in rice, Mol. Genet. Genom. 284 (3) (2010) 173–183, https://doi.org/10.1007/s00438-010-0557-0.

[50] S. Jayabalan, S. Pulipati, K. Ramasamy, D. Jaganathan, S.D. Venkatesan, G. Vijay, K. Kumari, K. Raju, G.N. Hariharan, G. Venkataraman, Analysis of genetic diversity and population structure using SSR markers and validation of a Cleavage amplified Polymorphic Sequences (CAPS) marker involving the sodium transporter *OsHKT1;5* in saline tolerant rice (*Oryza sativa* L.) landraces, Gene 713 (July) (2019) 143976, https://doi.org/10.1016/j.gene.2019.143976.

[51] X. Yin, A.K. Biswal, J. Dionora, K.M. Perdigon, C.P. Balahadia, S. Mazumdar, C. Chater, H.C. Lin, R.A. Coe, T. Kretzschmar, J.E. Gray, P.W. Quick, A. Bandyopadhyay, CRISPR-Cas9 and CRISPR-Cpf1 mediated targeting of a stomatal developmental gene EPFL9 in rice, Plant Cell Rep. 36 (5) (2017) 745–757, https://doi.org/10.1007/s00299-017-2118-z.

[52] X. Yin, A. Anand, P. Quick, A. Bandyopadhyay, Editing a stomatal developmental gene in rice with CRISPR/Cpf1., in: Yiping Qi (Ed.), Methods in Molecular Biology, Vol. 1917, Humana Press, New York, NY, 2019 https://doi.org/10.1007/978-1-4939-8991-1_19.

[53] Jing Wang, L. Zhou, H. Shi, M. Chern, H. Yu, H. Yi, M. He, J. Yin, X. Zhu, Y. Li, W. Li, J. Liu, J. Wang, X. Chen, H. Qing, Y. Wang, G. Liu, W. Wang, P. Li, et al., A single transcription factor promotes both yield and immunity in rice, Science 361 (6406) (2018) 1026–1028, https://doi.org/10.1126/science.aat7675.

[54] A. Sasaki, N. Yamaji, K. Yokosho, J.F. Ma, *Nramp5* is a major transporter responsible for manganese and cadmium uptake in rice, Plant Cell 24 (5) (2012) 2155–2167, https://doi.org/10.1105/tpc.112.096925.

[55] L. Tang, B. Mao, Y. Li, Q. Lv, L. Zhang, C. Chen, H. He, W. Wang, X. Zeng, Y. Shao, Y. Pan, Y. Hu, Y. Peng, X. Fu, H. Li, S. Xia, B. Zhao, Knockout of *OsNramp5* using the CRISPR/Cas9 system produces low Cd-accumulating *indica* rice without compromising yield, Sci. Rep. 7 (14438) (2017) 1–12, https://doi.org/10.1038/s41598-017-14832-9.

[56] J.D. Chang, S. Huang, N. Yamaji, W. Zhang, J.F. Ma, F.J. Zhao, *OsNRAMP1* transporter contributes to cadmium and manganese uptake in rice, Plant Cell Environ. 43 (10) (2020) 2476–2491, https://doi.org/10.1111/pce.13843.

[57] X.H. Liu, Y.S. Lyu, W. Yang, Z.T. Yang, S.J. Lu, J.X. Liu, A membrane-associated *NAC* transcription factor *OsNTL3* is involved in thermotolerance in rice, Plant Biotechnol. J. 18 (5) (2020) 1317–1329, https://doi.org/10.1111/pbi.13297.

[58] Y. Zeng, J. Wen, W. Zhao, Q. Wang, W. Huang, Rational improvement of rice yield and cold tolerance by editing the three genes *OsPIN5b, GS3*, and *OsMYB30* with the CRISPR–Cas9 system, Front. Plant Sci. 10 (1663) (2020) 1–13, https://doi.org/10.3389/fpls.2019.01663.

[59] A.K. Garg, J.-K. Kim, T.G. Owens, A.P. Ranwala, Ydo Choi, Lv Kochian, R.J. Wu, Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses, Proc. Natl. Acad. Sci. 99 (25) (2002), https://doi.org/10.1073/pnas.252637799, 15898 LP – 15903.

[60] H.W. Li, B.S. Zang, X.W. Deng, X.P. Wang, Overexpression of the *trehalose-6-phosphate synthase* gene *OsTPS1* enhances abiotic stress tolerance in rice, Planta 234 (5) (2011) 1007–1018, https://doi.org/10.1007/s00425-011-1458-0.

[61] M.J. Paul, A. Gonzalez-Uriarte, C.A. Griffiths, K. Hassani-Pak, The role of *trehalose 6-phosphate* in crop yield and resilience, Plant Physiol. 177 (May) (2018), https://doi.org/10.1104/pp.17.01634 pp.01634.2017.

[62] Z. Wang, X. Tian, Q. Zhao, Z. Liu, X. Li, Y. Ren, J. Tang, J. Fang, Q. Xu, Q. Bua, The E3 ligase drought hypersensitive negatively regulates cuticular wax

biosynthesis by promoting the degradation of transcription factor *ROC4* in rice, Plant Cell 30 (1) (2018) 228–244, https://doi.org/10.1105/tpc.17.00823.
[63] W. Wang, S. Peng, H. Liu, Y. Tao, J. Huang, K. Cui, L. Nie, The possibility of replacing puddled transplanted flooded rice with dry seeded rice in central China: a review, Field Crops Res. 214 (August) (2017) 310–320, https://doi.org/10.1016/j.fcr.2017.09.028.
[64] K. Shekhawat, S.S. Rathore, B.S. Chauhan, Weed management in dry direct-seeded rice: a review on challenges and opportunities for sustainable rice production, Agronomy 10 (1264) (2020) 1–19, https://doi.org/10.3390/agronomy10091264.
[65] A. Durand-Morat, L.L. Nalley, G. Thoma, The implications of red rice on food security, Glob. Food Sec. 18 (April) (2018) 62–75, https://doi.org/10.1016/j.gfs.2018.08.004.
[66] F.E. Dayan, A. Barker, R. Bough, M. Ortiz, H. Takano, S.O. Duke, Herbicide mechanisms of action and resistance., in: Comprehensive Biotechnology, 3rd edition, Vol. 4, Elsevier, 2019, https://doi.org/10.1016/B978-0-444-64046-8.00211-1.
[67] A. Forouzesh, E. Zand, S. Soufizadeh, S. Samadi Foroushani, Classification of herbicides according to chemical family for weed resistance management strategies-an update, Weed Res. 55 (4) (2015) 334–358, https://doi.org/10.1111/wre.12153.
[68] T.A. Gaines, S.O. Duke, S. Morran, C.A.G. Rigon, P.J. Tranel, Anita Küpper, F. E. Dayan, Mechanisms of evolved herbicide resistance, J. Biol. Chem. 295 (30) (2020) 10307–10330, https://doi.org/10.1074/jbc.REV120.013572.
[69] F.E. Dayan, S.O. Duke, Discovery for new herbicide sites of action by quantification of plant primary metabolite and enzyme pools, Engineering 6 (5) (2020) 509–514, https://doi.org/10.1016/j.eng.2020.03.004.
[70] V. Hoyos, G. Plaza, J.G. Vázquez-Garcia, C. Palma-Bautista, A.M. Rojano-Delgado, R. de Prado, Confirmation of multiple resistant *Chloris radiata* population, harvested in colombian rice fields, Agronomy 11 (496) (2021), https://doi.org/10.3390/agronomy11030496.
[71] M.I. Bzour, F.M. Zuki, M.S. Mispan, Introduction of imidazolinone herbicide and Clearfield® rice between weedy rice-control efficiency and environmental concerns, Environ. Rev. 26 (2) (2018) 181–198, https://doi.org/10.1139/er-2017-0096.
[72] F. Li, A. Shimizu, T. Nishio, N. Tsutsumi, H. Kato, Comparison and characterization of mutations induced by gamma-ray and carbon-ion irradiation in rice (*Oryza sativa* L.) using whole-genome resequencing, G3: Genes Genomes Genet. 9 (11) (2019) 3743–3751, https://doi.org/10.1534/g3.119.400555.
[73] V. Singh, S. Singh, H. Black, V. Boyett, S. Basu, D. Gealy, E. Gbur, A. Pereira, R. C. Scott, A. Caicedo, N.R. Burgos, Introgression of Clearfield™ rice crop traits into weedy red rice outcrosses, Field Crops Res. 207 (2017) 13–23, https://doi.org/10.1016/j.fcr.2017.03.004.
[74] J. Dauer, A. Hulting, D. Carlson, L. Mankin, J. Harden, C. Mallory-Smith, Gene flow from single and stacked herbicide-resistant rice (*Oryza sativa*): modeling occurrence of multiple herbicide-resistant weedy rice, Pest Manag. Sci. 74 (2) (2018) 348–355, https://doi.org/10.1002/ps.4711.
[75] J.R. Camacho, S.D. Linscombe, Y. Sanabria, P.A. Mosquera, J.H. Oard, Inheritance of Provisia™ rice resistance to quizalofop-p-ethyl under laboratory and greenhouse environments, Euphytica 215 (4) (2019) 83, https://doi.org/10.1007/s10681-019-2407-4.
[76] A. de Andrade, A. Tulmann-Neto, F.A. Tcacenco, R. Marschalek, A. Pereira, A. M. de Oliveira Neto, K.K. Scheuermann, E. Wickert, J.A. Noldin, Development of rice (*Oryza sativa*) lines resistant to aryloxyphenoxypropionate herbicides through induced mutation with gamma rays, Plant Breed. 137 (3) (2018) 364–369, https://doi.org/10.1111/pbr.12592.
[77] H. Lu, Q. Yu, H. Han, M.J. Owen, S.B. Powles, A novel psbA mutation (Phe274–Val) confers resistance to PSII herbicides in wild radish (*Raphanus raphanistrum*), Pest Manag. Sci. 75 (1) (2019) 144–151, https://doi.org/10.1002/ps.5079.
[78] Meiru Li, X. Li, Z. Zhou, P. Wu, M. Fang, X. Pan, Q. Lin, W. Luo, G. Wu, H. Li, Reassessment of the four yield-related genes *Gn1a, DEP1, GS3*, and *IPA1* in rice using a CRISPR/Cas9 system, Front. Plant Sci. 7 (377) (2016) 1–13, https://doi.org/10.3389/fpls.2016.00377.
[79] R.M. Pedroso, K. Al-Khatib, R. Alarcón-Reverte, A.J. Fischer, A psbA mutation (Val219 to Ile) causes resistance to propanil and increased susceptibility to bentazon in Cyperus difformis, Pest Manag. Sci. 72 (9) (2016) 1673–1680, https://doi.org/10.1002/ps.4267.
[80] J.J. Chen, S. Matsunaka, The *propanil hydrolyzing enzyme aryl acylamidase* in the wild rices of genus *Oryza*, Pestic. Biochem. Physiol. 38 (1) (1990) 26–33, https://doi.org/10.1016/0048-3575(90)90144-Q.
[81] S. Matsunaka, Propanil hydrolysis : inhibition in rice plants by insecticides, Science 160 (3834) (1967) 1360–1361, https://doi.org/10.1126/science.160.3834.1360.
[82] G. Pan, X. Zhang, K. Liu, J. Zhang, X. Wu, J. Zhu, J. Tu, Map-based cloning of a novel rice cytochrome P450 gene *CYP81A6* that confers resistance to two different classes of herbicides, Plant Mol. Biol. 61 (6) (2006) 933–943, https://doi.org/10.1007/s11103-006-0058-z.
[83] H. Saika, J. Horita, F. Taguchi-Shiobara, S. Nonaka, A. Nishizawa-Yokoi, S. Iwakami, K. Hori, T. Matsumoto, T. Tanaka, T. Itoh, M. Yano, K. Kaku, T. Shimizu, S. Toki, A novel rice cytochrome P450 gene, CYP72A31, confers tolerance to acetolactate synthase-inhibiting herbicides in rice and arabidopsis, Plant Physiol. 166 (3) (2014) 1232–1240, https://doi.org/10.1104/pp.113.231266.
[84] J. Zhang, Y. Xu, X. Wu, L. Zhu, A bentazon and sulfonylurea sensitive mutant: breeding, genetics and potential application in seed production of hybrid rice, Theor. Appl. Genet. 105 (1) (2002) 16–22, https://doi.org/10.1007/s00122-002-0874-8.
[85] W. Jiang, H. Zhou, H. Bi, M. Fromm, B. Yang, D.P. Weeks, Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice, Nucleic Acids Res. 41 (20) (2013) e188, https://doi.org/10.1093/nar/gkt780.
[86] R. Oliva, C. Ji, G. Atienza-Grande, J.C. Huguet-Tapia, A. Perez-Quintero, T. Li, J. S. Eom, C. Li, H. Nguyen, B. Liu, F. Auguy, C. Sciallano, V.T. Luu, G.S. Dossa, S. Cunnac, S.M. Schmidt, I.H. Slamet-Loedin, C. Vera Cruz, B. Szurek, et al., Broad-spectrum resistance to bacterial blight in rice using genome editing, Nat. Biotechnol. 37 (11) (2019) 1344–1350, https://doi.org/10.1038/s41587-019-0267-z.
[87] R.K. Varshney, I.D. Godwin, T. Mohapatra, J.D.G. Jones, S.R. McCouch, A SWEET solution to rice blight, Nat. Biotechnol. 37 (11) (2019) 1280–1282, https://doi.org/10.1038/s41587-019-0302-0.
[88] I.L. Quibod, G. Atieza-Grande, E.G. Oreiro, D. Palmos, M.H. Nguyen, S. T. Coronejo, E.E. Aung, C. Nugroho, V. Roman-Reyna, M.R. Burgos, P. Capistrano, S.G. Dossa, G. Onaga, C. Saloma, C.V. Cruz, R. Oliva, The Green Revolution shaped the population structure of the rice pathogen *Xanthomonas oryzae* pv. *oryzae*, ISME J. 14 (2) (2020) 492–505, https://doi.org/10.1038/s41396-019-0545-2.
[89] J.M. Lang, A.L. Pérez-Quintero, R. Koebnik, E. DuCharme, S. Sarra, H. Doucoure, I. Keita, J. Ziegle, J.M. Jacobs, R. Oliva, O. Koita, B. Szurek, V. Verdier, J. E. Leach, A pathovar of *Xanthomonas oryzae* infecting wild grasses provides insight into the evolution of pathogenicity in rice agroecosystems, Front. Plant Sci. 10 (507) (2019) 1–15, https://doi.org/10.3389/fpls.2019.00507.
[90] F. Wang, C. Wang, P. Liu, C. Lei, W. Hao, Y. Gao, Y.G. Liu, K. Zhao, Enhanced rice blast resistance by CRISPR/ Cas9-Targeted mutagenesis of the ERF transcription factor gene *OsERF922*, PLoS One 11 (4) (2016), e0154027, https://doi.org/10.1371/journal.pone.0154027.
[91] A. Macovei, N.R. Sevilla, C. Cantos, G.B. Jonson, I. Slamet-Loedin, T. Čermák, D. F. Voytas, I.R. Choi, P. Chadha-Mohanty, Novel alleles of rice *eIF4G* generated by CRISPR/Cas9-targeted mutagenesis confer resistance to Rice tungro spherical virus, Plant Biotechnol. J. 16 (11) (2018) 1918–1927, https://doi.org/10.1111/pbi.12927.
[92] Liyu Huang, R. Zhang, G. Huang, Y. Li, G. Melaku, S. Zhang, H. Chen, Y. Zhao, J. Zhang, Y. Zhang, F. Hu, Developing superior alleles of yield genes in rice by artificial mutagenesis using the CRISPR/Cas9 system, Crop J. 6 (5) (2018) 475–481, https://doi.org/10.1016/j.cj.2018.05.005.
[93] X. Fu, J. Xu, M. Zhou, M. Chen, L. Shen, T. Li, Y. Zhu, J. Wang, J. Hu, L. Zhu, Z. Gao, G. Dong, L. Guo, D. Ren, G. Chen, J. Lin, Q. Qian, G. Zhang, Enhanced expression of *QTL qLL9/DEP1* facilitates the improvement of leaf morphology and grain yield in rice, Int. J. Mol. Sci. 20 (866) (2019), https://doi.org/10.3390/ijms20040866.
[94] L. Shen, Y. Hua, Y. Fu, J. Li, Q. Liu, X. Jiao, G. Xin, J. Wang, X. Wang, C. Yan, K. Wang, Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice, Sci. China Life Sci. 60 (5) (2017) 506–515, https://doi.org/10.1007/s11427-017-9008-8.
[95] N. Takano-Kai, H. Jiang, A. Powell, S. McCouch, I. Takamure, N. Furuya, K. Doi, A. Yoshimura, Multiple and independent origins of short seeded alleles of *GS3* in rice, Breed. Sci. 63 (1) (2013) 77–85, https://doi.org/10.1270/jsbbs.63.77.
[96] L. Yang, W. Yueying, N. Jahan, H. Haitao, C. Ping, S. Lianguang, L. Haiyan, D. Guojun, H. Jiang, G. Zhenyu, Q. Qian, Z. Yu, G. Longbiao, Genome-wide association analysis and allelic mining of grain shape-related traits in rice, Rice Sci. 26 (6) (2019) 384–392, https://doi.org/10.1016/j.rsci.2018.09.002.
[97] Y. Sano, Differential regulation of *waxy* gene expression in rice endosperm, Theor. Appl. Genet. 68 (5) (1984) 467–473, https://doi.org/10.1007/BF00254822.
[98] F. Yunyan, Y. Jie, W. Fangquan, F. Fangjun, L. Wenqi, W. Jun, X. Yang, Z. Jinyan, Z. Weigong, Production of two elite glutinous rice varieties by editing *wx* gene, Rice Sci. 26 (2) (2019) 118–124, https://doi.org/10.1016/j.rsci.2018.04.007.
[99] Lichun Huang, N. Sreenivasulu, Q. Liu, Waxy editing: old meets new, Trends Plant Sci. 25 (10) (2020) 963–966, https://doi.org/10.1016/j.tplants.2020.07.009.
[100] Y. Xu, Q. Lin, X. Li, F. Wang, Z. Chen, J. Wang, W. Li, F. Fan, Y. Tao, Y. Jiang, X. Wei, R. Zhang, Q.H. Zhu, Q. Bu, J. Yang, C. Gao, Fine-tuning the amylose content of rice by precise base editing of the *Wx* gene, Plant Biotechnol. J. 19 (1) (2021) 11–13, https://doi.org/10.1111/pbi.13433.
[101] C. Shufen, C. Yicong, F. Baobing, J. Guiai, S. Zhonghua, L. Ju, T. Shaoqing, W. Jianlong, H. Peisong, W. Xiangjin, Editing of rice isoamylase gene ISA1 provides insights into its function in starch formation, Rice Sci. 26 (2) (2019) 77–87, https://doi.org/10.1016/j.rsci.2018.07.001.
[102] Juan Wang, Z. Chen, Q. Zhang, S. Meng, C. Wei, The NAC transcription factors *OsNAC20* and *OsNAC26* regulate starch and storage protein synthesis, Plant Physiol. 184 (4) (2020) 1775–1791, https://doi.org/10.1104/pp.20.00984.
[103] L. Zhang, B. Ma, Z. Bian, X. Li, C. Zhang, J. Liu, Q. Li, Q. Liu, Z. He, Grain size selection using novel functional markers targeting 14 genes in rice, Rice 13 (1) (2020) 1–16, https://doi.org/10.1186/s12284-020-00427-y.
[104] P. Tian, J. Liu, C. Mou, C. Shi, H. Zhang, Z. Zhao, Q. Lin, J. Wang, J. Wang, X. Zhang, X. Guo, Z. Cheng, S. Zhu, Y. Ren, C. Lei, H. Wang, J. Wan, *GW5-Like*, a homolog of *GW5*, negatively regulates grain width, weight and salt resistance in rice, J. Integr. Plant Biol. 61 (11) (2019) 1171–1185, https://doi.org/10.1111/jipb.12745.
[105] X.J. Song, T. Kuroha, M. Ayano, T. Furuta, K. Nagai, N. Komeda, S. Segami, K. Miura, D. Ogawa, T. Kamura, T. Suzuki, T. Higashiyama, M. Yamasaki, H. Mori, Y. Inukai, J. Wu, H. Kitano, H. Sakakibara, S.E. Jacobsen, M. Ashikari, Rare allele of a previously unidentified histone *H4 acetyltransferase* enhances

grain weight, yield, and plant biomass in rice, Proc. Natl. Acad. Sci. U.S.A. 112 (1) (2015) 76–81, https://doi.org/10.1073/pnas.1421127112.
[106] M. Ayaad, Z. Han, K. Zheng, G. Hu, M. Abo-Yousef, S.E.S. Sobeih, Y. Xing, Bin-based genome-wide association studies reveal superior alleles for improvement of appearance quality using a 4-way MAGIC population in rice, J. Adv. Res. 28 (2021) 183–194, https://doi.org/10.1016/j.jare.2020.08.001.
[107] K. Ishimaru, N. Hirotsu, Y. Madoka, N. Murakami, N. Hara, H. Onodera, T. Kashiwagi, K. Ujiie, B.I. Shimizu, A. Onishi, H. Miyagawa, E. Katoh, Loss of function of the *IAA*-glucose hydrolase gene *TGW6* enhances rice grain weight and increases yield, Nat. Genet. 45 (6) (2013) 707–711, https://doi.org/10.1038/ng.2612.
[108] J. Zou, S. Zhang, W. Zhang, G. Li, Z. Chen, W. Zhai, X. Zhao, X. Pan, Q. Xie, L. Zhu, The rice *HIGH-TILLERING DWARF1* encoding an ortholog of Arabidopsis *MAX3* is required for negative regulation of the outgrowth of axillary buds, Plant J. 48 (5) (2006) 687–698, https://doi.org/10.1111/j.1365-313X.2006.02916.x.
[109] J.M. Liu, S.J. Park, J. Huang, E.J. Lee, Y.H. Xuan, Bil Je, V. Kumar, R.A. Priatama, K.V. Raj, S.H. Kim, M.K. Min, J.H. Cho, T.H. Kim, A.K.N. Chandran, K.H. Jung, S. Takatsuto, S. Fujioka, C.D. Han, *Loose Plant Architecture1* (*LPA1*) determines lamina joint bending by suppressing auxin signalling that interacts with C-22-hydroxylated and 6-deoxo brassinosteroids in rice, J. Exp. Bot. 67 (6) (2016) 1883–1895, https://doi.org/10.1093/jxb/erw002.
[110] M. Qu, Z. Zhang, T. Liang, P. Niu, M. Wu, W. Chi, Z.Q. Chen, Z.J. Chen, S. Zhang, S. Chen, Overexpression of a methyl-CpG-binding protein gene *OsMBD707* leads to larger tiller angles and reduced photoperiod sensitivity in rice, BMC Plant Biol. 21 (1) (2021) 1–14, https://doi.org/10.1186/s12870-021-02880-3.
[111] H. Gao, M. Jin, X.M. Zheng, J. Chen, D. Yuan, Y. Xin, M. Wang, D. Huang, Z. Zhang, K. Zhou, P. Sheng, J. Ma, W. Ma, H. Deng, L. Jiang, S. Liu, H. Wang, C. Wu, L. Yuan, J. Wan, Days to *heading 7*, a major quantitative locus determining photoperiod sensitivity and regional adaptation in rice, Proc. Natl. Acad. Sci. U.S.A. 111 (51) (2014) 18399–18400, https://doi.org/10.1073/pnas.1422341112.
[112] F. Andrés, D.W. Galbraith, M. Talón, C. Domingo, Analysis of *Photoperiod Sensitivity5* sheds light on the role of phytochromes in photoperiodic flowering in rice, Plant Physiol. 151 (2) (2009) 681–690, https://doi.org/10.1104/pp.109.139097.
[113] J. Li, H. Chu, Y. Zhang, T. Mou, C. Wu, Q. Zhang, J. Xu, The rice *HGW* gene encodes a *ubiquitin-associated (UBA)* domain protein that regulates heading date and grain weight, PLoS One 7 (3) (2012) e34231, https://doi.org/10.1371/journal.pone.0034231.
[114] H. Mao, S. Sun, J. Yao, C. Wang, S. Yu, C. Xu, X. Li, Q. Zhang, Linking differential domain functions of the *GS3* protein to natural variation of grain size in rice, Proc. Natl. Acad. Sci. U.S.A. 107 (45) (2010) 19579–19584, https://doi.org/10.1073/pnas.1014419107.
[115] Ming Li, D. Tang, K. Wang, X. Wu, L. Lu, H. Yu, M. Gu, C. Yan, Z. Cheng, Mutations in the F-box gene LARGER PANICLE improve the panicle architecture and enhance the grain yield in rice, Plant Biotechnol. J. 9 (9) (2011) 1002–1013, https://doi.org/10.1111/j.1467-7652.2011.00610.x.
[116] J. Miao Liu, Q. Mei, C. Yun Xue, Z. Yuan Wang, D. Pin Li, Y. Xin Zhang, Y. Hu Xuan, Mutation of G-protein γ subunit DEP1 increases planting density and resistance to sheath blight disease in rice, Plant Biotechnol. J. 19 (3) (2021) 418–420, https://doi.org/10.1111/pbi.13500.
[117] X. Mao, Y. Zheng, K. Xiao, Y. Wei, Y. Zhu, Q. Cai, L. Chen, H. Xie, J. Zhang, OsPRX2 contributes to stomatal closure and improves potassium deficiency tolerance in rice, Biochem. Biophys. Res. Commun. 495 (1) (2018) 461–467, https://doi.org/10.1016/j.bbrc.2017.11.045.
[118] W. Tang, J. Ye, X. Yao, P. Zhao, W. Xuan, Y. Tian, Y. Zhang, S. Xu, H. An, G. Chen, J. Yu, W. Wu, Y. Ge, X. Liu, J. Li, H. Zhang, Y. Zhao, B. Yang, X. Jiang, et al., Genome-wide associated study identifies NAC42-activated nitrate transporter conferring high nitrogen use efficiency in rice, Nat. Commun. 10 (5279) (2019) 1–11, https://doi.org/10.1038/s41467-019-13187-1.
[119] J. Yu, W. Xuan, Y. Tian, L. Fan, J. Sun, W. Tang, G. Chen, B. Wang, Y. Liu, W. Wu, X. Liu, X. Jiang, C. Zhou, Z. Dai, D. Xu, C. Wang, J. Wan, Enhanced *OsNLP4-OsNiR* cascade confers nitrogen use efficiency by promoting tiller number in rice, Plant Biotechnol. J. 19 (1) (2021) 167–176, https://doi.org/10.1111/pbi.13450.
[120] K. Abe, E. Araki, Y. Suzuki, S. Toki, H. Saika, Production of high oleic/low linoleic rice by genome editing, Plant Physiol. Biochem. 131 (2018) 58–62, https://doi.org/10.1016/j.plaphy.2018.04.033.
[121] G.J. Tiwari, Q. Liu, P. Shreshtha, Z. Li, S. Rahman, RNAi-mediated down-regulation of the expression of OsFAD2-1: effect on lipid accumulation and expression of lipid biosynthetic genes in the rice grain, BMC Plant Biol. 16 (189) (2016), https://doi.org/10.1186/s12870-016-0881-6.
[122] A. Endo, H. Saika, M. Takemura, N. Misawa, S. Toki, A novel approach to carotenoid accumulation in rice callus by mimicking the cauliflower Orange mutation via genome editing, Rice 12 (1) (2019) 81, https://doi.org/10.1186/s12284-019-0345-3.
[123] H. Emons, W. Broothaerts, L. Bonfini, P. Corbisier, F. Gatto, et al., Challenges for the Detection of Genetically Modified Food or Feed Originating From Genome Editing, Publications Office of the European Union, Luxembourg, 2018.
[124] K.P. Purnhagen, E. Kok, G. Kleter, H. Schebesta, R.G.F. Visser, J. Wesseler, EU court casts new plant breeding techniques into regulatory limbo, Nat. Biotechnol. 36 (9) (2018) 799–800, https://doi.org/10.1038/nbt.4251.
[125] European Commission (EC), Study on the status of new genomic techniques under Union law and in light of the Court of justice ruling in case C-528/16. Commission Staff Working Document, 2021. Vol. SWD(2021), https://ec.europa.eu/food/sites/food/files/plant/docs/gmo_mod-bio_ngt_eu-study.pdf.
[126] J. Entine, D. Eriksson, M.S. Felipe, J.-H. Groenewald, D.L. Kershen, M. Lema, A. McHughen, A.L. Nepomuceno, R. Ohsawa, R.L. Ordonio, W.A. Parrott, K. P. Purnhagen, H. Quemada, C. Ramage, I. Slamet-Loedin, S.J. Smyth, M. S. Wasmer, D. Wray-Cahen, Regulatory approaches for genome edited agricultural plants in select countries and jurisdictions around the world, Transgenic Res. 8 (2021), https://doi.org/10.1007/s11248-021-00257-8.
[127] A. Gatica-Arias, The regulatory current status of plant breeding technologies in some Latin American and the Caribbean countries, Plant Cell Tissue Organ Cult. 141 (2) (2020) 229–242, https://doi.org/10.1007/s11240-020-01799-1.
[128] M. Ashraf, H.R. Athar, P.J.C. Harris, T.R. Kwon, Some prospective strategies for improving crop salt tolerance. Advances in Agronomy, Academic Press, 2008, pp. 45–110.
[129] W. Broothaerts, S. Jacchia, A. Angers, M. Petrillo, M. Querci, et al., New Genomic Techniques : State-of-the-Art Review, Publications Office of the European Union, Luxembourg, 2021.
[130] ENGL, Detection of food and feed plant products obtained by new mutagenesis techniques, 2019. JRC116289(March): 21, https://gmo-crl.jrc.ec.europa.eu/doc/JRC116289-GE-report-ENGL.pdf.
[131] K. Kawai, K. Kaku, N. Izawa, M. Shimizu, H. Kobayashi, et al., Herbicide sensitivities of mutated enzymes expressed from artificially generated genes of acetolactate synthase, J. Pestic. Sci. 33 (2) (2008) 128–137, https://doi.org/10.1584/jpestics.G07-29.
[132] H. Li, J. Li, J. Chen, L. Yan, L. Xia, Precise Modifications of Both Exogenous and Endogenous Genes in Rice by Prime Editing, Mol. Plant 13 (5) (2020) 671–674, https://doi.org/10.1016/j.molp.2020.03.011.
[133] C. Li, R. Zhang, X. Meng, S. Chen, Y. Zong, et al., Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors, Nat. Biotechnol. 38 (7) (2020) 875–882, https://doi.org/10.1038/s41587-019-0393-7.
[134] C. Li, Y. Zong, Y. Wang, S. Jin, D. Zhang, et al., Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion, Genome Biol. 19 (1) (2018) 1–9, https://doi.org/10.1186/s13059-018-1443-z.
[135] H. Maeda, K. Murata, N. Sakuma, S. Takei, A. Yamazaki, et al., A rice gene that confers broad-spectrum resistance to b-triketone herbicides, Science 365 (6451) (2019) 393–396, https://doi.org/10.1126/science.aax0379.
[136] D.B. Sagare, R. Abbai, A. Jain, P.K. Jayadevappa, S. Dixit, More and more of less and less: Is genomics-based breeding of dry direct-seeded rice (DDSR) varieties the need of hour? Plant Biotechnol. J. (2020) 2173–2186, https://doi.org/10.1111/pbi.13454.
[137] Z. Shimatani, S. Kashojiya, M. Takayama, R. Terada, T. Arazoe, et al., Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion, Nat. Biotechnol. 35 (5) (2017) 441–443, https://doi.org/10.1038/nbt.3833.
[138] Y. Sun, X. Zhang, C. Wu, Y. He, Y. Ma, et al., Engineering Herbicide-Resistant Rice Plants through CRISPR/Cas9-Mediated Homologous Recombination of Acetolactate Synthase, Mol. Plant 9 (4) (2016) 628–631, https://doi.org/10.1016/j.molp.2016.01.001.
[139] W. Xu, C. Zhang, Y. Yang, S. Zhao, G. Kang, et al., Versatile Nucleotides Substitution in Plant Using an Improved Prime Editing System, Mol. Plant 13 (5) (2020) 675–678, https://doi.org/10.1016/j.molp.2020.03.012.
[140] L. Liu, Y. Kuang, F. Yan, S. Li, B. Ren, et al., Developing a novel artificial rice germplasm for dinitroaniline herbicide resistance by base editing of OsTubA2, Plant Biotechnol. J. 19 (1) (2021) 5–7, https://doi.org/10.1111/pbi.13430.
[141] S.K. Sun, X. Xu, Z. Tang, Z. Tang, X.Y. Huang, et al., A molecular switch in sulfur metabolism to reduce arsenic and enrich selenium in rice grain, Nat. Commun. 12 (1) (2021) 1–14, https://doi.org/10.1038/s41467-021-21282-5.
[142] X. Liu, R. Qin, J. Li, S. Liao, T. Shan, et al., A CRISPR-Cas9-mediated domain-specific base-editing screen enables functional assessment of ACCase variants in rice, Plant Biotechnol. J. 18 (9) (2020) 1845–1847, https://doi.org/10.1111/pbi.13348.

plants

MDPI

Article

A Temporary Immersion System Improves Regeneration of In Vitro Irradiated Recalcitrant Indica Rice (*Oryza sativa* L.) Embryogenic Calli

Alejandro Hernández-Soto [1,2,*], Jason Pérez [2], Rebeca Fait-Zúñiga [2], Randall Rojas-Vásquez [3,4,5], Andrés Gatica-Arias [3,6], Walter Vargas-Segura [7] and Ana Abdelnour-Esquivel [2]

1 Doctorado en Ciencias Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia, Cartago P.O. Box 159-7050, Costa Rica
2 Biotechnology Research Center, Biology School, Costa Rica Institute of Technology, Cartago P.O. Box 159-7050, Costa Rica; jasperez@itcr.ac.cr (J.P.); rebe.fait@gmail.com (R.F.-Z.); aabdelnour@itcr.ac.cr (A.A.-E.)
3 Plant Biotechnology Laboratory, School of Biology, University of Costa Rica, San José P.O. Box 2060, Costa Rica; randallrojas16@gmail.com (R.R.-V.); andres.gatica@ucr.ac.cr (A.G.-A.)
4 Programa de Posgrado en Ciencias Agrícolas y Recursos Naturales (PPCARN), School of Agronomy, University of Costa Rica, San José P.O. Box 2060, Costa Rica
5 Vitroflora Labs S.A., Alajuela 20701, Costa Rica
6 Programa de Posgrado en Biología (PPB), School of Biology, University of Costa Rica, San José P.O. Box 2060, Costa Rica
7 Gamma Irradiation Laboratory, School of Physics, Costa Rica Institute of Technology, Cartago P.O. Box 159-7050, Costa Rica; walvargas@tec.ac.cr
* Correspondence: alhernandez@tec.ac.cr

Citation: Hernández-Soto, A.; Pérez, J.; Fait-Zúñiga, R.; Rojas-Vásquez, R.; Gatica-Arias, A.; Vargas-Segura, W.; Abdelnour-Esquivel, A. A Temporary Immersion System Improves Regeneration of In Vitro Irradiated Recalcitrant Indica Rice (*Oryza sativa* L.) Embryogenic Calli. *Plants* **2022**, *11*, 375. https://doi.org/10.3390/plants11030375

Academic Editor: Iyyakkannu Sivanesan

Received: 23 December 2021
Accepted: 26 January 2022
Published: 29 January 2022

Abstract: The development of gamma ray-mutated rice lines is a solution for introducing genetic variability in indica rice varieties already being used by farmers. In vitro gamma ray (^{60}Co) mutagenesis reduces chimeras and allows for a faster selection of desirable traits but requires the optimization of the laboratory procedure. The objectives of the present work were sequencing of *mat*K and *rbc*L, the in vitro establishment of recalcitrant rice embryogenic calli, the determination of their sensitivity to gamma radiation, and optimization of the generation procedure. All sequenced genes matched perfectly with previously reported *mat*K and *rbc*L *O. sativa* genes. Embryogenic calli induction improved using MS medium containing 2 mg L^{-1} 2,4-D, and regeneration was achieved with MS medium with 3 mg L^{-1} BA and 0.5 mg L^{-1} NAA. The optimized radiation condition was 60 Gy, (LD20 = 64 Gy) with 83% regeneration. An immersion system (RITA®, Saint-Mathieu-de-Tréviers, France) of either 60 or 120 s every 8 h allowed systematic and homogeneous total regeneration of the recalcitrant line. Other well-known recalcitrant cultivars, CR1821 and CR1113, also had improved regeneration in the immersion system. To our knowledge, this is the first study reporting the use of an immersion system to allow for the regeneration of gamma-ray mutants from recalcitrant indica rice materials.

Keywords: somatic embryogenesis; Cobalt-60; radiation-induced mutagenesis; temporary immersion systems (TIS)

1. Introduction

Rice is an important cereal that provides 20% of the world's energy, particularly in Asia, Africa, and Latin America [1]. The *Oryza* genus consists of 22 species, but only two are commonly planted, namely, *O. sativa* and *O. glaberrima* [2–6]. Farmers prefer only a few cultivars, depending on the country. The limited genetic variability of commercial materials can become an obstacle in increasing productivity given emerging conditions such as heat, salt stress, soil acidification, plague sensitivity, and weeds. Introducing variability with crossbreeding is slow and can result in the introduction of undesired traits.

Radiation methods to generate variability in seeds are standard techniques used since 1928 on vegetables, while other methods exist, such as ethyl methanesulfonate (EMS), and new breeding techniques to introduce specific genetically engineered mutations [7–12].

Plant-tissue culture represents an opportunity to overcome time limitations, land requirements, and the selection of the desired variability that results when using gamma-ray mutagenesis in seeds. Once irradiated, the seeds must be planted several times from M0 = seeds prior to mutagenic treatment; M1 = plants produced from material treated with the mutagen, to subsequent generations termed M2, M3, M4, to avoid chimeras and heterogenicity until exposed to stress-selection conditions, as has been done in the past [6,8]. In contrast, rice-tissue culture can produce a primitive cell aggregate or calli with embryogenic potential and consequently mutate and regenerate from one or a few cells with stressor selection from the beginning, such as NaCl or herbicide [11,13,14].

The latter is possible because plant cells are totipotent, which means that whole plants can develop from single cells [15]. We previously reported embryogenic calli mutation using gamma radiation for the Costa Rican cultivar CR-5272 for salt and drought tolerance; however, farmers no longer use this cultivar and instead use modern materials recalcitrant to tissue culture [14]. The establishment of embryogenic rice calli is influenced by the germplasm of origin and the 2,4-D concentration [15,16]. Embryogenic cells result when exposed to Murashige and Skoog (MS) medium supplemented with 2 to 2.5 mg L^{-1} 2,4-D, resulting in pro-embryos and somatic embryos [17–19]. Costa Rican cultivars such as CR-201, CR-1707, CR-1821, CR-8334, and CR-8341 have unpredictable and variable behavior, while CR-1113 and CR-5272 have predictable induction and regeneration in 2.5 mg L^{-1} 2,4-D [20].

Here, we faced three challenges. First, the identification of Lazarroz FL rice line with molecular markers, the chloroplast maturase gene K (*matK*) and ribulose-1,5-bisphosphate carboxylase small/large subunit (*rbcL*). Second, the improvement of our plant-tissue culture methods. Third, determining the radiosensitivity of embryogenic calli and further plant regeneration. We present a simple method to induce mutations using gamma rays in embryogenic calli of a recalcitrant cultivar, with an alternative immersion method that allowed our material to generate homogeneous and predictable in vitro plants after irradiation.

2. Results

2.1. Molecular Markers Used for the Identification of the Rice Cultivars

The chloroplast maturase gene K (*mat*K) and ribulose-1,5-bisphosphate carboxylase small/large subunit (*rbc*L), MZ558335 and MZ558334 sequences of Lazarroz FL showed perfect matches with the already published NCBI *Oryza sativa* indica demonstrated the identity of the non-irradiated material as expected. We detected three synonymous mutations in the *rbc*L sequence that are important for the characterization of the variety (Figure 1). Specifically, one synonymous SNP on the sixth glutamic acid triplet (GAA/GAG) indicates a putative origin of the germplasm ancestors from the Southeast Asia region because of its unique presence and matches with three cultivars of the region: the Pakistan cultivar NARC 17958 (GenBank KP827660.1), the Indonesia cultivar Pandak Kembang (GenBank MZ198248) and the Vietnam cultivar "Lua Khau Ky" isolate GBVN15800 (GenBank KR073275.1). We also detected two synonymous biallelic mutations at glycine 82 and 150 codifying triplets (GGC/GGA; GGT/GGC), which helped characterize and further identify the material. None of the mutations suggested biological importance since the open reading frame remained unaltered.

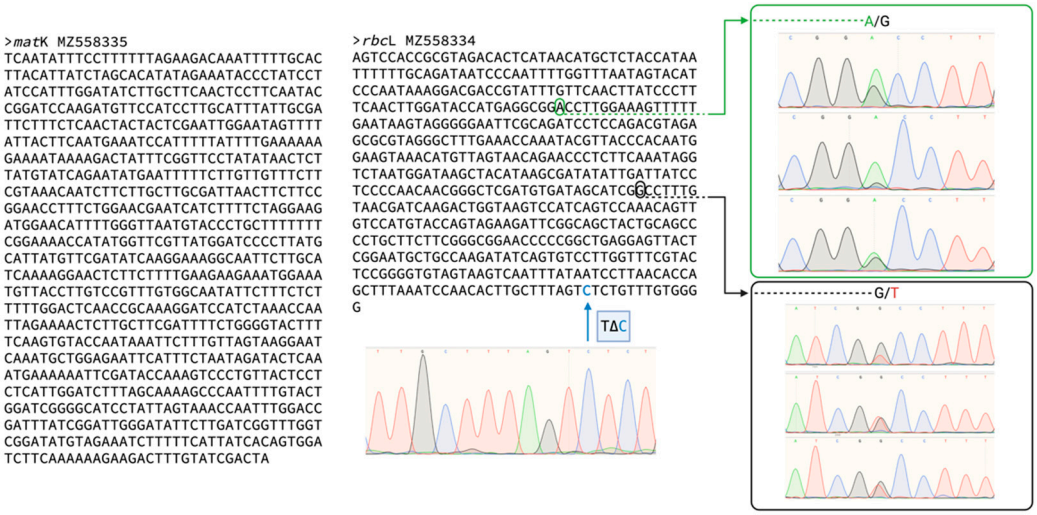

Figure 1. DNA markers used to identify the *mat*K and *rbc*L genes in non-irradiated Lazarroz FL rice variety. Note in green the synonymous SNP (C/T) and the biallelic synonymous mutations A/G and G/T (circled). None of the mutations had biological importance but helped in the genetic characterization of the cultivars.

2.2. Embryogenic Calli Induction

The embryogenic calli induction in Lazarroz FL was affected by the combination of the plant growth regulators used. Thus, induction percentages ranging from 12.77 to 71.44 were obtained, with significant differences between all treatments. The higher calli induction percentage (71.44) was achieved with 2 mg L^{-1} 2,4-D alone (Table 1). In our case, the positive induction at 2 mg L^{-1} of 2,4-D contrasted with a higher brown callus rate (3.55%) and although it is a small value, for the next steps of regeneration represented a challenge and particularly in our final irradiation goal, which also triggers browning (Table 1).

Table 1. Rice calli induction and browning rate from different induction treatments.

Treatment [1]	id	Embryogenic Calli (%)	Browning Rate (%)
2.5 mg L^{-1} 2,4-D	i	21.44 b	2.00 b
2.0 mg L^{-1} 2,4-D	ii	71.44 a	3.55 a
1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA	iii	12.77 d	0.66 c
2.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA	iv	16.77 c	0.21 c
2.0 mg L^{-1} 2,4-D + 0.25 mg L^{-1} TDZ	v	23.00 b	0.66 c

[1] All treatments had 30 replicates of 30 seeds per replicate (n = 900). Letters represent a significant difference ($p \leq 0.05$).

Rice embryogenic calli obtained from induction medium supplemented with 2 mg L^{-1} were produced after 15 days of culture from the scutellum of mature zygotic embryos (Figure 2A) and were composed of yellow friable aggregates (Figure 2B).

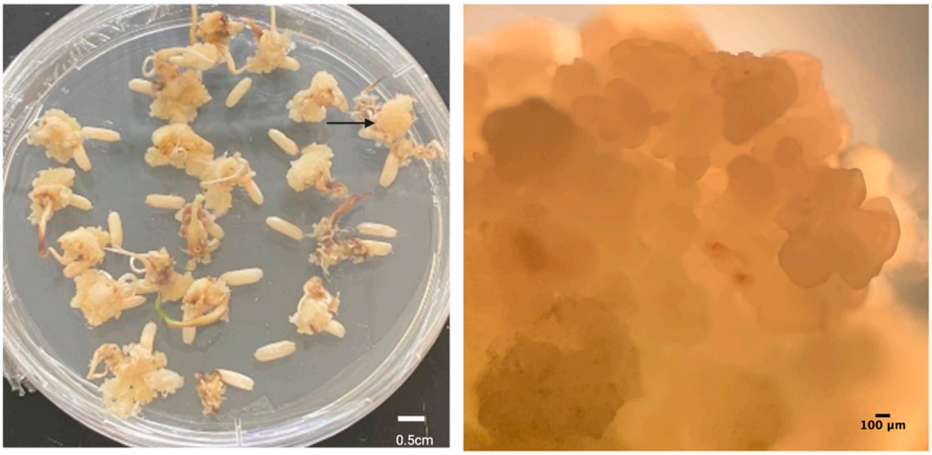

Figure 2. Calli induction of Lazarroz FL cultivar on MS medium with 2 mg L^{-1} 2,4-D after 15 days of culture under darkness. (**A**) Embryogenic calli obtained from the scutellum of mature zygotic embryos (arrow) (**B**) the compact and friable calli as observed under the stereoscope.

2.3. Regeneration on Semisolid Medium

The best regeneration rate of approximately 70% resulted from 0.5 mg of NAA+ 3 mg of 6-BA, with sprouting of 7.14% and browning of only 9.52%, for calli induced on 2 mg L^{-1} 2,4-D (Table 2). Other regeneration medium recipes also resulted in regeneration but with a higher browning and lower sprouting rate, and were consequently useless for our next step, gamma radiation mutagenesis.

Table 2. Rice calli response of Lazarroz FL cultivar after 4 weeks of culture on different regeneration media.

Induction Treatment [1]	Regeneration Treatment	Regeneration (%)	Sprouting (%)	Browning (%)
2 mg 2,4-D	0.5 mg NAA + 3 mg BA	69.04 a	7.14 ab	9.52 d
	0.5 mg NAA + 0.5 mg TDZ	38.09 c	2.38 b	61.90 a
	0.5 mg NAA + 0.5 mg Kinetin	47.61 b	9.52 a	23.80 c
	0.5 mg NAA + 0.5 mg BA	28.57 d	2.38 b	54.76 b
1 mg BA + 2 mg 2,4-D	0.5 mg NAA + 3 mg BA	28.29 b	0 a	58.43 b
	0.5 mg NAA + 0.25 mg TDZ	58.82 a	0 a	100 a
	0.5 mg NAA + 0.5 mg Kinetin	9.22 c	0 a	56.81 b
	0.5 mg NAA + 0.5 mg BA	12.82 c	0 a	44.26 c
1 mg BA + 1 mg 2,4-D	0.5 mg de NAA + 3 mg BA	58.45 ab	16.38 a	18. 69 bc
	0.5 mg de NAA + 0.5 mg TDZ	61.75 a	10.71 ab	27.93 a
	0.5 mg de NAA + 0.5 mg Kinetin	56.31 ab	15.92 a	20.01 ab
	0.5 mg de NAA + 0.5 mg BA	49.88 b	4.16 b	11.66 c
	0.5 mg de NAA + 3 mg BA	58.45 ab	16.38 a	18. 69 bc
2.5 mg 2,4-D	0.5 mg NAA + 3 mg BA	34.64 b	0 b	9.20 c
	0.5 mg NAA + 0.5 mg TDZ	51.41 a	9.61 a	22.96 a
	0.5 mg NAA + 0.5 mg Kinetin	43.62 ab	0 b	15.73 b
	0.5 mg NAA + 0.5 mg BA	18.00 c	0 b	15.19 b
2 mg 2,4-D + 0.25 mg TDZ	0.5 mg NAA + 3 mg BA	77.27 a	2.27 a	96.59 a
	0.5 mg NAA + 0.5 mg TDZ	50.25 b	0 a	86.36 b
	0.5 mg NAA + 0.5 mg Kinetin	73.86 a	3.40 a	82.95 b
	0.5 mg NAA + 0.5 mg BA	44.29 b	0 a	72.81 c

[1] All treatments had 6 replicates of 7 calli each replicate (n = 42). Letters represent significant differences for Tukey's test ($p \leq 0.05$) and the comparisons were made between treatments from same induction medium.

2.4. Gamma Radiation Mutagenesis

The effect of cobalt-60 (^{60}Co) gamma radiation on embryogenic indica rice calli was evaluated. A lethal dose (LD50) of the embryogenic calli was found to be 110 Gy, while the

20% lethal dose was 64 Gy, resulting in a 0 to 120 gray gradient exposure, with 200 calli per exposure (Table 3).

Table 3. Lethal effect of gamma radiation on the embryogenic rice calli of Lazarroz FL cultivar after 30 days of culture on regeneration medium, determined by probit model of survival (%) [1].

Lethal Gamma Rays Dose Model	Dose (Gy)	Lower Limit (Gy)	Upper Limit (Gy)
LD10	41.145	34.552	46.708
LD20	64.799	60.083	69.20
LD25	73.785	69.388	78.139
LD30	81.855	77.507	86.403
LD40	96.429	91.649	101.85
LD50	110.050	104.435	116.720

[1] All treatments had 10 replicates with 20 calli each (n = 200), $p \leq 0.05$. Data were compiled at 30 days post-radiation.

The best radiation/regeneration ratio was achieved at 60 Gy with 83% regeneration, allowing for a balance between gamma radiation at lethal dose 20 and regeneration (Figure 3). The increased regeneration achieved with 40 Gy (75.00%) and 60 Gy (83.85%) versus the control (69.04%) (Figure 3A) is a hormetic behavior previously reported in our lab [14].

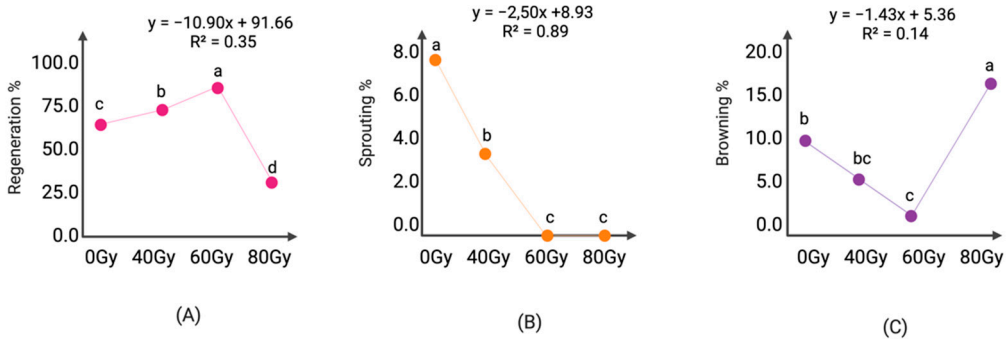

Figure 3. Correlation comparison of radiation dose influence on regeneration (**A**), sprouting (**B**) and browning rates (**C**) of calli of Lazarroz Fl cultivar, after 30 days of culture on regeneration medium. Letters a, b, bc, c and d represent significant differences for Tukey's test ($p \leq 0.05$). All treatments had 12 replicates of 7 calli each replicate (n = 84). Data were compiled at 15 days post-radiation.

Compared to the non-irradiated control (0 Gy), a significant decrease in the sprouting of embryogenic calli was observed, as the dose increased from 0 to 80 Gy. Sprouting of embryogenic calli irradiated with gamma rays (^{60}Co) decreased significantly ($p < 0.05$) occurred at doses higher than 60 Gy (Figure 3B). Moreover, Gamma-ray doses higher than 60 Gy severely affected browning rate (Figure 3C).

Gamma-irradiated calli produced plants after 45 days in the regeneration medium (Figure 4A) and fully in vitro plants (Figure 4B) at 60 days post-irradiation at 60 Gy.

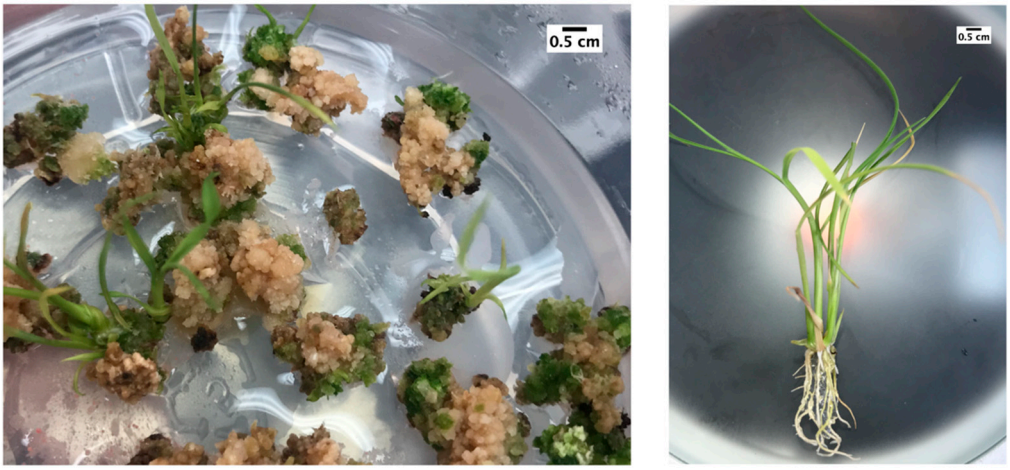

Figure 4. Response of 60 Gy irradiated calli Lazarroz FL cultivar after (**A**) 45 days and (**B**) 60 days of culture on regeneration medium.

2.5. Regeneration in Recipient for Automated Temporary Immersion (RITA® Saint-Mathieu-de-Tréviers, France)

Regeneration in the MS semisolid medium was irregular and not homogenous (Figure 5A). Instead, the RITA® immersion system (RITA®, Saint-Mathieu-de-Tréviers, France) provided predictable and homogeneous regeneration (Figure 5B).

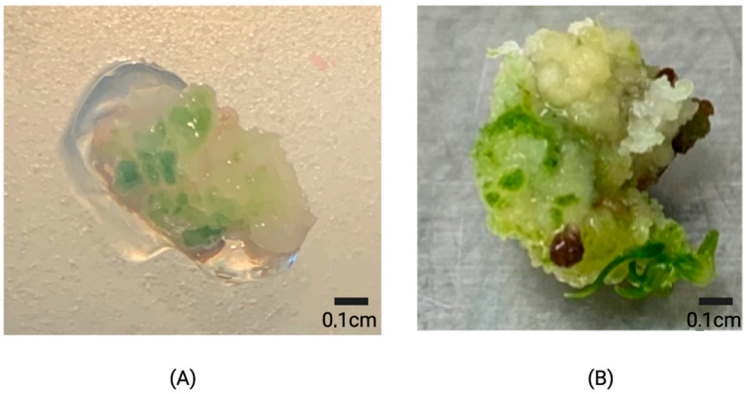

Figure 5. Regeneration in MS medium with 0.5 mg L^{-1} of NAA + 3 mg L^{-1} BA of an induced calli with 2 mg L^{-1} of 2,4-D after 15 days of induction. Regeneration in (**A**) semisolid medium and (**B**) RITA® (Saint-Mathieu-de-Tréviers, France).

The potential for temporary immersion system to regenerate recalcitrant materials (CR-5272, CR-1821, CR-1113 and Lazarroz FL) was evaluated. Our results showed no significant differences between the immersion time (60 vs. 120 s) on the regeneration capacity, sprouting and browning (Table 4). Nevertheless, a higher regeneration rate was obtained using the RITA® (Saint-Mathieu-de-Tréviers, France) in 60 s (100%) compared to regeneration on the best semi-solid medium (70%). Similarly, the sprouting rate was higher using the RITA® system independently of the immersion time compared to that obtained using the selected semi-solid medium (Tables 2 and 4).

Although, the immersion time (60 vs. 120 s) did not significantly affect the browning rate, and it was higher using an immersion of 120 s (97.56%) compared to 60 s (60.00%) and a semi-solid medium (9.52%) (Tables 2 and 4).

Table 4. Immersion regeneration, sprouting and browning rates [1].

Immersion Time	Regeneration	Sprouting	Browning Rate
60 s	100.00 a	25.00 a	60.00 a
120 s	97.56 a	31.71 a	97.56 a

[1] All treatments had 4 replicates of 10 calli per replicate (n = 40), $p \leq 0.05$. Data were compiled at 15 days post-radiation.

The potential for a temporary immersion system to regenerate recalcitrant materials was also validated for the well-known recalcitrant cultivars CR-5272, CR-1821 and CR-1113 (Figure 6).

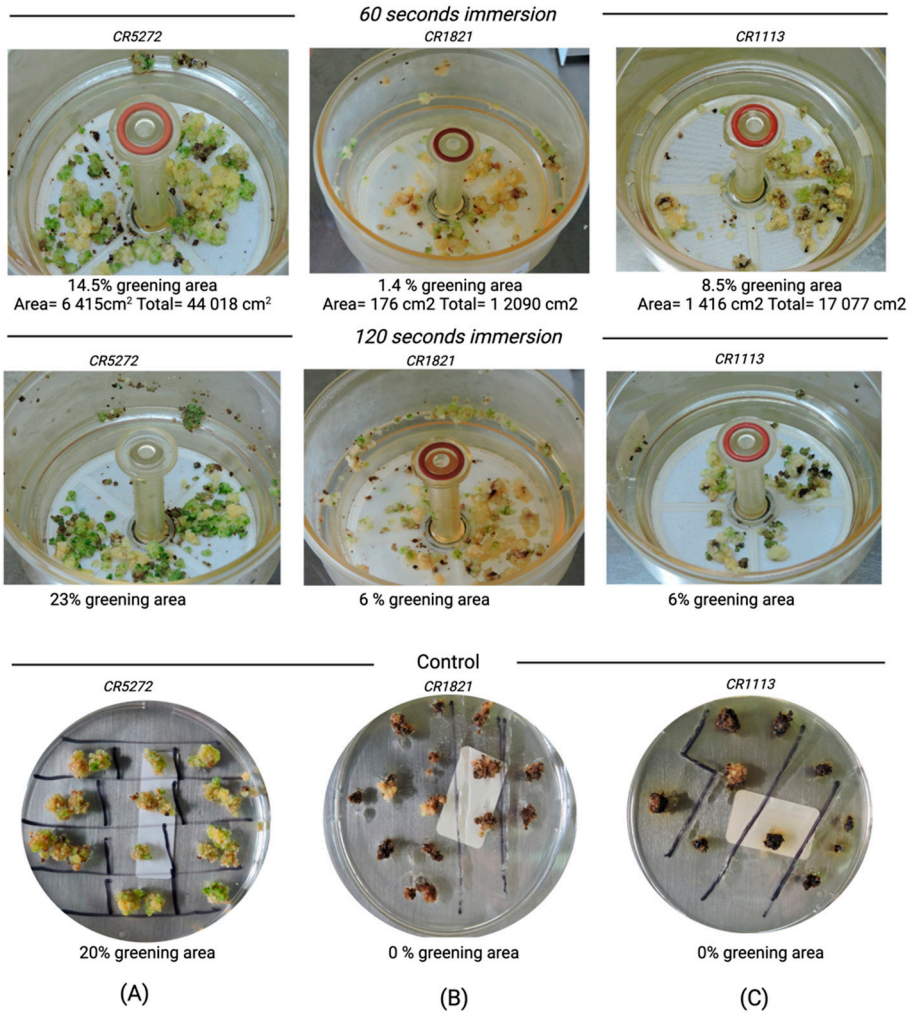

Figure 6. Regeneration of cultivars CR-5272 and recalcitrant CR-1821 and CR-1113 in MS medium with 0.5 mg L^{-1} of NAA + 3 mg L^{-1} of BA from calli induced with 2 mg L^{-1} of 2,4-D. (**A**) CR-5272 regeneration in RITA with immersion for 30 s or 60 s, and the semisolid medium control. (**B**) CR-1821 regeneration in RITA with immersion for 30 s or 60 s and the semisolid medium control. (**C**) CR-1113 regeneration in RITA with immersion for 30 s or 60 s and the semisolid medium control. Note that all RITA treatments contain calli with green areas that regenerate into plants. The absence of regeneration of the recalcitrant cultivars CR-1821 and CR-1113 was observed in the semisolid media.

3. Discussion

The present work optimized in vitro gamma ray (^{60}Co) mutagenesis in an embryogenic calli of a recalcitrant Costa Rican Lazarroz FL cultivar. The cultivar seems to be related to Southeast Asian rice cultivars based on its *rbc*L sequence pattern.

The best calli induction was observed for MS with 2mg of 2,4-D and regeneration with MS with 0.5 mg ANA + 3 mg BA. The calli induction step with a 2 mg L^{-1} of 2,4-D concentration was initially not expected because of previous local cultivar reports. Local cultivars CR-5272 and CR-1113 had a positive response at 2.5 mg L^{-1} of 2.4 D but a low performance at 2 mg L^{-1} of 2,4-D, while CR-201, CR-1707, CR-1821, CR-8334, and CR-8341 had recalcitrant and unpredictable in vitro behaviors [17,20]. Our result is similar to that obtained by other authors on Southeast Asian cultivars such as Malaysia MR219, where 2,4-D is critical and performs the best as an inducer at 2 mg L^{-1} [21,22]. On the contrary, a better calli induction occurred with higher concentrations of 2,4-D (from 2.5 mg to 3 mg L^{-1}) with other cultivars, such as MR220, GNY-53, and JP-5 [23,24].

Regeneration was improved using a temporal immersion system (RITA®, Saint-Mathieu-de-Tréviers, France) with either 60 or 120 s of immersion every 8 h, which achieved a predictable and more homogeneous regeneration response. The radiation dose with which to start mutagenesis was proposed as a lethal dose 20 at 60 Gy, as regeneration was not affected but remained as high as 80%. At 80 Gy, the regeneration fell to 30%, consistent with the oxidative damage provoked by radiation and corresponding to the lethal dose 30 of the embryogenic calli. We believe 60 Gy is an excellent condition to start mutagenesis, considering that in other in vitro plants, such as pineapple, potato, and banana, a 5–40 Gy dose of gamma irradiation was shown to be sufficient to produce variability [25]. The calculated radiation dose of the Lazarroz FL cultivar at 60 Gy corresponded to the LD20 and was different from our previous data achieved for CR-5272 with an LD50 of 60Gy [14].

Optimization of embryogenic calli in our recalcitrant Lazarroz FL cultivar became a challenge while at the same time an opportunity compared to our previous results from CR-5272 for the following reasons First, the alternative immersion method allowed our material to generate homogeneous and predictable in vitro plants after irradiation. The technique enabled regeneration for our Lazarroz FL cultivar and other recalcitrant rice lines CR-1821 and CR-1113, and consequently, is a great potential tool for regenerating gamma radiation mutated materials. The constant liquid and airflow can dilute oxidative compounds and facilitate more homogeneous exposure to nutrients. We validated the results with recalcitrant cultivars CR-1821 and CR-1113, which showed a total regeneration rate in contrast to the null regeneration in the conventional semisolid method. Plant cultivars' nonhomogeneous in vitro behavior is not fully understood, but recent discoveries provide insights into the genetic basis of the sucrose metabolism and calli browning. External phytohormones seem to trigger rice sucrose metabolism required for regeneration. The system appears to rely on the expression of endogenous cytokinin, auxin, and ABA signaling genes: ORYZA SATIVA RESPONSE REGULATOR 1 (ORR1), PIN-formed 1 (PIN1), and *late embryogenesis-abundant 1*(LEA-1) [26]. The expression of OsSRO1c, a regulator of oxidative stress, seems to be vital for avoiding calli browning in indica cultivars [27]. Tissue culture and gamma radiation produce oxidation via reactive oxygen species (ROS), usually contained in chloroplasts, peroxisomes, and mitochondria. ROS include superoxide (O_2^-), hydroxyl (OH^-) radicals, and hydrogen peroxide (H_2O_2) [28]. Tissue culture and gamma rays trigger ROS, consequently damaging the DNA by oxidation of the molecule into 8-oxo-7-hydroxyguanosine (8-oxo-dG) and further transversions of C/G and T/A [29].

Second, we can develop the desired mutations in a cultivar that farmers are already using, leading to faster breeding and adoption of a derived improved cultivar. Rice traits associated with specific genes are well known, which paves the way for producing novel cultivars based on mutation of desired or required conditions, such as biotic and abiotic stress tolerance [11]. We foresee the development of new traits such as NaCl, herbicide, and pH tolerance based on the corresponding selection agents and not limited by recalcitrant cultivars, which used to be our bottleneck to bring innovation.

4. Materials and Methods

4.1. Molecular Markers

A NucleoSpin™ Tissue Kit Macherey-Nagel (Düren, Germany) was used for DNA extraction from 1 mg of on non-irradiated lyophilized leaf tissue of Lazarroz FL. Thermo Fisher K1071 (Vilnius, Lithuania) was used for the subsequent PCR following the recommendations of the manufacturer. The primers used in this study are as follows: for rbcL, rbcLaf 5′ATGTCACCACAAACAGAGACTAAAGC3′ and rbcLar 5′GTAAAATCAAGTCCACCRCG-3′ or rbcLaf 5′ATGTCACCACAAACAGAGACTAAAGC3′ and rbcLr590 5′AGTCCACCGCGTAGACATTCAT-3′; for matK, matK-xf 5′TAATTTACGATCAATTCATTC-3′ and matKr 5′ACAAGAAAGTCGAAGTAT-3′. Briefly, the PCR master mix consisted of a mixture (50 µL) containing 1X Dream Taq Master mix Thermo Fisher (Vilnius, Lithuania), 20 µM of each primer, and 5 µL of DNA (50 ng/uL). The thermocycling program was 95 °C for 5 min, 40 cycles at 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, and a final cycle of 72 °C for 7 min.

4.2. Embryogenic Calli Induction

For initial assays, the palea and lemma of rice caryopsis of a commercial local indica cultivar were removed with No. 80 grit sandpaper. The obtained seeds were surface-sterilized as previously reported [14] through two incubations in 4% (*v*/*v*) NaOCl for 10 min each with constant agitation, using 10 mL of disinfectant solution for every gram of seeds. After the first and final incubation, the seeds were washed six to seven times with distilled sterilized water and were cultured in media composed of mineral salts and vitamins as described by Murashige and Skoog (MS), with 20 g L^{-1} sucrose and 0.1 g L^{-1} hydrolyzed casein. Treatments consisted of supplementing the basal medium with one of the following combinations of plant growth regulators: (i) 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) was used as a control, (ii) 2.0 mg L^{-1} 2,4-D, (iii) 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} 6-benzyladenine (6-BA), (iv) 2.0 mg L^{-1} 2,4-D + 1 mg L^{-1} 6-BA, and (v) 2.0 mg L^{-1} 2,4-D + 0.25 mg L^{-1} 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea (thidiazuron, TDZ) (Table 1). After adding plant growth regulators, the media volumes were adjusted as required, the pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl, and 5.4 g L^{-1} Gelzan® (Phytotechnology Laboratories®, Shawnee Mission, KS, USA) were added as a gelling agent. All previously mentioned chemicals were supplied by Phytotechnology Laboratories® (Shawnee Mission, KS, USA). An autoclave (1.2 ATM. cm^{-2} and 121 °C for 30 min) was used for the sterilizing medium and further dispensed on 94 × 16 mm vented polystyrene Petri dishes (Greiner Bio-One, Fisher-Scientific, Waltham, MA, USA) in a laminar flow chamber. For each treatment, at least 900 seeds were cultured after surface sterilization. Cultures were maintained in the dark at 26 ± 2 °C. Calli induction and browning rates (brown or necrotic/total calli) were recorded as response variables and analyzed in a completely randomized design with a generalized linear model with a Poisson distribution and logit link function. Post hoc analysis consisted of an Honest Significant Difference test on IBM SPSS version 27 [30] and differences between each combination of factors were recognized.

4.3. Regeneration on Semisolid Medium

Embryogenic calli obtained from each induction medium were transferred to different regeneration treatments. Basal regeneration medium was similar to that described by Sudhakar et al. [31], and was constituted by MS mineral salts and vitamins, 20 g L^{-1} sucrose and 0.3 g L^{-1} hydrolyzed casein. Treatments consisting of supplementing the basal medium with variations of growth regulators, and the control was supplemented with 0.5 mg L^{-1} α-Naphthaleneacetic Acid (NAA) + 3 mg L^{-1} 6-BA, while the second treatment was supplemented with 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} 6-BA and the third treatment was supplemented with 0.5 mg L^{-1} NAA + 1.5 mg L^{-1} Kinetin (KIN) and a fourth treatment consisted of supplementing basal medium with 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} TDZ. After adding growth regulators, pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl and 5.4 g L^{-1} Gelzan ® (Phytotechnology Laboratories®, Shawnee Mission, KS, USA) were added as

gelling agent. All previously mentioned reagents were supplied by Phytotechnology Laboratories® (Shawnee Mission, KS, USA). After a properly dissolving of gelling agent, 60 mL of media were dispensed on 475 mL polypropylene WNA Deli Containers, and afterwards, media were sterilized at 1.2 ATM.cm^{-2} and 121 °C for 30 min. For each treatment, 6 replicates of 7 calli were cultured on a factorial design. Factor 1 consisted of induction media and factor 2 was regeneration media. Cultures were maintained at an irradiance of 72 µmol s^{-1}m^{-2}, a 16 h light/8 h dark photoperiod was used and 26 ± 2 °C for a period of 4 weeks. Calli showing mature coleoptilar germinated embryos were determined as regenerated, calli with completely differentiated plantlets greater than 1 cm were categorized as sprouted, and necrotic calli with a dark coloration were identified as browning calli. These response variables were analyzed with a generalized linear model with a Poisson distribution and logit link function. Post hoc analysis consisted of an Honest Significant Difference test on IBM SPSS version 27 [31] and differences between each combination of factors were recognized.

4.4. Gamma Irradiation

Embryogenic calli irradiation was achieved with a gamma irradiator Ob-Servo Ignis type with 24 cobalt 60 source pencils (Institute of Isotopes Co, Ltd., Budapest, Hungary). To determine radiosensitivity and the median lethal dose (LD50), calli were irradiated at 0, 40, 60, 80, 100, 120 Gy. Ten repetitions, and 20 embryogenic calli per exposure were used. The survival rate was recorded after calli were transferred to the regeneration medium composed by basal regeneration medium supplemented with 0.5 mg L^{-1} NAA + 3 mg L^{-1} 6-BA, selected after previous experiments were analyzed. Culture conditions were the same indicated above and after 4 weeks lethal doses were calculated using probit analysis on IBM SPSS version 27 [31] based on calli death rates.

4.5. Regeneration in Recipient for Automated Temporary Immersion (RITA®, Saint-Mathieu-de-Tréviers, France)

The RITA® (Saint-Mathieu-de-Tréviers, France) temporary immersion system regeneration of the embryogenic calli consisted of 200 mL regeneration media previously described in 4.3, with 23 four-week-old calli per unit, different rice cultivars (Lazarroz FL, CR-5272, CR-1821, and CR-1113). Light and temperature culture conditions remained unchanged, with 60 or 120 s immersion used as treatments, every eight hours. After four weeks of culture, the variables evaluated were regeneration, sprouting, and browning, green area calculations with ImageJ version 1.52p [32].

5. Conclusions

Rice tissue culture is a tool for conventional and modern breeding, but is limited to the genotype response, particularly during the regeneration of recalcitrant varieties. Our results collected using an immersion system helped to overcome such difficulties and allowed for the induction of gamma-ray mutants. A temporary immersion system seems to help overcome calli browning while allowing the tissue to recover and consequently presenting a more efficient method. We foresee that having access to such methods could diminish the time to trigger innovation and focus on selecting mutants with desired traits in commercially used varieties.

Author Contributions: Conceptualization, A.H.-S., J.P., R.F.-Z., R.R.-V. and A.G.-A.; writing—original draft preparation, A.H.-S., J.P. and A.G.-A.; writing—review and editing, A.H.-S., J.P., A.G.-A. and A.A.-E.; visualization, A.H.-S. and J.P; investigation W.V.-S., R.F.-Z., R.R.-V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Research Vice-Rectory of TEC, Costa Rica, project number 1510-1022.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ali, J.; Nicolas, K.L.C.; Akther, S.; Torabi, A.; Ebadi, A.A.; Marfori-Nazarea, C.M.; Mahender, A. Improved Anther Culture Media for Enhanced Callus Formation and Plant Regeneration in Rice (*Oryza sativa* L.). *Plants* **2021**, *10*, 839. [CrossRef]
2. Wing, R.A.; Purugganan, M.D.; Zhang, Q. The Rice Genome Revolution: From an Ancient Grain to Green Super Rice. *Nat. Rev. Genet.* **2018**, *19*, 505–517. [CrossRef]
3. Chen, E.; Huang, X.; Tian, Z.; Wing, R.A.; Han, B. The Genomics of Oryza Species Provides Insights into Rice Domestication and Heterosis. *Annu. Rev. Plant Biol.* **2019**, *70*, 639–665. [CrossRef]
4. Kim, K.; Lee, S.C.; Lee, J.; Yu, Y.; Yang, K.; Choi, B.S.; Koh, H.J.; Waminal, N.E.; Choi, H.I.; Kim, N.H.; et al. Complete Chloroplast and Ribosomal Sequences for 30 Accessions Elucidate Evolution of Oryza AA Genome Species. *Sci. Rep.* **2015**, *5*, 15655. [CrossRef]
5. Nadir, S.; Xiong, H.B.; Zhu, Q.; Zhang, X.L.; Xu, H.Y.; Li, J.; Dongchen, W.; Henry, D.; Guo, X.Q.; Khan, S.; et al. Weedy Rice in Sustainable Rice Production. A Review. *Agron. Sustain. Dev.* **2017**, *37*, 46. [CrossRef]
6. Chauhan, B.S.; Jabran, K.; Mahajan, G. *Rice Production Worldwide*; Springer International Publishing: Cham, Switzerland, 2017; Volume 247. [CrossRef]
7. Viana, V.E.; Pegoraro, C.; Busanello, C.; Costa de Oliveira, A. Mutagenesis in Rice: The Basis for Breeding a New Super Plant. *Front. Plant Sci.* **2019**, *10*, 1326. [CrossRef]
8. Serrat, X.; Esteban, R.; Guibourt, N.; Moysset, L.; Nogués, S.; Lalanne, E. EMS Mutagenesis in Mature Seed-Derived Rice Calli as a New Method for Rapidly Obtaining TILLING Mutant Populations. *Plant Methods* **2014**, *10*, 5. [CrossRef]
9. Soriano, J.D. Mutagenic Effects of Gamma Radiation on Rice. *Bot. Gaz.* **1961**, *123*, 57–63. [CrossRef]
10. Romero, F.M.; Gatica-Arias, A. CRISPR/Cas9: Development and Application in Rice Breeding. *Rice Sci.* **2019**, *26*, 265–281. [CrossRef]
11. Hernández-Soto, A.; Echeverría-Beirute, F.; Abdelnour-Esquivel, A.; Valdez-Melara, M.; Boch, J.; Gatica-Arias, A. Rice Breeding in the New Era: Comparison of Useful Agronomic Traits. *Curr. Plant Biol.* **2021**, *27*, 100211. [CrossRef]
12. Oladosu, Y.; Rafii, M.Y.; Abdullah, N.; Hussin, G.; Ramli, A.; Rahim, H.A.; Miah, G.; Usman, M. Principle and Application of Plant Mutagenesis in Crop Improvement: A Review. *Biotechnol. Biotechnol. Equip.* **2016**, *30*, 1–16. [CrossRef]
13. Zain, C.R.C.M.; Kadhimi, A.A.; Alhasnawi, A.N.; Isahak, A.; Mohamad, A.; Doni, F.; Yusoff, W.M.W. Enhancing of Drought-Tolerant Rice (*Oryza sativa*) Variety MRQ74 through Gamma Radiation and in vitro Pathway. *Biotechnology* **2016**, *15*, 125–134. [CrossRef]
14. Abdelnour-Esquivel, A.; Perez, J.; Rojas, M.; Vargas, W.; Gatica-Arias, A. Use of Gamma Radiation to Induce Mutations in Rice (*Oryza sativa* L.) and the Selection of Lines with Tolerance to Salinity and Drought. *In Vitro Cell. Dev. Biol.—Plant* **2020**, *56*, 88–97. [CrossRef]
15. Fehér, A. Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? *Front. Plant Sci.* **2019**, *10*, 536. [CrossRef]
16. Binte Mostafiz, S.; Wagiran, A. Efficient Callus Induction and Regeneration in Selected *Indica* Rice. *Agronomy* **2018**, *8*, 77. [CrossRef]
17. Vega, R.; Vásquez, N.; Espinoza, A.M.; Gatica, A.M.; Valdez-Melara, M. Histology of Somatic Embryogenesis in Rice (*Oryza sativa* cv. 5272). *Rev. Biol. Trop.* **2009**, *57* (Suppl. S1), 141–150. [CrossRef]
18. Che Radziah, C.M.Z.; Naji Alhasnawi, A.; Kadhimi, A.A.; Isahak, A.; Mohamad, A.; Farshad Ashraf, M.; Doni, F.; Mohtar Wan Yusoff, W. Development of a Technique for Callus Induction and Plant Regeneration in *Oryza sativa* L. Var. MRQ74 and MR269. *Adv. J. Food Sci. Technol.* **2017**, *13*, 128–137. [CrossRef]
19. Murashige, T.; Skoog, F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant.* **1962**, *15*, 474–497. [CrossRef]
20. Valdez, M.; Muñoz, M.; Vega, J.R.; Espinoza, A.M. Plant Regeneration of Indica Rice (*Oryza sativa*) Cultivars from Mature Embryo-Derived Calli. *Rev. Biol. Trop.* **1997**, *44–45*, 13–21. [CrossRef]
21. Pawar, B.; Kale, P.; Bahurupe, J.; Jadhav, A.; Kale, A.; Pawar, S. Proline and Glutamine Improve in vitro Callus Induction and Subsequent Shooting in Rice. *Rice Sci.* **2015**, *22*, 283–289. [CrossRef]
22. Abiri, R.; Maziah, M.; Shaharuddin, N.A.; Yusof, Z.N.B.; Atabaki, N.; Hanafi, M.M.; Sahebi, M.; Azizi, P.; Kalhori, N.; Valdiani, A. Enhancing Somatic Embryogenesis of Malaysian Rice Cultivar MR219 Using Adjuvant Materials in a High-Efficiency Protocol. *Int. J. Environ. Sci. Technol.* **2017**, *14*, 1091–1108. [CrossRef]
23. Hussain, Z.; Khan, M.H.; Bano, R.; Rashid, H.; Chaudhry, Z. Protocol Optimization for Efficient Callus Induction and Regeneration in Three Pakistani Rice Cultivars. *Pak. J. Bot.* **2010**, *42*, 879–887.
24. Ming, N.J.; Mostafiz, S.B.; Johon, N.S.; Zulkifli, N.S.A.; Wagiran, A. Combination of Plant Growth Regulators, Maltose, and Partial Desiccation Treatment Enhance Somatic Embryogenesis in Selected Malaysian Rice Cultivar. *Plants* **2019**, *8*, 144. [CrossRef]

25. Kashtwari, M.; Mansoor, S.; Wani, A.A.; Najar, M.A.; Deshmukh, R.K.; Baloch, F.S.; Abidi, I.; Zargar, S.M. Random Mutagenesis in Vegetatively Propagated Crops: Opportunities, Challenges and Genome Editing Prospects. *Mol. Biol. Rep.* **2021**, *1*, 1–21. [CrossRef]
26. Lee, S.T.; Huang, W.L. Cytokinin, Auxin, and Abscisic Acid Affects Sucrose Metabolism Conduce to de Novo Shoot Organogenesis in Rice (*Oryza sativa* L.) Callus. *Bot. Stud.* **2013**, *54*, 5. [CrossRef]
27. Zhang, K.; Su, J.; Xu, M.; Zhou, Z.; Zhu, X.; Ma, X.; Hou, J.; Tan, L.; Zhu, Z.; Cai, H.; et al. A Common Wild Rice-Derived BOC1 Allele Reduces Callus Browning in Indica Rice Transformation. *Nat. Commun.* **2020**, *11*, 443. [CrossRef]
28. Debnath, S.; Chandel, R.K.; Devi, K.; Khan, Z. Mechanism and Molecular Response of Induced Genotoxicity and Oxidative Stress in Plants. In *Induced Genotoxicity and Oxidative Stress in Plants*; Khan, Z., Ansari, M.Y.K., Shahwar, D., Eds.; Springer: Singapore, 2021. [CrossRef]
29. Xie, X.; He, Z.; Chen, N.; Tang, Z.; Wang, Q.; Cai, Y. The Roles of Environmental Factors in Regulation of Oxidative Stress in Plant. *BioMed Res. Int.* **2019**, *2019*, 21–27. [CrossRef]
30. IBM Corp. *Released 2020. IBM SPSS Statistics for Windows*; Version 27.0; IBM Corp: Armonk, NY, USA, 2020.
31. Sudhakar, D.; Duc, L.T.; Bong, B.B.; Tinjuangjun, P.; Maqbool, S.B.; Valdez, M.; Jefferson, R.; Christou, P. An Efficient Rice Transformation System Utilizing Mature Seed-Derived Explants and a Portable, Inexpensive Particle Bombardment Device. *Transgenic Res.* **1998**, *7*, 289–294. [CrossRef]
32. Schneider, C.; Rasband, W.; Eliceiri, K. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675. [CrossRef]

Research Article

Tolerance to aryloxy-phenoxy-propionate (APP) as a model for Lazarroz FL rice *in vitro* gamma irradiation variability selection

Alejandro Hernández-Soto [1,2,*], Daniela Méndez-Navarro [2], Jason Pérez [2], Andrés Gatica-Arias [3], Walter Vargas-Segura [4] Fabián Echeverria-Beirute[1], and Ana Abdelnour-Esquivel [2]

[1] Doctorado en Ciencias Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia, Cartago P.O. Box 159-7050, Costa Rica; fecheverria@itcr.ac.cr (F.E.B)

[2] Biotechnology Research Center, Biology School, Costa Rica Institute of Technology, Costa Rica PO Box 159-7050 Cartago, Costa Rica; mdanim_77@hotmail.com (D.M.-N); jasperez@itcr.ac.cr (J.P.); aabdelnour@itcr.ac.cr (A.A.-E.)

[3] Plant Biotechnology Laboratory, School of Biology, University of Costa Rica, P.O. Box 2060, San José, Costa Rica; andres.gatica@ucr.ac.cr (A.G.-A.)

[4] Gamma Irradiation Laboratory, School of Physics, Costa Rica Institute of Technology, Costa Rica, PO Box 159-7050 Cartago, Costa Rica; walvargas@tec.ac.cr

* Correspondence: alhernandez@tec.ac.cr

Abstract: *In vitro* gamma ray (^{60}Co) mutagenesis is a powerful tool to achieve variability in commercial rice lines used by farmers, such as Lazarroz FL. We previously reported the optimized *in vitro* gamma mutagenesis system for Lazarroz FL Indica callus. As a continuation, in the present study, we targeted the *ACC2* gene mutagenesis that provides tolerance to aryloxy-phenoxy-propionate (APP) fluazifop-P-butyl as a model to show the system's potential to create variability while providing a solution for weed management. The DL50 of fluazifop-P-butyl was calculated in calli as DL50= 6,93 mg/L (0,425 mg/L - 15,743 mg/L, R2 = 0,402, 1000n) and regenerated *vitroplants* at an LD50 of 3.771 mg/L (R2 = 1, 290n). We used 5 mg/L fluazifop-P-butyl as a selection agent and the second round of selection of 10 mg/L (3000 *vitroplants*) resulted in one survivor plant when using calli as a starting material. The putative tolerant plant also tolerated 150 mg/L in the greenhouse. The *ACC2* gene was sequenced, and a heteroecious mutation, T2222I/T2222M, was discovered that may be linked to tolerance. We improved the *in vitro* system by using seeds as a gamma irradiation starting point instead of embryogenic calli, followed by calli induction, regeneration, and exposure to the selection agent. The modification allowed higher gamma doses with an LD50 of 350 Gy and one to thirty-one putative tolerant plants. The *in vitro* model showed that gamma-ray mutants from recalcitrant indica rice materials are possible, and the use of selection agents such APP can help create variability useful for breeding a more resilient rice.

Keywords: Mutagenesis, Biotechnology, Plant Tissue Culture, Fluazifop-P-butyl

Citation: Hernández-Soto, A.; Méndez-Navarro, D.; Pérez, J.; Rojas-Vásquez, R.; Gatica-Arias, A.; Vargas, W.; Echeverria-Beirute,F; Abdelnour-Esquivel, A. Tolerance to aryloxy-phenoxy-propionate (APP) as a model for Lazarroz FL rice *in vitro* gamma irradiation variability selection. *Research Square,* **2022**. https://doi.org/10.21203/rs.3.rs-1950230/v1

Posted: 11 August 2022

1. Introduction

Rice (*Oryza sativa* L.) is a crucial crop responsible for 20% of calories of half the human population and is facing constraints of yield related to biotic factors, such as pathogens, and abiotic stresses, such as salinity, drought, heat or cold [1-2]. Breeding can introduce desired traits based on genetic variability related to heat, salt, soil acidification stress, and plague tolerance to the few cultivars preferred by farmers in specific geographic areas [3-6]. Rice-induced genetic variability obtained by seed irradiation is well known and has been used since it was proven in 1928 on vegetables; however, it is slow and requires land, resources, and human capabilities to find desired traits [7-10]. Plant tissue culture provides a platform to produce gamma mutations from primitive embryogenic cells [11-13].

We previously reported a system to create variability using *in vitro* gamma radiation at an optimized dose of 60 Gy in embryogenic calli of Lazarroz indica rice [14]. The proposed model required a method to narrow the *in vitro* selection while correlating with desired mutations. In this paper, we suggest resistance to stressors such as the herbicide aryloxy-phenoxy-propionate (APP) to regenerate complete plants from tolerant mutated cells as a model for such challenges. The herbicide is commonly used to control weeds in rice by acting on the *ACC2* enzyme, which is related to fatty acid synthesis, while mutations in the carboxyl transferase domain of *ACC2* correlate with APP tolerance, as described below [15-21].

The herbicides acting on the ACC2 enzyme include at least two main groups, aryloxy-phenoxy-propionates (APP) and cyclohexanediones [15]. In grasses, there are two types of ACCases, cytosolic and plastidic. The second ACC2 is affected by APPs in rice. The APPs include clodinafop-propargyl, cyhalo-fop-butyl, fenoxapropethyl, metamifop, diclofop-meth fenthiaprop, quizalofop-ethyl, haloxyfop-R-methyl, and fluazifop-P-butyl. Fluazifop is a molecule of 327.25 g mol-1 that can control grasses at a dose of 210 g ai Ha^{-1} and 420 g ai Ha^{-1} preemergent with low residual effects [15-16]. Specific mutations in ACC2 enzymes correlate with APP tolerance, as described below.

The enzyme acetyl-CoA carboxylase 2 ACC2 (EC 6.4.1.2, UniProt: A2Y2U1) is located on chromosome 5 at amino acids 14,067,726-14,079,652 and is delivered to the plastid. Mutations in the carboxyl transferase domain of ACC2 between amino acids 1,781-2,078 and 2,027-2,096 are related to APPS tolerance, such as Ile1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg, and Gly-2096-Ala [16-19]. Mutations such as A2004 V provide wheat with tolerance to 10 g ai Ha^{-1} of quizalofop [16,17]. Similarly, *Oryza japonica* ACCase 2 (*LOC_Os05g22940*), mutations on I1781 V, C2088R, and W2027C, also provide tolerance to APPs [21]. Finally, an indica rice with a mutation tolerant to APPs with the mutation I1781 L has been available in the USA market since 2018 [22].

This paper aims to improve our previous system and obtain more predictable traits that correlate with specific mutations. Here, we present a nonreported mutation, T2222I/T2222M, that may be linked to tolerance to APPs and present an improvement of our earlier *in vitro* system by using seeds instead of calli during gamma irradiation. We also show aryloxy-phenoxy-propionate (APP) fluazifop-P-butyl tolerance as a model to demonstrate the system's potential for incorporating features that correlate with mutations. The obtained mutant can be incorporated into the breeding program to look for functional variability.

2. Results

2.1 Fluazifop-P-butyl toxicity to embryogenic calli

The toxicity of fluazifop-P-butyl to Lazarroz FL embryogenic calli was calculated based on calli browning to be LD50=6.93 mg -L R2=0.402 (Table 1). A higher brown callus rate of 79% resulted in over 10 mg -L of the selection APP agent corresponding with the calculated LD75=17.8 mg (Table 1). We decided to test regenerated *vitroplants* from calli instead of directly exposed calli to fluazifop-P-butyl because of the absence of regeneration of nonbrowned surviving calli (139n) and the variability of the results (Table 2).

Table 1. Nonirradiated Lazarroz FL calli toxicity to fluazifop-P-butyl

Treatment[1]	Total	Non brown	Brown	Browning rate (%)[2]	LD_{50}[1] (mg L^{-1})	LD_{75}[1] (mg L^{-1})
0 mg L^{-1} Fluazifop-P-butyl	200	120	80	40		
1 mg L^{-1} Fluazifop-P-butyl	200	97	103	48	6.93	17.8
10 mg L^{-1} Fluazifop-P-butyl	200	42	158	79	(0.42-15.74)	(4.1-35.2)
100 mg L^{-1} Fluazifop-P-butyl	200	0	200	100		

[1] Probit statistical analysis resulted in R^2=0.402, y=-0.42+0.6x
[2] Brown or necrotic/total calli

2.2 Vitroplant toxicity to fluazifop-P-butyl

The Fluazifop-P-butyl toxicity to Lazarroz FL *vitroplants* regenerated from calli was calculated based on necrosis of plants at 21 days and resulted in LD50=3.771 mg -L and LD75= 4.287 mg -L, R2=1 (Table 2, Figure 1A, 1B, 1C).

Table 2. Nonirradiated Lazarroz FL rice *vitroplant* toxicity to fluazifop-P-butyl

Treatment[1]	Total	Alive	Necrotic	Mortality Rate (%)	LD_{50}[1] (mg L^{-1})	LD_{75}[1] (mg L^{-1})
0 mg L^{-1} Fluazifop-P-butyl	55	55	0	0		
1 mg L^{-1} Fluazifop-P-butyl	40	40	0	0		
4 mg L^{-1} Fluazifop-P-butyl	71	36	35*	49		
5 mg L^{-1} Fluazifop-P-butyl	58	0	58*	100	3.771	4.287
10 mg L^{-1} Fluazifop-P-butyl	88	0	88*	100		
25 mg L^{-1} Fluazifop-P-butyl	57	0	57*	100		
100 mg L^{-1} Fluazifop-P-butyl	70	0	70*	100		

[1] Probit statistical analysis resulted in R^2=1, y=-4.59+7.6x
* Necrotic *vitroplants* were subcultured into fresh medium to verify mortality

The necrotic plants were subcultured into fresh medium for 21 days to verify mortality, resulting in null sprouting, regeneration, or green spots but remaining necrotic (Figure 1D).

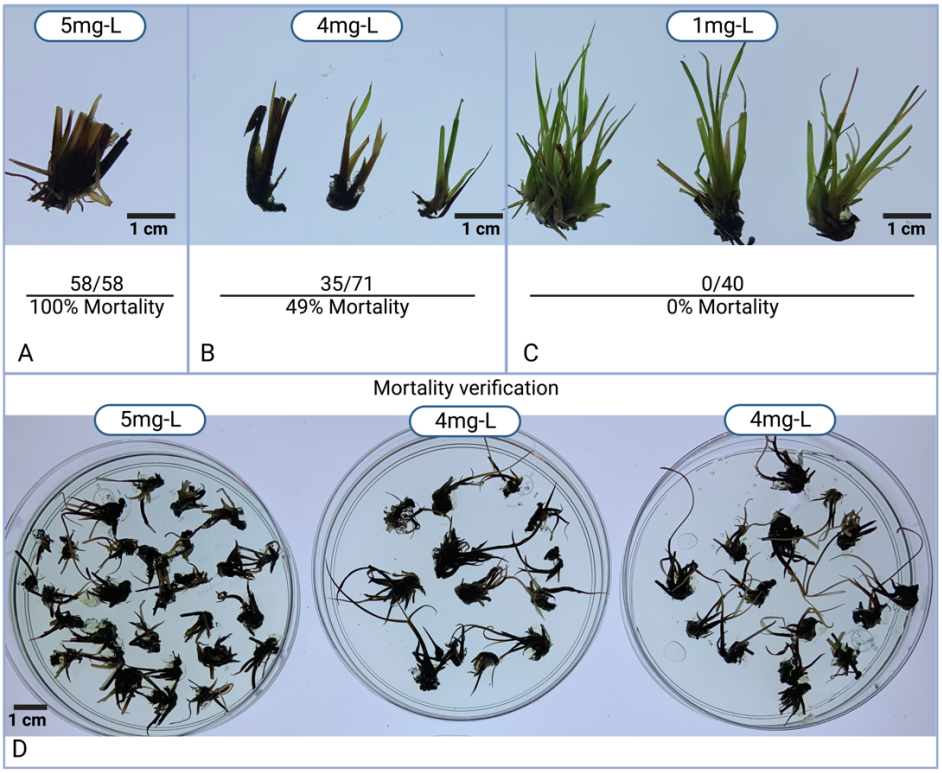

Figure 1. Regenerated calli of *vitroplants* of the Lazarroz FL cultivar on MS medium with 3 mg L^{-1} BA under fluazifop-P-butyl stress after 21 days of culture. **A**) 5 mg L^{-1} fluazifop-P-butyl, (**B**) 4 mg L^{-1} fluazifop-P-butyl, (**C**) 1 mg L^{-1} fluazifop-P-butyl, (**D**) necrotic *vitroplants* subcultured in fresh medium for 21 days to verify mortality after exposure to 5 mg L^{-1} and 4 mg L^{-1} fluazifop-P-butyl stress, as shown in (A) and (B). Note that all remained necrotic and had no sprouting or green spots.

2.3. Gamma radiation mutagenesis and plant regeneration

We decided to use 5 mg L^{-1} as the fluazifop-P-butyl dose to select putative tolerant mutants to avoid false-positives. We selected 100 putative resistant lines out of 8000 *vitroplants* regenerated from embryogenic calli irradiated at 60 Gy (^{60}Co) that survived when exposed to 5 mg L^{-1} fluazifop-P-butyl stress (Figure 2). The plants grew normally and put through a second selection round to validate their tolerance. The second round of selection consisted of a higher dose of 10 mg-L fluazifop-P-butyl with the survival of only one putative tolerant plant out of 100 (Figure 2).

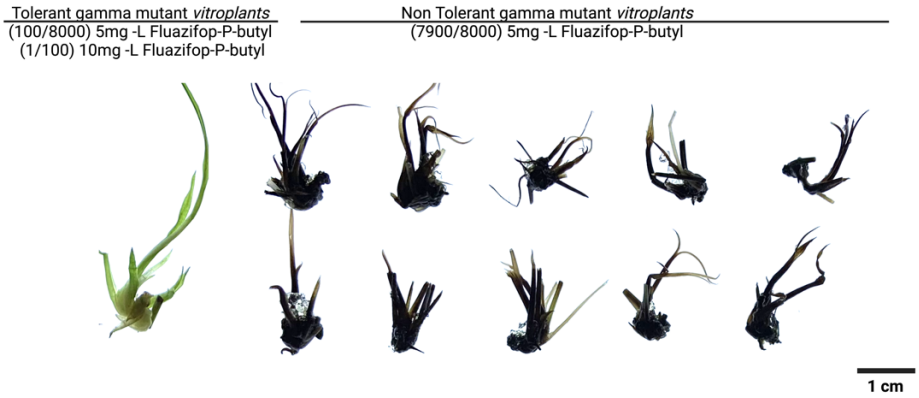

Figure 2. Tolerant *vitroplant* of the Lazarroz FL cultivar obtained by gamma mutagenesis on calli at 60 Gy on MS medium with two rounds of selections, the first at 5 mg L−1 of fluazifop-P-butyl stress after 21 days of culture, 21 days of recovery with no stress agent, and another round of 21 days of 10 mg L^{-1} of fluazifop-P-butyl stress. Note that nontolerant plants became necrotic at 5 mg L−1 fluazifop-P-butyl stress after 21 days of culture, while only 1 out of 100 putative tolerant *vitroplants* remained green during the second round of selection at 10 mg L^{-1} fluazifop-P-butyl stress.

2.4. Molecular markers used for the identification of the rice cultivars

We sequenced and published exon 32 of the acetyl-CoA carboxylase 2 gene (*ACCase2*) MZ558337 and compared it with nonirradiated Lazarroz FL plants to validate perfect matches with our control. The control sequence perfectly matched our accession, and the reference genes of *Oryza sativa indica* at chromosome five demonstrated the identity and absence of mutations as expected.

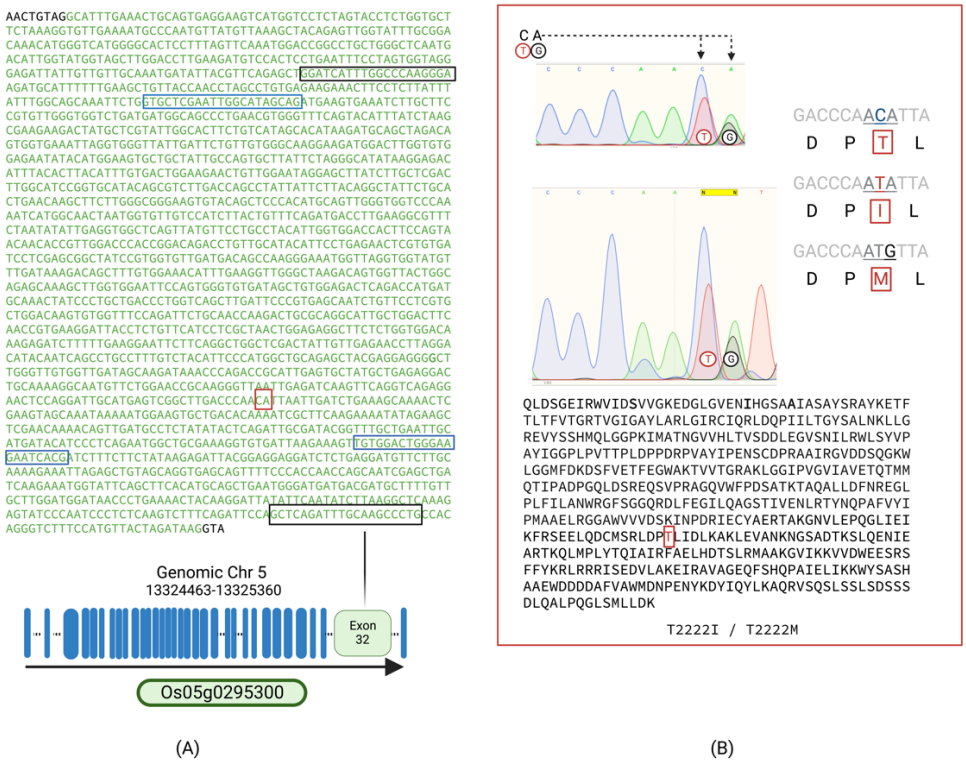

Figure 3. DNA markers used to identify exon 32 of the *ACC2* gene in the nonirradiated Lazarroz FL rice variety. Note in (A) the sequence of exon 32 is shown in green, and the locations of the primers used for PCR and sequencing are shown in black and blue boxes. In the red box is the site corresponding to the biallelic mutation. (B) Sequencing electropherogram and corresponding amino acid mutation putative changes.

The sequence of exon 32 of the acetyl-CoA carboxylase 2 gene (*ACCase2*) of the fluazifop-P-butyl tolerant *vitroplant,* showed one mutation in the expected domain related to tolerance. The mutation may have resulted in a change in the amino acid T2222I/T2222M (Figure 3). The plant remained tolerant in a greenhouse at a dose of 150 mg/L.

We also sequenced the *mat*K and *rbcL* genes of the mutants that matched almost perfectly with the control reported sequences MZ558335 and MZ558334, respectively. We noted that a biallelic G/T in *mat*K may change the amino acid isoleucine into another nonpolar amino acid or methionine. We also noted a biallelic *rbc*L GT/AG mutation that could change the amino acid tyrosine into another uncharged polar amino acid serine. The results are consistent with our mutation in the *ACC2* gene of 1 per 1000 bp (Figure 4).

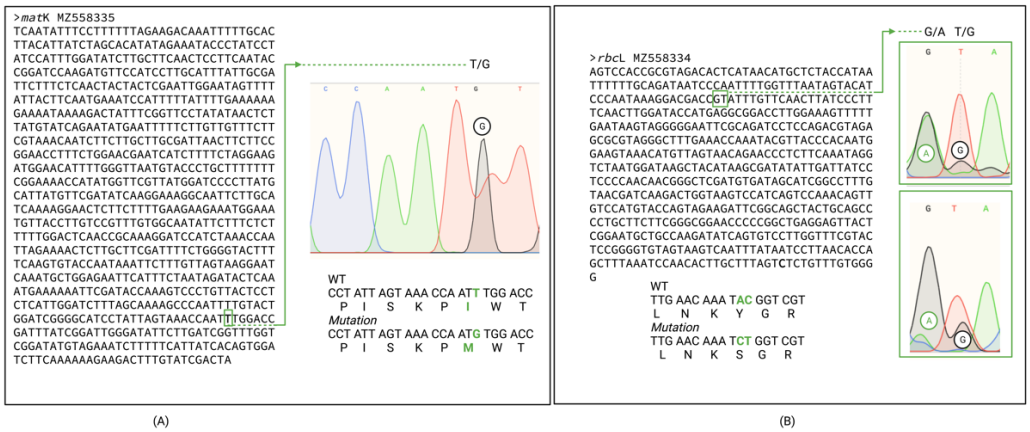

Figure 4. DNA markers (A) *mat*K and (B) *rbcL* genes of the mutants in comparison with the control reported sequences MZ558335 and MZ558334. Note in green the biallelic mutations G/T in *mat*K resulting in isoleucine change to methionine and GT/AG coding for tyrosine that may change into serine in a mutation in *rbcL*.

Our results were positive, but we still had room for improvement since the number of putative mutants was low. We tried a different approach that allowed us to increase the gamma dose and, consequently, the mutation rate. We used seeds instead of calli for gamma exposure and kept the same tissue culture methods as previously reported [14]. The system improved in terms of having a larger window for optimization that allows for using a range of gamma rays from 50 to 350 Gy with 31 tolerant *vitroplants* after the two rounds of fluazifop-P-butyl selection at 5 and 10 mg L^{-1} (Figure 5).

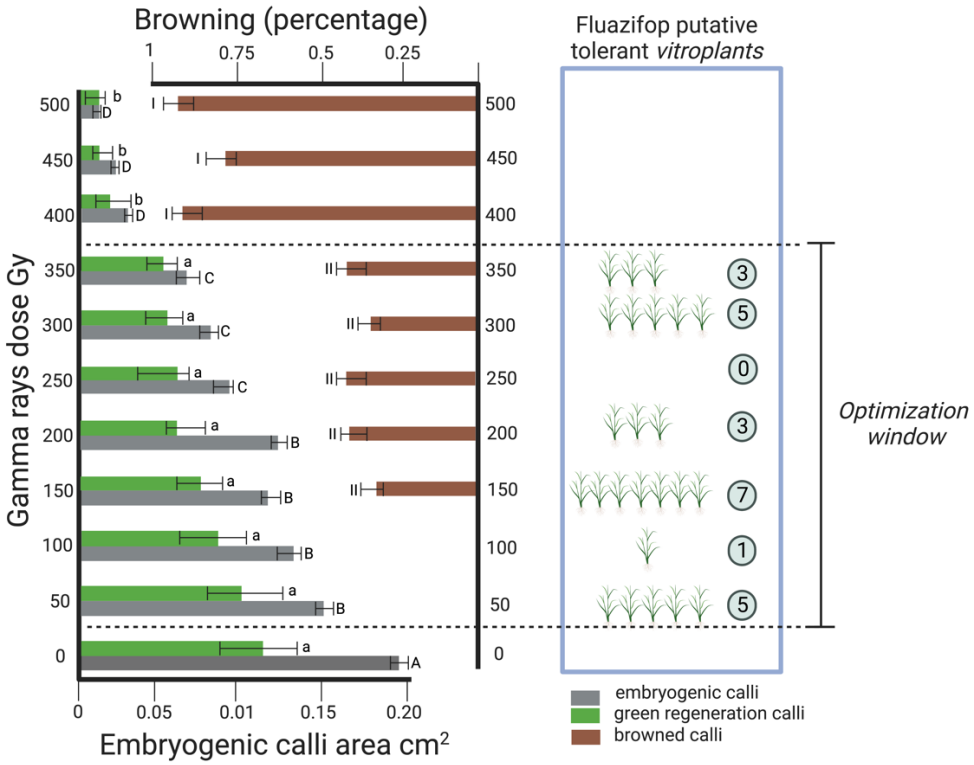

Figure 5. Gamma radiation optimization using Lazzaroz FL seeds as a starting material and further calli induction, regeneration, and selection in two rounds of fluazifop-P-butyl selection at 5 and 10 mg L^{-1}. Note the optimization window of gamma exposure ranges

from 50 to 350 Gy, allowing predictable calli induction and regeneration, as well as putative tolerant *vitroplants*.

3. Discussion

In previous experiences, gamma radiation allowed the development of salt and drought tolerance in cultivar CR-5272 [9]. Nevertheless, such a trait was useless for farmers and breeders who no longer use that variety for planting or breeding programs. It was also not a good trait for optimization because salt tolerance can be a multigenic trait. We investigated Lazarroz and developed its protocols for gamma radiation variability [14]. Lazarroz is a commercial cultivar used and preferred by farmers, representing 45% of planted rice in Costa Rica [23]. The present work resulted in an optimized *in vitro* gamma-ray (60Co) on the *ACC2* mutagenesis system based on APPs selection from a recalcitrant Costa Rican Lazarroz FL cultivar. This trait will help breeding programs to manage weeds.

The selected tissue and fluazifop-P-butyl dose were vital in optimizing the mutation system. We were aware that selection on calli reduces time, but we preferred using regenerated *vitroplants* for optimization because of the following. We used calli browning to calculate mortality, but the interval was not as accurate as *vitroplants*. The LD50 on calli resulted in 6,93 mg L−1 R2 = 0,402, 1000 n, compared with a more precise LD50 of 3.771 mg for regenerated *vitroplants.* We believe browning triggered by radiation and tissue culture can have an oxidative background noise that masks the accurate lethal dose [24,25]. Our second issue was that calli regeneration was problematic, having nonbrown calli unable to regenerate after irradiation and stress selection; 139 calli that survived at 1 mg (97n) and 10 mg exposition (42n) did not regenerate (Table 1).

For the first attempt we used 60 Gy irradiation on calli and direct selection with 5 mg L^{-1} fluazifop-P-butyl but had no regeneration after 21 d (500 n, data not presented). Direct calli selection is an option with a recovery time after irradiation and a low selection agent dose to allow regeneration. Other authors used such rationale in rice but with the CRISPR/Cas9 technique at a maximum amount of 2 μM of a similar molecule haloxyfop-R-methyl (0,654 mg L^{-1}) for calli selection and further validation in a greenhouse of 150 mg L^{-1} [21,26]. In maize (*Zea mays* L.) and other grass, such as *Paspalum vaginatum*, selection was possible on calli with a different molecule acting on *ACC2* at 10 μM of sethoxydim [27-29].

In our case, a recovery step was preferred on regeneration medium for 21 days when having green calli and subsequent selection with fluazifop-P-butyl at 5 mg L^{-1}. The rationale is that investing 21 days in this previous step guaranteed predictable regeneration. The putative mutants had a second selection round that helped us better select stable mutants linked to tolerance. We considered a mutation stable when micropropagation resulted in 100% clonal offspring with tolerance to a higher concentration of the herbicide, 10 mg L^{-1}. We had 100 mutants, but only one had clonal offspring resistance. The plant had one mutation in the expected domain that has never been reported to be linked with APPs tolerance, T2222I/T2222M. It seems that such a putative mutation can potentially be a real linked mutation because of the nature of the mutation and the dose used. Threonine's uncharged polar nature differs from the hydrophobic nature of isoleucine or methionine. The tolerance at 150 mg/L is the dose of selection used by other authors linked to *ACCase* mutations [21]. New mutations are being discovered, such as I1879S, P1927Y and W2097G, very near our site of mutation [30]. However, we still cannot fully validate such a hypothesis because tolerance could also result from another detoxification mechanism, and we have not yet studied detoxification mediated by cytochrome P450, glutathione S-transferase, reduced absorption or reduced xylem or phloem translocation [31,32].

The proposed system of using APP tolerance could allow the selection of small genomic mutations of approximately 1 per 1000 bp, which seems to be valid and a starting point for working with cultivars that farmers are using. We foresee using whole genome

sequencing and analysis of the offspring of our mutant to accurately understand the mutation percentage and potential to create variability for the breeding program.

Our finding of using seeds instead of calli, which improved our method from 1 putative plant tolerant to APPs to 31 plants, could help create variability for even more difficult tissue culture cultivars for rice and other plants. The tissue was consistently damaged as the gamma dose increased, having brown nonembryogenic calli, which is common in stressed indica rice cells [33]. Having a window between 50 and 350 Gy also allows flexibility in the kind of mutations resulting from the DNA damage response (DDR) as well as the desired or required dose for a given trait [34].

4. Materials and Methods

4.1. Fluazifop-P-butyl toxicity to embryogenic calli

Embryogenic calli induction was achieved as previously described starting with seeds followed by disinfection with 4% (*v*/*v*) NaOCl and water [14]. The calli induction media was composed of mineral salts and vitamins as described by Murashige and Skoog (MS), with 20 gL^{-1} sucrose, 0.1 gL^{-1} hydrolyzed casein and 2.5 mgL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl, and 5.4 gL^{-1} Gelzan® was added as a gelling agent.

To determine the median lethal dose (LD50) of fluazifop-P-butyl, 200 calli per treatment were subcultured into 0, 1, 10, and 100 mgL^{-1} fluazifop-P-butyl in basal regeneration medium supplemented with 0.5 mg L^{-1} NAA + 3 mg L^{-1} 6-BA. All previously mentioned chemicals were supplied by Phytotechnology Laboratories® (Shawnee Mission, KS, USA). An autoclave (1.2 ATM. cm^{-2} and 121 °C for 30 min) was used for the sterilizing medium and further dispensed on 94 × 16 mm vented polystyrene Petri dishes (Greiner Bio-One, Fisher Scientific, Waltham, MA, USA) in a laminar flow chamber. Calli browning rates (brown or necrotic/total calli) were recorded as response variables and analyzed in a completely randomized design with a generalized linear model with a Poisson distribution and logit link function. Lethal doses were calculated using probit analysis on IBM SPSS version 27 [35] based on calli browning rates.

4.2. Fluazifop-P-butyl toxicity to vitroplants

The determination of the toxicity of fluazifop-P-buty for *vitroplants* was calculated based on regenerated *vitroplants* from embryogenic calli as described next. Embryogenic calli obtained as described in 4.1 were transferred to regeneration medium as described by Sudhakar et al. [36] and were constituted by MS mineral salts and vitamins, 20 gL^{-1} sucrose and 0.3 gL^{-1} hydrolyzed casein supplemented with 0.5 mg L^{-1} NAA + 3 mg L^{-1} 6-BA. To determine the median lethal dose (LD50), the 42-day regenerated vitroplants were subcultured into regenerated plants containing 0, 1, 4, 5, 10, 25 and 100 mg L^{-1} fluazifop-P-butyl. After adding the growth regulators NAA and BA, the pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl, and 5.4 g L^{-1} Gelzan® (Phytotechnology Laboratories®, Shawnee Mission, KS, USA) was added as a gelling agent. All previously mentioned reagents were supplied by Phytotechnology Laboratories® (Shawnee Mission, KS, USA). After properly dissolving the gelling agent, 60 mL of media was dispensed on 475 mL polypropylene WNA Deli Containers supplied by Phytotechnology Laboratories® (Shawnee Mission, KS, USA), and afterward, media was sterilized at 1.2 ATM.cm^{-2} and 121 °C for 30 min. Necrotic plants were considered dead after transplanting into fresh medium with no fluazifop-P-butyl for 100 days and remained necrotic. Lethal doses were calculated using probit analysis on IBM SPSS version 27 [35] based on calli browning rates.

4.3. Gamma irradiation of embryogenic calli

Embryogenic calli irradiation was achieved at 60 Gy with an Ob-Servo Ignis type gamma irradiator with 24 cobalt 60 source pencils (Institute of Isotopes Co, Ltd., Budapest,

Hungary). We tested 1000 embryogenic calli exposed to 60 Gy and transferred them to basal regeneration medium supplemented with 0.5 mg L^{-1} NAA + 3 mg L^{-1} 6-BA. A total of 8000 regenerated *vitroplants* were exposed to the same medium containing 5 mg L^{-1} fluazifop-P-butyl for 21 days. The putative tolerant *vitroplants* had 21 days of recovery in medium with no selection agent, followed by another round of selection of a higher dose of 10 mg-L fluazifop-P-butyl. The tolerant plant was taken to the greenhouse for acclimatization, grown until the 3-5 leaf stage and exposed to 150 ml/L spray fluazifop-P-butyl to validate tolerance, similar to Liu *et al.* [21].

4.4. Gamma irradiation of seeds

Seed irradiation was achieved at 0 to 500 Gy with an Ob-Servo Ignis type gamma irradiator as described above. We tested 100 seeds per dose, followed by callus induction and regeneration as described previously. Selection of the putative mutants was performed as described in 4.3.

4.5. Molecular markers

A NucleoSpin™ Tissue Kit Macherey-Nagel (Düren, Germany) was used for DNA extraction from 1 mg of nonirradiated lyophilized leaf tissue of Lazarroz FL. Thermo Fisher K1071 (Vilnius, Lithuania) was used for the subsequent PCR following the recommendations of the manufacturer. The primers used in this study are as follows: for 1844 bp amplification of *ACC2* Exon 32, *ACC2*-DF 5′-GGATCATTTGGCCCAAGGGA-3′ and *ACC2*-DR 5′-AGGGCTTGCAAATCTGAGCT-3′; for 1465 bp amplification of *ACC2* Exon 32, *ACC2*-F 5′-GTGCTCGAATTGGCATAGCAG-3′ and *ACC2*-R 5′-CGTGATTCTTCCCAGTCCACA-3′. The PCR master mix consisted of a mixture (50 μL) containing 1X Dream Taq Master mix Thermo Fisher (Vilnius, Lithuania), 20 μM of each primer, and 5 μL of DNA (50 ng/μL). The thermocycling program was 95 °C for 5 min, 40 cycles at 95 °C for 45 s, 55 °C for 100 s and 72 °C for 1 min, and a final cycle of 72 °C for 7 min.

PCR and sequencing of the *mat*K and *rbcL* genes of the mutants were performed as reported for the control sequences MZ558335 and MZ558334 [14].

5. Conclusions

In our previous work [14], we suggested the potential of gamma radiation to trigger innovation of rice lines used by farmers. Here, we proved the model with a specific trait based on chemical selection and showed the potential way to start searching for other mutations of interest on desired traits for commercially used varieties.

Author Contributions: Conceptualization, A.H.-S., D.M-N., J.P., and A.G.-A.; writing—original draft preparation, A.H.-S., J.P. and A.G.-A.; writing—review and editing, A.H.-S., J.P., A.G.-A., F.E.B, and A.A.-E.; visualization, A.H.-S. D.M-N. and J. P; investigation W.V.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Research Vice-Rectory of TEC, Costa Rica, project number 1510-1022.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.Ins

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Chauhan, B.S.; Jabran, K.; Mahajan, G. *Rice Production Worldwide*; Springer International Publishing: Cham, Switzerland, 2017; Volume 247. https://doi.org/10.1007/978-3-319-47516-5.
2. Wing, R.A.; Purugganan, M.D.; Zhang, Q. The Rice Genome Revolution: From an Ancient Grain to Green Super Rice. *Nat. Rev. Genet.* **2018**, *19*, 505–517. https://doi.org/10.1038/s41576-018-0024-z.
3. Hernández-Soto, A.; Echeverría-Beirute, F.; Abdelnour-Esquivel, A.; Valdez-Melara, M.; Boch, J.; Gatica-Arias, A. Rice Breeding in the New Era: Comparison of Useful Agronomic Traits. Curr. Plant Biol. **2021**, 27, 100211. https://doi.org/10.1016/j.cpb.2021.100211.

4. Nadir, S.; Xiong, H.B.; Zhu, Q.; Zhang, X.L.; Xu, H.Y.; Li, J.; Dongchen, W.; Henry, D.; Guo, X.Q.; Khan, S.; et al. Weedy Rice in Sustainable Rice Production. A Review. *Agron. Sustain. Dev.* **2017**, *37*, 46. https://doi.org/10.1007/s13593-017-0456-4.
5. Romero, F.M.; Gatica-Arias, A. CRISPR/Cas9: Development and Application in Rice Breeding. *Rice Sci.* **2019**, *26*, 265–281. https://doi.org/10.1016/j.rsci.2019.08.001.
6. Soriano, J.D. Mutagenic Effects of Gamma Radiation on Rice. *Bot. Gaz.* **1961**, *123*, 57–63.
7. Viana, V.E.; Pegoraro, C.; Busanello, C.; Costa de Oliveira, A. Mutagenesis in Rice: The Basis for Breeding a New Super Plant. *Front. Plant Sci.* **2019**, *10*, 1326. https://doi.org/10.3389/fpls.2019.01326.
8. Oladosu, Y.; Rafii, M.Y.; Abdullah, N.; Hussin, G.; Ramli, A.; Rahim, H.A.; Miah, G.; Usman, M. Principle and Application of Plant Mutagenesis in Crop Improvement: A Review. *Biotechnol. Biotechnol. Equip.* **2016**, *30*, 1–16. https://doi.org/10.1080/13102818.2015.1087333.
9. Zain, C.R.C.M.; Kadhimi, A.A.; Alhasnawi, A.N.; Isahak, A.; Mohamad, A.; Doni, F.; Yusoff, W.M.W. Enhancing of Drought-Tolerant Rice (Oryza sativa) Variety MRQ74 through Gamma Radiation and in vitro Pathway. *Biotech.* **2016**, 15, 125–134. https://doi.org/10.3923/biotech.2016.125.134.
10. Abdelnour-Esquivel, A.; Perez, J.; Rojas, M.; Vargas, W.; Gatica-Arias, A. Use of Gamma Radiation to Induce Mutations in Rice (*Oryza sativa* L.) and the Selection of Lines with Tolerance to Salinity and Drought. *In Vitro Cell. Dev. Biol.—Plant* **2020**, *56*, 88–97. https://doi.org/10.1007/s11627-019-10015-5.
11. Fehér, A. Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? *Front. Plant Sci.* **2019**, *10*, 536. https://doi.org/10.3389/fpls.2019.00536.
12. Binte Mostafiz, S.; Wagiran, A. Efficient Callus Induction and Regeneration in Selected *Indica* Rice. *Agronomy* **2018**, *8*, 77. https://doi.org/10.3390/agronomy8050077.
13. Vega, R.; Vásquez, N.; Espinoza, A.M.; Gatica, A.M.; Valdez-Melara, M. Histology of Somatic Embryogenesis in Rice (*Oryza sativa* cv. 5272). *Rev. Biol. Trop.* **2009**, *57* (Suppl. S1), 141–150. https://doi.org/10.15517/rbt.v57i0.21291.
14. Hernández Soto, A.; Pérez, J.; Fait Zúñiga, R.; Rojas Vásquez, R.; Gatica Arias, A.; Vargas Segura, W.; Abdelnour Esquivel, A. A Temporary Immersion System Improves Regeneration of In Vitro Irradiated Recalcitrant Indica Rice (Oryza Sativa L.) Embryogenic Calli. Plants **2022**, 11 (3), 375. https://doi.org/10.3390/plants11030375.
15. Lancaster, Z. D.; Norsworthy, J. K.; Scott, R. C. Residual Activity of ACCase-Inhibiting Herbicides on Monocot Crops and Weeds. *Weed Technology* **2018**, *32* (4), 364–370. https://doi.org/10.1017/wet.2018.13.
16. Delye, C.; Zhang, X.-Q.; Michel, S.; Matejicek, A.; Powles, S. B. Molecular Bases for Sensitivity to Acetyl-Coenzyme. Plant Physiol. **2005**, 137 (March), 794–806. https://doi.org/10.1104/pp.104.046144.as.
17. Powles, S. B.; Yu, Q. Evolution in Action: Plants Resistant to Herbicides. *Annu. Rev. Plant Biol.* **2010**, 61, 317-347. https://doi.org/10.1146/annurev-arplant-042809-112119.
18. de Andrade, A.; Tulmann-Neto, A.; Tcacenco, F. A.; Marschalek, R.; Pereira, A.; de Oliveira Neto, A. M.; Scheuermann, K. K.; Wickert, E.; Noldin, J. A. Development of Rice (*Oryza Sativa*) Lines Resistant to Aryloxyphenoxypropionate Herbicides through Induced Mutation with Gamma Rays. *Plant Breeding* **2018**, *137* (3), 364–369. https://doi.org/10.1111/pbr.12592.
19. Jin, M.; Chen, L.; Wang, X.; Tang, X. Development of Herbicide Resistance Genes and Their Application in Rice. *The Crop Journal* **2022**, *10* (1), 26–35. https://doi.org/10.1016/j.cj.2021.05.007.
20. Zhang, R.; Liu, J.; Chai, Z.; Chen, S.; Bai, Y.; Zong, Y.; Chen, K.; Li, J.; Jiang, L.; Gao, C. Generation of Herbicide Tolerance Traits and a New Selectable Marker in Wheat Using Base Editing. *Nat. Plants* **2019**, *5* (5), 480–485. https://doi.org/10.1038/s41477-019-0405-0.
21. Liu, X.; Qin, R.; Li, J.; Liao, S.; Shan, T.; Xu, R.; Wu, D.; Wei, P. A CRISPR-Cas9-Mediated Domain-Specific Base-Editing Screen Enables Functional Assessment of ACCase Variants in Rice. *Plant Biotechnology Journal* **2020**, *18* (9), 1845–1847. https://doi.org/10.1111/pbi.13348.
22. Camacho, J. R.; Linscombe, S. D.; Sanabria, Y.; Mosquera, P. A.; Oard, J. H. Inheritance of Provisia™ Rice Resistance to Quizalofop-p-Ethyl under Laboratory and Greenhouse Environments. *Euphytica* **2019**, *215* (4), 83. https://doi.org/10.1007/s10681-019-2407-4.
23. Orozco, A. Z.; Carrodeguas, A. Pacífico Central , Costa Rica Morpho-Agronomic Variability in Rice Genotypes in the Central Pacific, Costa Rica. *Ciencia y Agricultura* **2022**, *19* (1), 1–15. https://doi.org/https://doi.org/10.19053/01228420.v19.n1.2022.12567
24. Xie, X.; He, Z.; Chen, N.; Tang, Z.; Wang, Q.; Cai, Y. The Roles of Environmental Factors in Regulation of Oxidative Stress in Plant. *BioMed Res. Int.* **2019**, *2019*, 21–27. https://doi.org/10.1155/2019/9732325
25. Debnath, S.; Chandel, R.K.; Devi, K.; Khan, Z. Mechanism and Molecular Response of Induced Genotoxicity and Oxidative Stress in Plants. In *Induced Genotoxicity and Oxidative Stress in Plants*; Khan, Z., Ansari, M.Y.K., Shahwar, D., Eds.; Springer: Singapore, **2021**. https://doi.org/10.1007/978-981-16-2074-4_8.
26. Li, C.; Zong, Y.; Wang, Y.; Jin, S.; Zhang, D.; Song, Q.; Zhang, R.; Gao, C. Expanded Base Editing in Rice and Wheat Using a Cas9-Adenosine Deaminase Fusion. *Genome Biology* **2018**, *19* (1), 1–9. https://doi.org/10.1186/s13059-018-1443-z.
27. Heckart, D. L.; Parrott, W. A.; Raymer, P. L. Obtaining Sethoxydim Resistance in Seashore Paspalum. *Crop Science* **2010**, *50* (6), 2632–2640. https://doi.org/10.2135/cropsci2010.02.0080.
28. Parker, W. B.; Somers, D. A.; Wyse, D. L.; Keith, R. A.; Burton, J. D.; Gronwald, J. W.; Gengenbach, B. G. Selection and Characterization of Sethoxydim-Tolerant Maize Tissue Cultures. *Plant Physiology* **1990**, *92* (4), 1220–1225. https://doi.org/10.1104/pp.92.4.1220
29. Ostlie, M.; Haley, S. D.; Anderson, V.; Shaner, D.; Manmathan, H.; Beil, C.; Westra, P. Development and Characterization of Mutant Winter Wheat (Triticum Aestivum L.) Accessions Resistant to the Herbicide Quizalofop. *Theoretical and Applied Genetics* **2014**, *128* (2), 343–351. https://doi.org/10.1007/s00122-014-2434-4.
30. Xu, R.; Liu, X.; Li, J.; Qin, R.; Wei, P. Identification of Herbicide Resistance OsACC1 Mutations via in Planta Prime-Editing-Library Screening in Rice. *Nat. Plants* **2021**, *7* (7), 888–892. https://doi.org/10.1038/s41477-021-00942-w.
31. Gaines, T. A.; Duke, S. O.; Morran, S.; Rigon, C. A. G.; Tranel, P. J.; Küpper, A.; Dayan, F. E. Mechanisms of Evolved Herbicide Resistance. JBC **2020**, 295 (30), 10307–10330. https://doi.org/10.1074/jbc.REV120.013572.
32. Takano, H. K.; Ovejero, R. F. L.; Belchior, G. G.; Maymone, G. P. L.; Dayan, F. E. ACCase-Inhibiting Herbicides: Mechanism of Action, Resistance Evolution and Stewardship. Sci. Agric. **2020**, 78 (1), 1–11. https://doi.org/10.1590/1678-992x-2019-0102.
33. Binte Mostafiz, S.; Wagiran, A. Efficient Callus Induction and Regeneration in Selected *Indica* Rice. *Agronomy* **2018**, *8*, 77. https://doi.org/10.3390/agronomy8050077
34. Kim, J. H.; Ryu, T. H.; Lee, S. S.; Lee, S.; Chung, B. Y. Ionizing Radiation Manifesting DNA Damage Response in Plants: An Overview of DNA Damage Signaling and Repair Mechanisms in Plants. *Plant Science* **2019**, *278*, 44–53. https://doi.org/10.1016/j.plantsci.2018.10.013.

35. IBM Corp. *Released 2020*. *IBM SPSS Statistics for Windows*, Version 27.0; IBM Corp: Armonk, NY, USA, 2020.
36. Sudhakar, D.; Duc, L.T.; Bong, B.B.; Tinjuangjun, P.; Maqbool, S.B.; Valdez, M.; Jefferson, R.; Christou, P. An Efficient Rice Transformation System Utilizing Mature Seed-Derived Explants and a Portable, Inexpensive Particle Bombardment Device. *Transgenic Res.* **1998**, *7*, 289–294. https://doi.org/10.1023/A:1008870012568.

fermentation

MDPI

Article

NTH2 1271_1272delTA Gene Disruption Results in Salt Tolerance in *Saccharomyces cerevisiae*

Alejandro Hernández-Soto [1,*], José Pablo Delgado-Navarro [2], Miguel Benavides-Acevedo [3], Sergio A. Paniagua [4] and Andres Gatica-Arias [3,5,6]

[1] Doctorado en Ciencias Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia, Cartago P.O. Box 159-7050, Costa Rica
[2] Costa Rica Institute of Technology, Biology School, Biotechnology Research Center (CIB), Cartago P.O. Box 159-7050, Costa Rica; jp2996@hotmail.com
[3] Laboratory of Plant Biotechnology, School of Biology, University of Costa Rica, San José P.O. Box 11501-2060, Costa Rica; miguel.benavides@ucr.ac.cr (M.B.-A.); andres.gatica@ucr.ac.cr (A.G.-A.)
[4] Centro Nacional de Alta Tecnología CeNAT, Laboratorio Nacional de Nanotecnología LANOTEC, San José P.O. Box 1174-1200, Costa Rica; sergian@gmail.com
[5] Research Center in Cellular and Molecular Biology (CIBCM), University of Costa Rica, San José P.O. Box 11501-2060, Costa Rica
[6] Programa de Posgrado en Biología (PPB), School of Biology, University of Costa Rica, San José P.O. Box 11501-2060, Costa Rica
* Correspondence: alhernandez@itcr.ac.cr

Abstract: Trehalose is a common energy reservoir, and its accumulation results in osmotic protection. This sugar can accumulate through its synthesis or slow degradation of the reservoir by trehalase enzymes. *Saccharomyces cerevisiae* contains two neutral trehalases, *NTH1* and *NTH2*, responsible for 75% and 25% of the enzymatic metabolism. We were interested in the loss-of-function of both enzymes with CRISPR/Cas9. The later *NTH2* was of great importance since it is responsible for minor metabolic degradation of this sugar. It was believed that losing its functionality results in limited osmotic protection. We constructed an osmotolerant superior yeast capable of growing in 0.85 M NaCl after independent *nth2 1271_1272delTA* mutation by CRISPR/Cas9 technology, compared with *nth1 893_894insT* and wild type. We suggest that this yeast model could give clues to breeding commercial yeast resulting in non-GMO salinity-tolerant strains.

Keywords: mutation; precision biotechnology; stress tolerance; osmotolerance

Citation: Hernández-Soto, A.; Delgado-Navarro, J.-P.; Benavides-Acevedo, M.; Paniagua, S.-A.; Gatica-Arias, A. *NTH2* 1271_1272delTA Gene Disruption Results in Salt Tolerance in *Saccharomyces cerevisiae*. *Fermentation* **2022**, *8*, 166. https://doi.org/10.3390/fermentation8040166

Academic Editor: Alessandro Robertiello

Received: 17 January 2022
Accepted: 30 March 2022
Published: 5 April 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a stress-tolerance-related sugar [1]. This nonreductive disaccharide, composed of two molecules of D-glucose, is also common in the metabolism of bacteria, fungi, plants, and some invertebrate animals [2–4]. Trehalose protects against structural disorders during water removal. It has been suggested that the hydroxyl groups of trehalose form hydrogen bonds with polar lipids and proteins instead of water, protecting these hydroxyl groups from structural disorder during water removal [5]. Trehalose can also work indirectly by stabilizing heat shock proteins during the refolding of damaged proteins [6]. Trehalose acts as a cryoprotectant to avoid aggregation by stabilizing proteins in a chaperone-like manner [5,7–10].

The intracellular accumulation of this disaccharide is due to the trehalose synthesis complex: *TPS1*, *TPS2*, *TPS3*, and T*SL1* [11]. However, the *NTH1*, *NTH2*, and *ATH1* genes are responsible for degrading trehalose and are also positively regulated under stress conditions, creating a metabolic cycle of carbohydrate regulation whose understanding is still controversial [12,13]. A high intracellular concentration of this molecule results in

protecting the structures of biomolecules and the blockage of proteases while allowing better recovery under stress [12,14,15]. Otherwise, the excessive accumulation of trehalose obstructs the active transport of glucose and inhibits growth in yeast [1,16].

Saccharomyces cerevisiae was used as our model organism for a proof-of-concept study to achieve salt-tolerant phenotypic traits using CRISPR/Cas9 editing and independent disruption of *NTH2* compared to wild type and *NTH1* disruption. A CRISPR-derived mutant is considered conventional in many legal frameworks like Brazil, allowing further breeding of commercial strains with no regulatory constraints [17]. We avoided other techniques such as homolog recombination because it could result in a genetically modified organism (GMO) limiting its industrial use.

Three enzymes are responsible for trehalose degradation in yeast: *NTH1* and *NTH2* are cytosolic neutral pH orthologs, while the third ortholog, *ATH1*, is a vacuolar acid pH enzyme [12,18]. The neutral trehalases *NTH1* and *NTH2* are responsible for 75% and 25% of the intracellular hydrolysis of carbohydrates, respectively [19]. We selected salt tolerance since yeast tends to decrease in viability under sodium chloride osmotic stress conditions. The viability of *S. boulardii* decreased at 0.4 M NaCl [20], and *S. cerevisiae* showed decreased viability at 0.68 M NaCl (4% *m*/*v*) [21].

Previous research on trehalases has dismissed the importance of *NTH2* because of its low expression and has instead focused on *NTH1* [7,22,23]. It was proposed that *NTH1* is the only protein that performs hydrolysis activity but that it works together with *NTH2* for hydrolysis recovery after exposure to high temperatures [14,15,24]. The *NTH1* and *NTH2* genes encode functional trehalases, with *NTH2* playing a protagonist role in the stationary phase [18]. It has also been proposed that eliminating the *NTH1* gene and overexpressing tps1 (trehalose-6-phosphate synthase) increases trehalose and improves survival in high ethanol concentrations [7,22]. The deletion of all three of these enzymes results in intracellular trehalose accumulation and improved salt stress recovery [12]. The triple deletion of trehalases in yeast leads to tolerance of high concentrations of ethanol and high temperatures and higher viability after freezing [25]. Eliminating the *NTH1* and *NTH2* genes together in *S. cerevisiae* results in up to 180 days of resistance under stress conditions [26]. However, it seemed that eliminating the *NTH2* gene resulted in no salt tolerance [13]. Finally, accumulating trehalose resulting from neutral trehalases being knocked out then affects the growth of yeast [16].

In this article, we report a CRISPR/Cas9-specific *NTH2 1271_1272delTA* disruption in *S. cerevisiae* that results in the capacity of the yeast to grow in 0.85 M NaCl and tolerate 1.2M NaCl in comparison with non-mutant and mutant *NTH1 893_894insT* strains. We propose that disrupting *NTH2* alone with our design in yeast can trigger stress tolerance, although the mechanism remains unknown. To our knowledge, there is no formal report linking the disruption of *NTH2* alone to stress tolerance in *S. cerevisiae*. The knowledge generated herein is potentially valuable to improve industrial yeast or serve as a model to develop osmotic stress tolerance in other organisms using a similar rationale.

2. Materials and Methods

2.1. The Strain, Medium and Growth Conditions

S. cerevisiae CEN.PK2-1C (*MATa; his3D1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2*) was used for all experiments. Cultures were grown in YPD medium (2% yeast extract, 1% peptone, and 2% dextrose), YPD agar (2% yeast extract, 1% peptone, 2% dextrose, and 2.2% agar) or YPAD (2% yeast extract, 1% peptone, 2% dextrose, and 40 mg/L adenine hemisulfate) at 30 °C at 30 °C at 200 rpm on an orbital shaker (Digisystem Laboratory Instruments Inc.). All reagents were purchased from Thermo Fisher Scientific® (Thermo Fisher, Carlsbad, CA, USA).

2.2. Preparation of the CRISPR Plasmid

Plasmid *bRA89*, containing *Streptococcus pyogenes* Cas9, a single guide scaffold, and hygromycin B resistance, was purchased from Addgene [27]. The single guide targeting the gene *NTH1 (YDR001C,* strain *ATCC 204508 / S288c)* and *NTH2* (YBR001C, strain R64-1-1.80) was designed using the CRISPRdirect Platform [28]. Recognition of the single guide insertion for the *NTH2* gene (5′-TGCTATTAAAGAATATAAAG [AGG]-3′) and *NTH1* (5′-GGTTACCCTTATGCTGTTCC [TGG]-3′) were cloned into bRA89 at Genscript, just below the RNA scaffold section, using *BplI* restriction sites. Sanger sequencing confirmed the insertion of the single guide section next to the 5′ end of the RNA scaffold. More details are provided in the Supplementary Materials (Figure S1, Figure S2).

2.3. S. cerevisiae Competent Cell Preparation and Transformation

Competent cells were prepared and transformed using the lithium acetate (LiAc) method with the following modifications [29]. An aliquot of approximately 1×10^7 cells/mL (16 h culture) was inoculated in 50 mL of YPD medium and incubated until reaching an OD (600 nm) of 0.5. Then, the biomass was harvested by centrifugation (4000 rpm) for 10 min at room temperature, washed twice in 10 mL of sterile distilled water, and resuspended in 1.5 mL of sterile lithium acetate buffer (1 volume of TE 10X buffer, pH 7.5; 1 volume of 1 M 10X LiAc, pH 7.5 and 8 volumes of distilled water). Freshly prepared competent yeast cells (200 µL) were mixed with 1µg of the final plasmid and combined with 200 µg of denatured Salmon Sperm DNA (Thermo Fisher, Carlsbad, CA, USA) and 1 mL of fresh PEG buffer (8 volumes of PEG 3350 50% and 1 volume of TE 10X buffer, pH 7.5; 1 volume of 1 M LiAc 10X, pH 7.5). The solution was incubated at 150 rpm for 30 min at 25 °C and then heated to 42 °C for exactly 15 min. Subsequently, the solution was resuspended in 200 µL of YPAD medium and incubated for another 45 min at 25 °C. Finally, the cells were cultured on YPD agar plates supplemented with hygromycin B Phytotechnology Laboratories® (*Shawnee Mission, Kansas, USA*) at 500 µg mL^{-1} for 72 h at 28 °C in an incubator.

2.4. Selection and Sequencing Confirmation of Mutants

Thirty (30) randomly selected strains were grown on YPD overnight media. Subsequently, the manufacturer's instructions obtained genomic DNA using the ReliaPrepTM gDNA Tissue Miniprep System (Promega, USA). Specific primers flanking the target sgRNA site of the *NTH2* gene were designed and named *nth2f*: 5′-GCAA-GAGGTATGGTGGAGCA-3′ and *nth2r*: 5′-TTCAGCTAGCTCCTCCCAGT-3′ (Tm 55 °C; 539 bp); while *NTH1* primers flanking the target sgRNA site were also designed and named *nth1f*: 5′-ACCCCCGGTTTACTAGCATTG-3′ and *nth1r*: 5′-TAAGGTAAC-GCCGTGTTTCGA-3′ (Tm 55 °C; 528 bp). Sanger sequencing of PCR products was performed in Macrogen at Rockville, MD, USA, to confirm the mutation and absence of off-targets. We selected two isolates with the same mutation on the *NTH2*-disrupted gene and intact *NTH1* gene, named *nth2 1271_1272delTA*. Similarly, we selected two isolates with the *NTH1*-disrupted gene and intact *NTH2* gene, named *nth1 893_894insT*.

2.5. nth1 893_894insT and nth2 1271_1272delTA Strain Phenotypes

The methylene blue staining technique allowed examination of the viability of the wild-type and mutated yeast strains in 0.85 M NaCl versus the control (0 M NaCl) on Potato Dextrose (PD) (20%Potato, 2% dextrose) for 48 h at 28 °C. Growth curves of the samples were generated with 0 and 0.85 M NaCl in the YPD medium. The test was carried out with a SPECTROstarNANO plate reader from BMG LABTECH at 200 rpm and 30 °C for 24 h, with four repetitions per variant. The growth curve comparisons resulted from *T*-test analysis with and without saline stress conditions of the wild-type and mutated strains. The comparison also consisted of seriated dilutions on agar PD plate-based comparison after two weeks of growth under stress (0.85 M and 1.2M NaCl). Trehalose content

was extracted following the protocol described by Divate et al. [22] with the following variations. The cells were grown after 0.85 M NaCl versus the control (0 M NaCl) on PD for 48 h at 28 °C, collected by centrifugation at 7000× g for 5 min and dried at 100°C for 12 h. A pellet of 50mg was mixed with 1 mL ethanol (99.5%) and incubated in a boiling water bath for 1 h. HPLC-RID determined the content, P-SA-MQ-006 provided by CITA-UCR, as follows: The ethanolic extract was centrifuged and filtered through a 0.20 μm regenerated cellulose micropore (17761-Q, Minisart-RC15®, Sartorius AG, Göttingen, Germany), the filtrate was collected in a 2 mL vial for HPLC. AC Chromatographic separation was performed using Agilent Technologies 1260 Infinity liquid chromatograph equipped with Suplecogel 8Ca high resolution column (300 mm × 7.8 mm, 8 μm, PN 59247-U), quaternary pump (G1311B), column compartment (G1316A), automatic liquid sampling module (ALS, G7129A) and refractive index detector (G1362A) (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of ultrapure water (type I, 0.055 μS cm−1 at 25 ◦C, 5 μg L−1 TOC) obtained using A10 Milli-Q Advantage and Elix 35 purification system (Merck KgaA, Darmstadt, Germany). Solvent flow, column compartment temperature, detector cell temperature, and injection volume were constant during the elution at 0.40 mL min^{-1}, 80 °C, 40 °C, and 10 μL, respectively. The area under the curve (AUC) of the trehalose signal in the samples was interpolated in the calibration curve of the certified reference standard (Sigma-Aldrich, PHR1344-500 mg), in a concentration range of 0.025 to 0.25 g/100mL.

2.6. Statistical Software

Minitab v.19.1.1 supported statistical analysis and RStudio v.1.1.423 was used for visualization.

2.7. Scanning Electron Microscopy (SEM)

Wild-type and mutated yeast grown in 0.00 and 0.85M NaCl and fixed with Karnovsky fixative (2.5% *w*/*v* of glutaraldehyde, 0.1 M of paraformaldehyde in phosphate buffer pH 7.4, during 48 h at 4 °C) were mounted on carbon tape and sputtered with gold using a Denton Vacuum Desk V sputter system at 20 mA for 300 s. Images were taken using a JSM-6390LV (JEOL, Tokyo, Japan) scanning electron microscope with an accelerating voltage of 15 kV under high vacuum. Scanning electron microscopy images at different resolutions of the wild-type and *NTH2*-mutated yeasts and subsequent cell area calculations were analyzed with ImageJ version 1.52p.

2.8. Transmission Electron Microscopy (TEM)

Wild-type and mutated yeast grown with and without NaCl were treated with Karnovsky fixative for six days. The yeast was then centrifuged at 3000 rpm for 5 min and rinsed with 0.1 M phosphate buffer solution (pH 7.2) for three 15 min wash/centrifugation cycles. The yeast samples were then stirred for one hour in 2% *w*/*v* OsO_4 in 0.1 M phosphate buffer solution, followed by three wash cycles as per the previous step but with type 3 water instead of buffer. The yeasts were finally isolated via centrifugation at 3000 rpm for 10 min and subsequently embedded in a solution of agar/agarose 4% w/v in a hot water bath (50 °C). After cooling to 37 °C, one drop of the solution was added to an Eppendorf tube containing 100 μL of the yeast sediment and mixed thoroughly. After cooling, the solids were removed and cut into 3 mm^3 segments. Dehydration was performed by rinsing in an ascending gradient of acetone (30% to 100% *v*/*v*) followed by infiltration with Spurr resin: acetone 50:50 overnight and three successive infiltrations with pure Spurr resin for two hours each. The resulting solids were transferred to BEEM embedding capsules. Polymerization was achieved in an oven at 70 °C for 24 h. Ultrafine segments (approximately 70 nm) were cut with a Leica EM UC7 ultramicrotome with a diamond knife and supported on Cu TEM grids (200 mesh). The sections were stained with 4% *m*/*v* uranyl acetate in 50% *v*/*v* ethanol for 15 min and washed five times in DI water, followed

by soaking for 10 min in Reynold's stain and rinsing five times in water. Once dry, the grids were mounted in a JEOL JEM 2011 TEM and observed at 120 kV at magnifications of 3000–20,000×.

3. Results

3.1. Confirmation of NTH1-NTH2 Gene-Mutated Cells

NTH1 and *NTH2* disruption were confirmed in the randomly selected strains. We selected two isolates of *NTH2* mutant strains named *nth2 1271_1272delTA* containing the same mutation, a deletion of two bases, TA, located at positions 1271-1272, three bases downstream from the PAM site expected. A PCR of the target sequence (Figure 1A) and Sanger sequencing confirmed that the deletion was present in only the strains treated with the CRISPR/Cas9 technique directed through the single guide RNA complementary to section 1258 to 1276 of the *NTH2* compared with the chromosome 2 sequence. Sequencing also confirmed that the open reading frame disruption results from forming the TAA triplet in the respective open reading frame, creating a stop codon at amino acid 424 (Figure 1B). Additionally, the integrity of the *NTH1* gene was verified in the *NTH2*-mutated strain by PCR and sequencing of the specific mutation zone. The *S. cerevisiae NTH1* gene was not altered due to CRISPR/Cas9 genome editing in the strain *nth2 1271_1272delTA.*

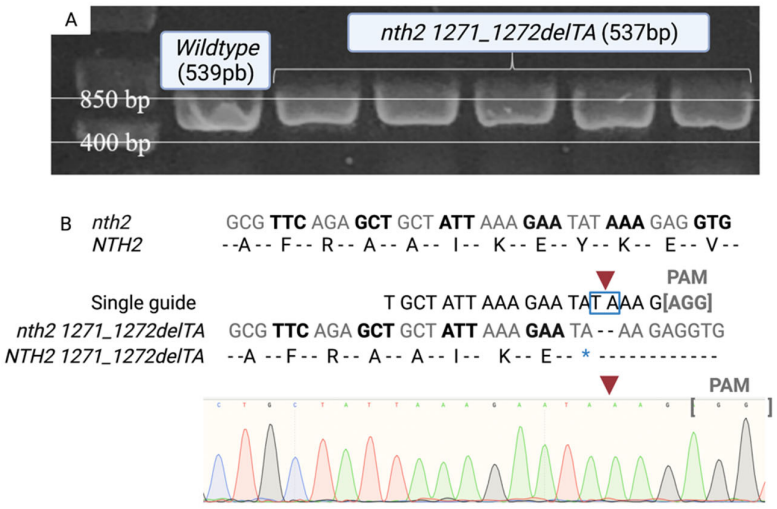

Figure 1. Confirmation of *NTH2* gene disruption in *S. cerevisiae* by CRISPR/Cas9. (**A**). Amplification of the segment containing the expected mutation site on a 1% *m/v* agarose gel; (**B**). Sequencing of the *S. cerevisiae NTH2* gene. In the box, it can be observed how the deletion 1271_1272delTA caused the Y424X mutation in the *nth2 1271_1272delTA* strain. Image created with BioRender.com (accessed on March 30th, 2022).

The *NTH1* mutant strains named *nth1 893_894insT*, consisted of three strains containing an insertion of one base, T, located at a position three bases downstream from the PAM site, as expected. The insertion results in disruption of the open reading frame three triplets downstream TAG, resulting in a truncated 301 AA protein with SWW* instead of PGGR (Figure 2).

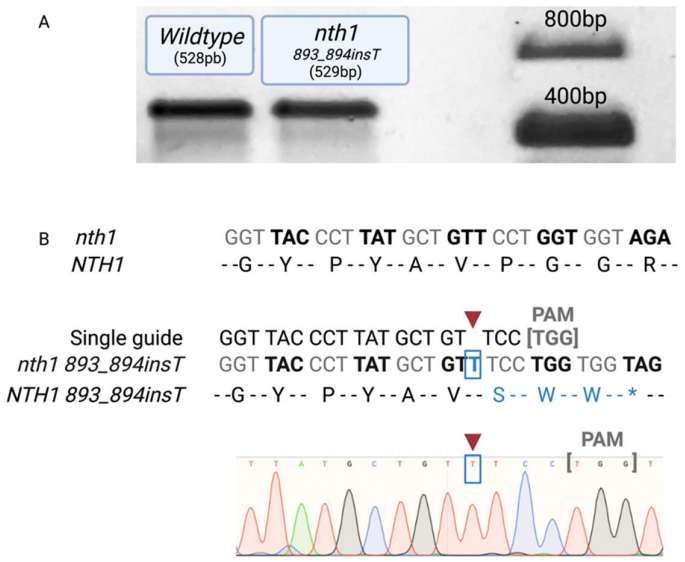

Figure 2. Confirmation of *NTH1* gene disruption in *S. cerevisiae* by CRISPR/Cas9. (**A**). Amplification of the segment containing the expected mutation site on a 1% *m/v* agarose gel; (**B**). Sequencing of the *S. cerevisiae NTH1* gene revealed the insertion of a T in position 893 resulting in a truncated 301 AA protein with SWW* instead of PGGR. Image created with BioRender.com (accessed on March 30th, 2022).

Mutation on the *NTH1* and *NTH2* genes showed disruption and absence of the active sites for a putative first ORF protein translated, specifically amino acid residues D478, E674 for *NTH1*, and D507 and E703 for *NTH2*. The *nth2 1271_1272delTA* may produce a small truncated 423 amino acid protein instead of the complete enzyme, lacking biochemical function. Similarly, the insertion in *NTH1* results in a knockout of three triplets downstream of TAG, resulting in a truncated 301 AA with no disaccharide binding site. The truncated sequence of mutants *nth1 893_894insT* and *nth2 1271_1272delTA* may still contain the Ca2+ binding domain if the three-dimensional conformation is unchanged.

When comparing the resulting putative Open Reading Frames, we noted that phosphorylation activation of the enzymes remains in the amino terminal that corresponds to the first disrupted ORF of both *nth1 893_894insT* and *nth2 1271_1272delTA*. A putative, predicted second ORF corresponding to the carboxyterminal end is different for each mutant. In the case of *nth1 893_894insT,* the second ORF may have all the binding sites (R302, N346, E424, R473, G476), active sites (D507, E703), and substrate binding sites (338-339WD, 384-386RSQ) in the second ORF. In the case of *nth2 1271_1272delTA*, the sites may be spliced by having one binding site (G505) and the active sites in the second ORF (D478, E674), but most binding sites (R331, N375, R384) and all substrate binding sites (309-310WD, 355-357RSQ) in the first ORF (Table 1).

Table 1. Predicted Open Reading Frames (ORF) of mutants in comparison with the wild-type *NTH1* and *NTH2* genes.

Gene	Open Reading Frames
NTH1 wild type	MSQVNTSQGPVAQGRQRRLSSLSEFNDPFSNAEVYYGPPTDPRKQKQAK- PAKINRTRTMSVFDNVSPFKKTGFGKLQQTRRGSEDDTYSSSQGNRRFFIEDVDKTLNELLAAEDTDKNYQITIEDTGPKVLKVGTANSY GYKHINIRGTYMLSNLLQELTIAKSFGRHQIFLDEAR- INENPVNRLSRLINTQFWNSLTRRVDLNNVGEIAKDTKIDTPGAKNPRIYVPYDCPEQYEFYVQASQMHPSLKLEVEYLPKKITAEYVKS VNDTPGLLALAMEEHFNPSTGEKTLIGYPYAVPGGRFNELYGWDSYMMALGLLE- ANKTDVARGMVEHFIFEINHYGKILNANRSYYLCRSQPPFLTEMALVVFKKLGGRSNPDAVDLLKRAFQASIKEYKTVWTASPRLDPET GLSRYHPNGLGIPPETESDHFDTVLLPYASKHGVTLDEFKQLYNDGKIKEPKLDEFFLH- DRGVRESGHDTTYRFEGVCAYLATIDLNSLLYKYEIDIADFIKEFCDDKYEDPLDHSITTSAMWKEMAKIRQEKITKYMWDDESGFFFDY NTKIKHRTSYESATTFWALWAGLATKEQAQKMVEKALPKLEMLGGLAACTERSRGPISIS- RPIRQWDYPFGWAPHQILAWEGLRSYGYLTVTNRLAYRWLFMMTKAFVDYNGIVVEKYDVTRGTDPHRVEAEYGNQGADFKGAAT EGFGWVNASYILGLKYMNSHARRALGACIPPISFFSSLRPQERNLYGL
nth1 893_894insT, ORF1	MSQVNTSQGPVAQGRQRRLSSLSEFNDPFSNAEVYYGPPTDPRKQKQAK- PAKINRTRTMSVFDNVSPFKKTGFGKLQQTRRGSEDDTYSSSQGNRRFFIEDVDKTLNELLAAEDTDKNYQITIEDTGPKVLKVGTANSY GYKHINIRGTYMLSNLLQELTIAKSFGRHQIFLDEAR- INENPVNRLSRLINTQFWNSLTRRVDLNNVGEIAKDTKIDTPGAKNPRIYVPYDCPEQYEFYVQASQMHPSLKLEVEYLPKKITAEYVKS VNDTPGLLALAMEEHFNPSTGEKTLIGYPYAVSWW
nth1 893_894insT, ORF2	MLFPGGRFNELYGWDSYMMALGLLEANKTDVARGMVEHFIFEINHYG- KILNANRSYYLCRSQPPFLTEMALVVFKKLGGRSNPDAVDLLKRAFQASIKEYKTVWTASPRLDPETGLSRYHPNGLGIPPETESDHFDT VLLPYASKHGVTLDEFKQLYNDGKIKEPKLDEFFLHDRGVRESGHDTTYRFEGVCAYLATID- LNSLLYKYEIDIADFIKEFCDDKYEDPLDHSITTSAMWKEMAKIRQEKITKYMWDDESGFFFDYNTKIKHRTSYESATTFWALWAGLAT KEQAQKMVEKALPKLEMLGGLAACTERSRGPISISRPIRQWDYPFGWAPHQILAWEGLRSY- GYLTVTNRLAYRWLFMMTKAFVDYNGIVVEKYDVTRGTDPHRVEAEYGNQGADFKGAATEGFGWVNASYILGLKYMNSHARRALG ACIPPISFFSSLRPQERNLYGL
NTH2 wild type	MVDFLPKVTEINPPSEGNDGEDNIKPLSSGSEQRPLKEEGQQG- GRRHHRRLSSMHEYFDPFSNAEVYYGPITDPRKQSKIHRLNRTRTMSVFNKVSDFKNGMKDYTLKRRGSEDDSFLSSQGNRRFYIDNVD LALDELLASEDTDKNHQITIEDTGPKVIKVGTANSNGFKHVNVRGTYMLSNLLQELTIAKS- FGRHQIFLDEARINENPVDRLSRLITTQFWTSLTRRVDLYNIAEIARDSKIDTPGAKNPRIYVPYNCPEQYEFYIQASQMNPSLKLEVEYLP KDITAEYVKSLNDTPGLLALAMEEHVNPSTGERSLVGYPYAVPGGRFNE- LYGWDSYLMALGLIESNKVDVARGMVEHFIFEIDHYSKILNANRSYYLCRSQPPFLTDMALLVFEKIGGKNNPNAIQLLKRAFRAAIKE YKEVWMSSPRLDSLTGLSCYHSDGIGIPPETEPDHFDTILLPYAEKYNVTLEKLRYLYNEG- MIKEPKLDAFFLHDRAVRESGHDTTYRFEGVCAYLATIDLNSLLYKYEKDIAFVIKEYFGNEYKDENDGTVTDSEHWEELAELRKTRIN KYMWDEDSGFFFYYNTKLKCRTSYESATTFWSLWAGLATEEQAKITVEKALPQLEMLG- GLVACTEKSRGPISIDRPIRQWDYPFGWAPHQILAWKGLSAYGYQQVATRLAYRWLYMITKSFVDYNGMVVEKYDVTRGTDPHRVDA EYGNQGADFKGVATEGFGWVNTSYLLGLKYMNNHARRALAACSPPLPFFNSLKPSEKKLYYL
nth2 1271_1272delTA, ORF1	MVDFLPKVTEINPPSEGNDGEDNIKPLSSGSEQRPLKEEGQQG- GRRHHRRLSSMHEYFDPFSNAEVYYGPITDPRKQSKIHRLNRTRTMSVFNKVSDFKNGMKDYTLKRRGSEDDSFLSSQGNRRFYIDNVD LALDELLASEDTDKNHQITIEDTGPKVIKVGTANSNGFKHVNVRGTYMLSNLLQELTIAKS- FGRHQIFLDEARINENPVDRLSRLITTQFWTSLTRRVDLYNIAEIARDSKIDTPGAKNPRIYVPYNCPEQYEFYIQASQMNPSLKLEVEYLP KDITAEYVKSLNDTPGLLALAMEEHVNPSTGERSLVGYPYAVPGGRFNE- LYGWDSYLMALGLIESNKVDVARGMVEHFIFEIDHYSKILNANRSYYLCRSQPPFLTDMALLVFEKIGGKNNPNAIQLLKRAFRAAIKE
nth2 1271_1272delTA, ORF2	MSSPRLDSLTGLSCYHSDGIGIPPETEPDHFDTILLPYAEKYNVTLEKLRYLYNEG- MIKEPKLDAFFLHDRAVRESGHDTTYRFEGVCAYLATIDLNSLLYKYEKDIAFVIKEYFGNEYKDENDGTVTDSEHWEELAELRKTRIN KYMWDEDSGFFFYYNTKLKCRTSYESATTFWSLWAGLATEEQAKITVEKALPQLEMLG- GLVACTEKSRGPISIDRPIRQWDYPFGWAPHQILAWKGLSAYGYQQVATRLAYRWLYMITKSFVDYNGMVVEKYDVTRGTDPHRVDA EYGNQGADFKGVATEGFGWVNTSYLLGLKYMNNHARRALAACSPPLPFFNSLKPSEKKLYYL

*Binding site, *NTH1* in red R302, N346, E424, R473, G476; NTH2 R331, N375, R384, G505; Active site in green *NTH1:* D478, E674; *NTH2*:D507, E703; Substrate binding underline *NTH1:* 338-339WD, 384-386RSQ; *NTH2* 309-310WD, 355-357RSQ; Phosphorylation site of activation *NTH1* S20, S21, S60, S83, *NTH2* R49, S52, R109, S112.

3.2. Behavior of the nth2 1271_1272delTA Strain under Salinity Stress

The *nth2 1271_1272delTA* strain has increased tolerance and can survive in high concentrations of NaCl (0.85M NaCl). We noted that the *nth2 1271_1272delTA* strains were slightly smaller than the control under the light microscopy, although we were not able

to detect any statistical difference. We validated that the cells remained the same and had no statistical differences from the control under high osmolarity conditions and also with the scanning electron microscopy. Yeast dimensions were determined from the scanning electron microscopy images (Figure 3A). The sizes of the cells remained statistically and phenotypically identical to the wild-type strain and were not collapsed, although we noted that they were slightly smaller (Figure 3B).

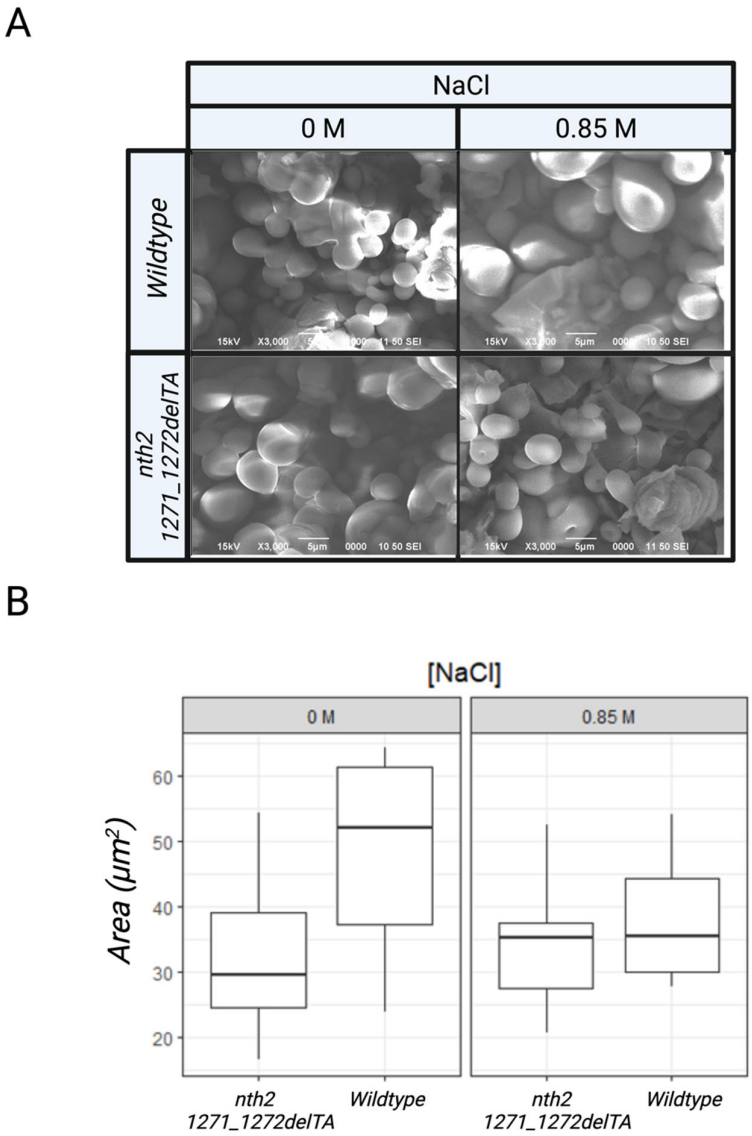

Figure 3. Analysis of the sizes of the wild-type and *nth2 1271_1272delTA* yeasts. (**A**). Scanning electron microscopy views of the wild-type CEN.PK2-1C strain and mutated strain *nth2 1271_1272delTA* grown in 0 and 0.85 M NaCl; (**B**). Box plots representing the sizes of the wild-type and *nth2 1271_1272delTA* strains of yeast under nonstress and stress (NaCl) conditions. No significant difference was observed ($p < 0.05$).

The *nth2 1271_1272delTA* strain cells were also not different under transmission electron microscopy analysis (data not shown). Organelles and structures such as vacuoles, nucleus, mitochondrion, cell membrane, and cell wall had no differences compared to the

wild-type CEN.PK2-1C strain. We noted no organelle disruption nor structural changes in the tolerant strain (for more details check the Supplementary Materials).

The *nth2 1271_1272delTA* strain was viable in a maximum concentration of 0.85 M NaCl and gave a standard growth curve and an average growth rate of 0.2327 ± 0.0057 h^{-1} (Figure 4). When statistically analyzing the specific growth rates ($p<0.05$), the *nth2 1271_1272delTA* strain under stress conditions had a growth rate of 0.2179 ± 0.0061 h^{-1} and behaved in the same way as the wild-type strain under normal conditions 0.2255 ± 0.0037 h^{-1}. The behavior of the wild-type strain in NaCl solution presented a significant decrease of 0.1580 ± 0.0009 h^{-1}.

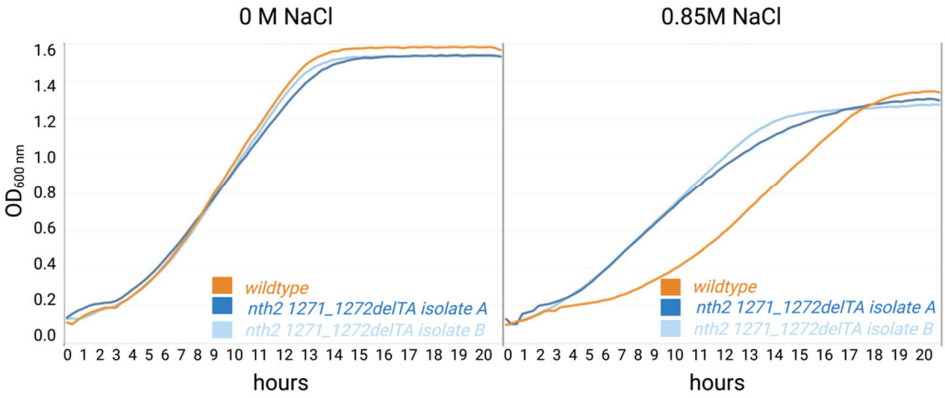

Figure 4. Growth curves of *S. cerevisiae nth2 1271_1272delTA* strains in the presence and absence of osmotic stress (0.85 M NaCl) by indirect measurements of optical density at 600 nm after 20 h of growth under stress. The curve was built with the mean of four independent samples per hour for each condition. The growth rate was calculated for the wild type in non-stress conditions of 0.2255 ± 0.0037 h^{-1} versus 0.1580 ± 0.0009 h^{-1} in 0.85M NaCl stress, and *nth2 1271_1272delTA* 0.2327 ± 0.0057 h^{-1} versus 0.2179 ± 0.0061 h^{-1} in 0.85M NaCl stress.

We expected a slight salt tolerance because of the neglected reported activity of *NTH2*. The latter was reasonable because the strain still had functional NTH1 enzymes that metabolize trehalose. However, the data obtained showed that the *nth2 1271_1272delTA* strains were superiorly tolerant. The *nth2 1271_1272delTA* strains had an average growth curve in a 0.85M NaCl liquid medium, with two isolates having the same mutation and behavior. Instead, the wild type had a slower growth curve (Figure 4).

We mutated the homolog gene *NTH1* resulting in *nth1 893_894insT* strains to compare it with *nth2 1271_1272delTA*. The *nth1 893_894insT* strains showed no tolerance to 0.85 and 1.2M NaCl when grown on agar plate-based comparison after two weeks (Figure 5). Growth on 0.85M and 1.2M was detected after three days for *nth2 1271_1272delTA*, mutants but it took a week for the control and *nth1 893_894insT* mutants. We could not detect *nth1 893_894insT* salt tolerance when growing the mutant in 0.85 M NaCl liquid media (data not presented).

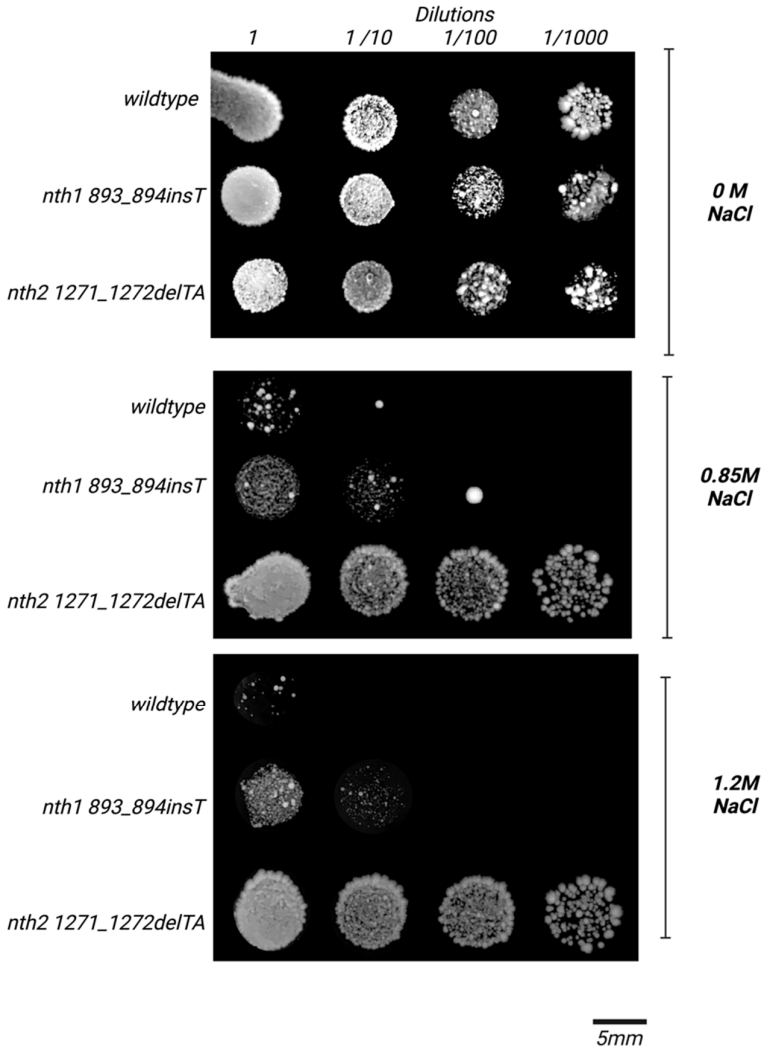

Figure 5. Agar plate comparison of yeast strains: *nth1 893_894insT*, *nth2 1271_1272delTA,* and wild-type after two weeks of growth under 0, 0.85 M and 1.2M NaCl stress. Note serial dilutions of yeast starting in OD_{600} = 1**.**

We noted no difference in the intracellular content of trehalose in *nth2 1271_1272delTA* in comparison with the control in the stationary phase after 48h of growth with or without stress. However, in *nth1 893_894insT*, the trehalose content was low with or without NaCl. (Table 2).

Table 2. Intracellular trehalose content of yeast cells under non-stress and stress conditions.

Strain[1]	Intracellular Content of Trehalose	
	0 M NaCl	**0.85 M NaCl**
S. cerevisiae CENPK2 (control)	(150 ± 22) mg 100 mL^{-1}	(118 ± 18) mg/100 mL
S. cerevisiae CENPK2 *nth2 1271_1272delTA*	(139 ± 21) mg 100 mL^{-1}	(107 ± 16) mg/100 mL
S. cerevisiae CENPK2 *nth1 893_894insT*	(34.8 ± 5,2) mg 100 mL^{-1}	(33.8 ± 5,1) mg/100 mL

[1] All cells grew with the same conditions, trehalose content determined by HPLC.

4. Discussion

The salinity tolerance of the *S. cerevisiae* strain CENPK2 increased when the *NTH2* gene was disrupted using CRISPR/Cas9-mediated genome editing compared with wild type and *NTH1* disruption. The *nth2 1271_1272delTA* yeast strains grew in 0.85 M NaCl with no detectable changes in behavior other than stress tolerance. Although deletion of *NTH1* and *NTH1-NTH2* together was known to result in stress tolerance, to our knowledge, this is the first report of using the CRISPR/Cas9 technique to disrupt *NTH2* alone that results in remarkable stress tolerance, as confirmed by an automated measurement system. The result is similar to a predictive model suggesting such tolerance for *NTH2* kanMX4 deletion and a neglectable tolerance for *NTH1*-disrupted strains [30].

The use of CRISPR/Cas9 resulted in the expected specific mutations of the *S. cerevisiae* of both *NTH2* and *NTH1* independent genes, three bases downstream of the PAM section (NGG) with the *S. pyogenes* Cas9 enzyme [31,32]. In the case of *NTH2*, the double-strand break resulted in the deletion of two nucleotides after nonhomologous end joining (NHEJ), introducing the "TAA" stop codon in the respective open reading frame (Figure 1). The *nth2 1271_1272delTA* strain had a deletion of two nucleotides, TA, located at positions 1271-1272, which resulted in a stop codon Y424X mutation of the *NTH2* gene; notably, no changes in the *NTH1* gene were observed. The anticipated tridimensional structure of the putative 423 amino acid Open Reading Frame (ORF) of *nth2 1271_1272delTA* indicated that this modified enzyme should be inactive due to the absence of the active residues ASP507 and GLU703. Similarly, the *NTH1 mutant*, *nth1 893_894insT* contained an insertion of one T base three bases downstream of the PAM site as expected and resulted in an early stop codon three triplets downstream.

In this study, *nth2 1271_1272delTA* strains remained viable and had better tolerance to 0.85 M NaCl than the wild-type and *nth1 893_894insT* strains. The behavior and size of *S. cerevisiae nth2 1271_1272delTA* did not change compared to the wild type and had normal variability depending on the generations and growth stage. Yeast tends to shrinkage and collapse in NaCl osmotic stress without plasmolysis, but its primary difference is in mitochondrial fragmentation [33]. We validate that *nth2 1271_1272delTA* did not collapse under stress and was identical to the control with no stress and had no fragmentation of its organelles using transmission electron microscopy.

The *nth2 1271_1272delTA* strain showed exponential growth under NaCl stress conditions (0.2179 ± 0.0061 h^{-1}) very similar to the wild-type strain without the presence of the osmotic agent (0.2255 ± 0.0037 h^{-1}). The results indicate that this mutation provides the yeast with a greater tolerance to saline conditions without significantly affecting its specific growth rate than the wild-type strain under salinity stress (0.1580 ± 0.0009 h^{-1}).

Our data differ from previous reports that have proposed that eliminating the *NTH2* gene resulted in no salt tolerance [13]. However, the results are not comparable due to methodological differences of complete deletion of the gene versus a point mutation. We used 0.85M NaCl stress from the beginning, an automatic growing system under constant salt stress sampling every 15 min beginning at time 0 (Figure 4) and validated the tolerance in the semisolid plate (Figure 5). The growth rate is also not comparable with our control strain CENPK2 having half the growth rate in non-stress conditions of 0.2255 ± 0.0037 h^{-1} versus 0.1580 ± 0.0009 h^{-1} in stress, and *nth2 1271_1272delTA* 0.2327 ± 0.0057 h^{-1} versus 0.2179 ± 0.0061 h^{-1} in stress. We believe that these automatic results and visual colony growth can capture the behavior of the mutation while reducing human error [33].

We foresee the disruption of *NTH2* to provide stress tolerance in an industrial strain, because undisrupted *NTH1* can provide the metabolic equilibrium as described next. *NTH1* and *NTH2* are required and regulated for fueling growth. *NTH1* is phosphorylated by Cdk1(S66) and PKA1 (S20, S21, S60, S83) to be activated, and is required for fueling biosynthesis during S, G2, and M [34]. *NTH2* contains an N terminal phosphorylation region (R49, S52, R109, S112) and is expressed at a high level in the stationary phase after glucose exhaustion [15]. *NTH2* and *NTH1* are downregulated at the exponential phase and have a higher expression at the stationary phase [19,35]. The presence of salinity stress

causes trehalose accumulation in *S. cerevisiae* and higher ethanol osmotolerance [13,25]. Heat stress (40C), $CuSO_4$, $NaAsO_2$, H_2O_2, cycloheximide (CHX) but not NaCl (1.5M) trigger the expression of *NTH1* and, in practice, its disruption is unrequired for salt tolerance [36]. It is also known that strains with *NTH2* disruption, previously named YBR0106, grow normally in YEP glycerol and were associated with increased sensitivity against heat shock at 50°C [14], while *Δnth1* grows poorly in YEP glycerol and cannot mobilize endogenous trehalose [24]. *NTH1* disruption may provide some stress tolerance but not related to NaCl, as previously reported [22]. However, disruption of *NTH1* may not be useful for industry because these mutants cannot hydrolyze trehalose after returning from a heat stress temperature of 40 °C to an average growth temperature of 30 °C [15].

We do not fully understand the tolerance since there were no detectable changes in trehalose in the stationary phase of *nth2 1271_1272delTA* and the control as described next. We also did not note differences between the mutant *nth2 1271_1272delTA* (107 ± 16) mg/100 mL and the control (118 ± 18mg/100 mL) under stress conditions (0.85M NaCl). Instead, *nth1 893_894insT* mutants had a stable low concentration of trehalose in stress (33.8 ± 5.1mg 100 mL^{-1}) and non-stress (34.8 ± 5.2 mg 100 mL^{-1}) conditions in comparison with the control in stress (118 ± 18mg 100 mL^{-1}) and non-stress conditions (150 ± 22 mg 100 mL^{-1}). We expected no improvement of *nth1 893_894insT mutants* in salt as previously reported [12]. In addition, no important change of intracellular trehalose was previously reported when *NTH2* is eliminated under osmotic NaCl stress [12,37]. However, in previous reports testing the relationship of *NTH1* and *NTH2* with pressure tolerance, the trehalose content was slightly high but not statistically different in *Δnth2* in stationary phase (*Δnth2*=316 ± 66 μg/mg of protein, wt = 257 ± 47 μg mL^{-1}, *Δnth1*=519 ± 80 μg mL^{-1}). Notably, *Δnth2* acquired a barotolerance dismissed by the authors (*Δnth2*=5.0 ± 2.0, wild type = 3.4 ± 1.0, *Δnth1* = 0.3 ± 0.09). Instead, the authors focused on *Δnth1* sensitivity although having a higher concentration of trehalose [37]. High trehalose concentration can protect from pressure but requires hydrolysis mediated by *NTH1* because it interferes with the reactivation of the cell [37,38]. A high trehalose concentration is insufficient for stress tolerance, but its correct use as an energy reservoir seems essential. Yeast cells subjected to 50 MPa of pressure results in the immediate induction of the *TPS1* gene (at 0′,5′,10′,15′ was 2.41, 3.92, 4.15, 4.16) triggering trehalose synthesis, while *NTH1* and *NTH2* are induced primarily post-pressurization (at 0′,5′,10′,15′ was *NTH1*=0.41, 2.07, 2.78, 3.14; *NTH2*=1.07, 2.23, 3.21, 3.73) [39].

NTH2-disrupted mutants can mobilize and use trehalose. Its mutation results in an increased acid trehalase activity [19], meaning that the metabolic stability of the strain is not compromised. In addition, no significant change in intracellular trehalose occurs when *NTH2* is eliminated under osmotic NaCl stress such as in our results [12,22]. The latter also means that trehalose negatively affects growth, for overaccumulation is unfeasible [16].

Interestingly, in *Cryptococcus neoformans*, the disruption of *NTH2* alone increased the survival ability of the yeast, but the deletion of *NTH1-NTH2* was negative for the microorganism [40]. Similarly, a database of yeast mutants growth modeling completed with kanMX4 interrupting NTH2 in haploid BY4741 background predicts tolerance to salt stress, such as our results [30].

In our *NTH2* mutation model, an alternative explanation is that *nth2 1271_1272delTA* translates the gene into two ORFs considering that yeast produces alternative ORFs [41–43]. In that case, the ORFs from *nth2 1271_1272delTA* may not be active but may be able to bind the substrate. The protein fragments could transitorily protect trehalose from catabolism. The first ORF, containing binding sites R331, N375, R384, 309–10WD, and 355–357RSQ but not the active sites, could bind to the trehalose and protect the molecule from the enzymatic activity of *NTH1.*

Breeding industrial yeast can result in cost-effectiveness or reductions in fermentation. In the case of stress tolerance traits, yeast is constantly exposed to ethanol toxicity, oxidative stress, temperature stress, and osmotic stress, diminishing its capacity to

produce ethanol [44,45]. Our data also show that the osmotic tolerance of the *nth2 1271_1272delTA* disruption strain mediated by CRISPR is superior and could represent a solution for the fermentation industry without compromising its metabolism, phenotype, or behavior [46,47].

5. Conclusions

The *S. cerevisiae NTH2* gene was disrupted with the CRISPR/Cas9 technique, resulting in a *nth2 1271_1272delTA* phenotypically normal strain that could grow under osmotic stress (0.85 M sodium chloride).

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/fermentation8040166/s1, Figure S1: TEM images of the CEN.PK2-1C wild-type strain and mutated *Δnth2* strain of *S. cerevisiae* grown in 0 M and 0.85 M NaCl. The scale bar represents 0.5 µm in all cases (5000× magnification). N: nucleus, V: vacuole, M: mitochondrion, CM: cell membrane, CW: cell wall.; Figure S2. DNA alignment of the sequences of the NTH1 and NTH2 genes, including the gRNA position and primers used in this study. sgNTH1= single guide NTH1, sgNTH2= single guide NTH2, nth1f=forward NTH1 primer, nth2f =forward NTH2 primer, nth1r = reverse NTH1 primer, nth2r =reverse NTH2 primer; Figure S3. Representation of the gRNA and scaffold. A. The bRA89 plasmid with the corresponding Bpl1 sites used for replacement of gRNA. B. The final NTH2-sgRNA with the scaffold. C. The final NTH1 sgRNA with the scaffold.

Author Contributions: Conceptualization, A.H.-S., J.P.D.-N. and A.G.-A.; writing—original draft preparation, A.H.-S., J.P.D.-N., M.B.-A., S.A.P. and A.G.-A.; writing—review and editing, A.H.-S., J.P.D.-N., M.B.-A., S.A.P. and A.G.-A.; visualization, A.H.-S., J.P.D.-N., M.B.-A., S.A.P. and A.G.-A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financed by the Espacio de Estudios Avanzados de la Universidad de Costa Rica (Space for Advanced Studies at the University of Costa Rica), grant number 801-B7-294, UCREA.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: This article is part of the Doctoral Thesis of the first author, Doctorado en Ciencia Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica (TEC), Universidad Nacional, Universidad Estatal a Distancia, Cartago, Costa Rica. We thank Fabián Echeverría-Beirute of DOCINADE-ITCR for the assistance in reviewing and editing the manuscript. We also thank Ethel Sánchez-Chacón for TEM sample preparation and the Laboratorio Institucional de Microscopía of Tecnológico de Costa Rica for ultramicrotome cuts.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Zhang, X.; Zhang, Y.; Li, H. Regulation of trehalose, a typical stress protectant, on central metabolisms, cell growth and division of *Saccharomyces cerevisiae* CEN.PK113-7D. *Food Microbiol.* **2020**, *89*, 103459. https://doi.org/10.1016/j.fm.2020.103459.
2. Paul, M.J.; Gonzalez-Uriarte, A.; Griffiths, C.A.; Hassani-Pak, K. The Role of Trehalose 6-Phosphate in Crop Yield and Resilience. *Plant Physiol.* **2018**, *177*, 12–23. https://doi.org/10.1104/pp.17.01634.
3. Fichtner, F.; Lunn, J.E. The Role of Trehalose 6-Phosphate (Tre6P) in Plant Metabolism and Development. *Annu. Rev. Plant Biol.* **2021**, *72*, 737–760. https://doi.org/10.1146/annurev-arplant-050718-095929.
4. Cai, X.; Seitl, I.; Mu, W.; Zhang, T.; Stressler, T.; Fischer, L.; Jiang, B. Biotechnical production of trehalose through the trehalose synthase pathway: Current status and future prospects. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2965–2976. https://doi.org/10.1007/s00253-018-8814-y.
5. Rapoport, A.; Golovina, E.A.; Gervais, P.; Dupont, S.; Beney, L. Anhydrobiosis: Inside yeast cells. *Biotechnol. Adv.* **2019**, *37*, 51–67. https://doi.org/10.1016/j.biotechadv.2018.11.003.
6. Glatz, A.; Pilbat, A.-M.; Németh, G.L.; Vince-Kontár, K.; Jósvay, K.; Hunya, Á.; Udvardy, A.; Gombos, I.; Péter, M.; Balogh, G.; et al. Involvement of small heat shock proteins, trehalose, and lipids in the thermal stress management in *Schizosaccharomyces pombe*. *Cell Stress Chaperon* **2016**, *21*, 327–338. https://doi.org/10.1007/s12192-015-0662-4.
7. Dong, J.; Chen, D.; Wang, G.; Zhang, C.; Du, L.; Liu, S.; Zhao, Y.; Xiao, D. Improving freeze-tolerance of baker's yeast through seamless gene deletion of NTH1 and PUT1. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 817–828. https://doi.org/10.1007/s10295-016-1753-7.

8. Tapia, H.; Koshland, D.E. Trehalose Is a Versatile and Long-Lived Chaperone for Desiccation Tolerance. *Curr. Biol.* **2014**, *24*, 2758–2766. https://doi.org/10.1016/j.cub.2014.10.005.
9. Olsson, C.; Jansson, H.; Swenson, J. The Role of Trehalose for the Stabilization of Proteins. *J. Phys. Chem. B* **2016**, *120*, 4723–4731. https://doi.org/10.1021/acs.jpcb.6b02517.
10. Bosch, S.; De Beaurepaire, L.; Allard, M.; Mosser, M.; Heichette, C.; Chrétien, D.; Jegou, D.; Bach, J.-M. Trehalose prevents aggregation of exosomes and cryodamage. *Sci. Rep.* **2016**, *6*, 36162. https://doi.org/10.1038/srep36162.
11. Babazadeh, R.; Lahtvee, P.-J.; Adiels, C.B.; Goksör, M.; Nielsen, J.B.; Hohmann, S. The yeast osmostress response is carbon source dependent. *Sci. Rep.* **2017**, *7*, 990. https://doi.org/10.1038/s41598-017-01141-4.
12. Garre, E.; Matallana, E. The three trehalases Nth1p, Nth2p and Ath1p participate in the mobilization of intracellular trehalose required for recovery from saline stress in *Saccharomyces cerevisiae*. *Microbiology* **2009**, *155*, 3092–3099. https://doi.org/10.1099/mic.0.024992-0.
13. Mahmud, S.A.; Nagahisa, K.; Hirasawa, T.; Yoshikawa, K.; Ashitani, K.; Shimizu, H. Effect of trehalose accumulation on response to saline stress in *Saccharomyces cerevisiae*. *Yeast* **2009**, *26*, 17–30. https://doi.org/10.1002/yea.1646.
14. Nwaka, S.; Kopp, M.; Holzer, H. Expression and Function of the Trehalase Genes NTH1 and YBR0106 in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1995**, *270*, 10193–10198. https://doi.org/10.1074/jbc.270.17.10193.
15. Nwaka, S.; Holzer, H. Molecular Biology of Trehalose and the Trehalases in the Yeast *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res. Mol. Biol.* **1997**, *58*, 197–237. https://doi.org/10.1016/s0079-6603(08)60037-9.
16. Gibney, P.A.; Schieler, A.; Chen, J.C.; Rabinowitz, J.D.; Botstein, D. Characterizing the in vivo role of trehalose in *Saccharomyces cerevisiae* using the *AGT1* transporter. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 6116–6121. https://doi.org/10.1073/pnas.1506289112.
17. Hallerman, E.M.; Bredlau, J.P.; Camargo, L.S.A.; Dagli, M.L.Z.; Karembu, M.; Ngure, G.; Romero-Aldemita, R.; Rocha-Salavarrieta, P.J.; Tizard, M.; Walton, M.; et al. Towards progressive regulatory approaches for agricultural applications of animal biotechnology. *Transgenic Res.* **2022**, *3*, 1–33. https://doi.org/10.1007/s11248-021-00294-3.
18. Wang, P.-M.; Zheng, D.-Q.; Chi, X.-Q.; Li, O.; Qian, C.-D.; Liu, T.-Z.; Zhang, X.-Y.; Du, F.-G.; Sun, P.-Y.; Qu, A.-M.; et al. Relationship of trehalose accumulation with ethanol fermentation in industrial *Saccharomyces cerevisiae* yeast strains. *Bioresour. Technol.* **2014**, *152*, 371–376. https://doi.org/10.1016/j.biortech.2013.11.033.
19. Jules, M.; Beltran, G.; François, J.; Parrou, J.L. New Insights into Trehalose Metabolism by *Saccharomyces cerevisiae*: NTH2 Encodes a Functional Cytosolic Trehalase, and Deletion of TPS1 Reveals Ath1p-Dependent Trehalose Mobilization. *Appl. Environ. Microbiol.* **2008**, *74*, 605–614. https://doi.org/10.1128/aem.00557-07.
20. Avila-Reyes, S.; Camacho-Diaz, B.; Acosta-Garcia, M.; Jimenez-Aparicio, A.; Hernandez-Sanchez, H. Effect of salt and sugar osmotic stress on the viability and morphology of *Saccharomyces boulardii*. *Int. J. Environ. Agric. Biotechnol.* **2016**, *1*, 593–602. https://doi.org/10.22161/ijeab/1.3.43.
21. Logothetis, S.; Walker, G.; Nerantzis, E. Effect of salt hyperosmotic stress on yeast cell viability. *Zb. Matic Srp. Přír. Nauk.* **2007**, *113*, 271–284. https://doi.org/10.2298/zmspn07132711.
22. Divate, N.R.; Chen, G.-H.; Divate, R.D.; Ou, B.-R.; Chung, Y.-C. Metabolic engineering of *Saccharomyces cerevisiae* for improvement in stresses tolerance. *Bioengineered* **2017**, *8*, 524–535. https://doi.org/10.1080/21655979.2016.1257449.
23. Sun, X.; Zhang, C.-Y.; Wu, M.-Y.; Fan, Z.-H.; Liu, S.-N.; Zhu, W.-B.; Xiao, D.-G. MAL62 overexpression and NTH1 deletion enhance the freezing tolerance and fermentation capacity of the baker's yeast in lean dough. *Microb. Cell Factories* **2016**, *15*, 1–8. https://doi.org/10.1186/s12934-016-0453-3.
24. Nwaka, S.; Mechler, B.; Destruelle, M.; Holzer, H. Phenotypic features of trehalase mutants in *Saccharomyces cerevisiae*. *FEBS Lett.* **1995**, *360*, 286–290. https://doi.org/10.1016/0014-5793(95)00105-i.
25. Mahmud, S.A.; Hirasawa, T.; Shimizu, H. Differential importance of trehalose accumulation in *Saccharomyces cerevisiae* in response to various environmental stresses. *J. Biosci. Bioeng.* **2010**, *109*, 262–266. https://doi.org/10.1016/j.jbiosc.2009.08.500.
26. Tapia, H.; Young, L.; Fox, D.; Bertozzi, C.R.; Koshland, D. Increasing intracellular trehalose is sufficient to confer desiccation tolerance to *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 6122–6127. https://doi.org/10.1073/pnas.1506415112.
27. Anand, R.; Beach, A.; Li, K.; Haber, J. Rad51-mediated double-strand break repair and mismatch correction of divergent substrates. *Nature* **2017**, *544*, 377–380. https://doi.org/10.1038/nature22046.
28. Naito, Y.; Hino, K.; Bono, H.; Ui-Tei, K. CRISPRdirect: Software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics* **2015**, *31*, 1120–1123. https://doi.org/10.1093/bioinformatics/btu743.
29. Gietz, R.D.; Schiestl, R.H. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2007**, *2*, 31–34. https://doi.org/10.1038/nprot.2007.13.
30. Fernandez-Ricaud, L.; Warringer, J.; Ericson, E.; Pylvänäinen, I.; Kemp, G.J.L.; Nerman, O.; Blomberg, A. PROPHECY—a database for high-resolution phenomics. *Nucleic Acids Res.* **2004**, *33*, D369–D373. https://doi.org/10.1093/nar/gki126.
31. Doudna, J.A.; Charpentier, E. The new frontier of genome engineering with CRISPR-Cas9. *Science* **2014**, *346*, 1258096. https://doi.org/10.1126/science.1258096.
32. Wang, H.; La Russa, M.; Qi, L.S. CRISPR/Cas9 in Genome Editing and Beyond. *Annu. Rev. Biochem.* **2016**, *85*, 227–264. https://doi.org/10.1146/annurev-biochem-060815-014607.
33. Morris, G.J.; Winters, L.; Coulson, G.E.; Clarke, K.J. Effect of Osmotic Stress on the Ultrastructure and Viability of the Yeast *Saccharomyces cerevisiae*. *Microbiol.* **1986**, *132*, 2023–2034. https://doi.org/10.1099/00221287-132-7-2023.
34. Ewald, J.C.; Kuehne, A.; Zamboni, N.; Skotheim, J.M. The Yeast Cyclin-Dependent Kinase Routes Carbon Fluxes to Fuel Cell Cycle Progression. *Mol. Cell* **2016**, *62*, 532–545. https://doi.org/10.1016/j.molcel.2016.02.017.
35. Yi, C.; Wang, F.; Dong, S.; Li, H. Changes of trehalose content and expression of relative genes during the bioethanol fermentation by *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **2016**, *62*, 827–835. https://doi.org/10.1139/cjm-2015-0832.
36. Zähringer, H.; Burgert, M.; Holzer, H.; Nwaka, S. Neutral trehalase Nth1p of *Saccharomyces cerevisiae* encoded by theNTH1gene is a multiple stress responsive protein. *FEBS Lett.* **1997**, *412*, 615–620. https://doi.org/10.1016/s0014-5793(97)00868-5.
37. Iwahashi, H.; Nwaka, S.; Obuchi, K. Evidence for Contribution of Neutral Trehalase in Barotolerance of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **2000**, *66*, 5182–5185. https://doi.org/10.1128/aem.66.12.5182-5185.2000.
38. Singer, M.A.; Lindquist, S. Thermotolerance in *Saccharomyces cerevisiae*: The Yin and Yang of trehalose. *Trends Biotechnol.* **1998**, *16*, 460–468. https://doi.org/10.1016/s0167-7799(98)01251-7.
39. Bravim, F.; Lippman, S.I.; da Silva, L.F.; Souza, D.T.; Fernandes, A.A.R.; Masuda, C.A.; Broach, J.R.; Fernandes, P.M.B. High hydrostatic pressure activates gene expression that leads to ethanol production enhancement in a *Saccharomyces cerevisiae* distillery strain. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 2093–2107. https://doi.org/10.1007/s00253-012-4356-x.

40. Botts, M.R.; Huang, M.; Borchardt, R.K.; Hull, C.M. Developmental Cell Fate and Virulence Are Linked to Trehalose Homeostasis in *Cryptococcus neoformans*. *Eukaryot. Cell* **2014**, *13*, 1158–1168. https://doi.org/10.1128/ec.00152-14.
41. Blevins, W.R.; Ruiz-Orera, J.; Messeguer, X.; Blasco-Moreno, B.; Villanueva-Cañas, J.L.; Espinar, L.; Díez, J.; Carey, L.B.; Albà, M.M. Uncovering de novo gene birth in yeast using deep transcriptomics. *Nat. Commun.* **2021**, *12*, 1–13. https://doi.org/10.1038/s41467-021-20911-3.
42. Cakiroglu, S.A.; Zaugg, J.B.; Luscombe, N.M. Backmasking in the yeast genome: Encoding overlapping information for protein-coding and RNA degradation. *Nucleic Acids Res.* **2016**, *44*, 8065–8072. https://doi.org/10.1093/nar/gkw683.
43. Orr, M.W.; Mao, Y.; Storz, G.; Qian, S.-B. Alternative ORFs and small ORFs: Shedding light on the dark proteome. *Nucleic Acids Res.* **2020**, *48*, 1029–1042. https://doi.org/10.1093/nar/gkz734.
44. Saini, P.; Beniwal, A.; Kokkiligadda, A.; Vij, S. Response and tolerance of yeast to changing environmental stress during ethanol fermentation. *Process Biochem.* **2018**, *72*, 1–12. https://doi.org/10.1016/j.procbio.2018.07.001.
45. Pratt, P.L.; Bryce, J.H.; Stewart, G.G. The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology. *J. Inst. Brew.* **2003**, *109*, 218–228. https://doi.org/10.1002/j.2050-0416.2003.tb00162.x.
46. Muysson, J.; Miller, L.; Allie, R.; Inglis, D.L. The Use of CRISPR-Cas9 Genome Editing to Determine the Importance of Glycerol Uptake in Wine Yeast During Icewine Fermentation. *Fermentation* **2019**, *5*, 93. https://doi.org/10.3390/fermentation5040093.
47. Vilela, A. An Overview of CRISPR-Based Technologies in Wine Yeasts to Improve Wine Flavor and Safety. *Fermentation* **2021**, *7*, 5. https://doi.org/10.3390/fermentation7010005.

8. Conclusiones y recomendaciones

- El proyecto logró establecer un sistema de inducción de variabilidad genética *in vitro* para la variedad comercial de arroz Lazarroz FL utilizando Fluazifop-P-Butil como un agente de selección en la optimización del sistema según se había planteado en el objetivo general (OG). Los detalles del sistema se detallan a continuación.

- El medio óptimo para la inducción de callo embriogénico consiste de medio MS con 2 mg L^{-1} 2,4-D. La regeneración consiste en un medio MS con 3 mg L^{-1} BAP y 0.5 mg L^{-1} NAA. La primera condición de selección de la dosis de irradiación fue 60 Gy (DL20 = 64 Gy). El uso de un sistema de inmersión temporal (RITA®) de 120segundos cada 8h permite una regeneración homogénea y predecible. (OE1, OE2)

- La dosis correcta del cálculo de la dosis letal de Fluazifop-P-Butil en callo fue de DL50= 6,93 mg / L (0,425 mg / L - 15,743 mg / L, R2 = 0,402, 1000n); y en *vitro plantas* fue DL50 3.771mg / L (R2 = 1, 290n). (OE2)

- El sistema se optimizó mediante dos etapas de selección que consisten en una primera exposición de pre-selección a 5 mg/L y una segunda etapa de 10 mg/L Fluazifop-P-butil. Un lote de 8000 *vitro plantas* regeneradas a partir de callo irradiado a 60Gy tuvo con un único sobreviviente. El mutante dispone de una mutación bialélica en el dominio carboxiterminal del gen blanco ACC2 T2222I/T2222M que podría estar relacionado con la tolerancia. El mutante también dispone de mutaciones bialélicas en los genes control G/T *mat*K y GT/AG en *rbcL*. Lo anterior es consistente con la tasa de mutación en los tres genes analizados y corresponde aproximadamente a una mutación por cada 1000 pb. En invernadero, una planta de tres hojas aclimatada del mutante T2222I/T2222M tolera 150mg/L de Fluazifop-P-butil (OE3).

- El sistema se mejoró mediante el uso de semillas en lugar de callo como tejido inicial *in vitro* para la irradiación, lo que permitió el uso de ventanas de aplicación de rayos gama entre 50 a 350Gy. La semilla irradiada responde a la inducción de callo, regeneración y tiene un incremento en el resultado con respecto a la versión original del sistema, con un total de 31 mutantes putativos a partir de 4000 *vitro plantas*. (OG)

- En la pasantía del proyecto de Doctorado se evaluó CRISPR-CAS9 en genes homólogos de arroz en levadura relacionados con tolerancia a salinidad. La mutación *nth2 1271_1272delTA* resultó en levaduras capaces de tolerar 0.85 M de Cloruro de Sodio. El modelo es útil en la búsqueda de mutaciones dirigidas en arroz.

- Se recomienda el análisis de la M2 de la planta tolerante con la mutación ACC2 T2222I/T2222M para evaluar si la descendencia dispone o no de tolerancia, así como la secuenciación de genoma completo de la planta mutante en M1 y/o al menos una planta M2 según sea posible.
- Se recomienda el análisis de 31 plantas putativas nuevas en invernadero y su descendencia M2.

9. Referencias

Abdelnour-Esquivel, A., Perez, J., Rojas, M., Vargas, W., & Gatica-Arias, A. (2020). Use of gamma radiation to induce mutations in rice (*Oryza sativa* L.) and the selection of lines with tolerance to salinity and drought. *In Vitro Cellular & Developmental Biology - Plant*, *56*(1), 88–97. https://doi.org/10.1007/s11627-019-10015-5

Anders, S., Cowling, W., Pareek, A., Gupta, K. J., Singla-Pareek, S. L., & Foyer, C. H. (2021). Gaining Acceptance of Novel Plant Breeding Technologies. *Trends in Plant Science*, *26*(6), 575–587. https://doi.org/10.1016/j.tplants.2021.03.004

Bai, C., Rivera, S. M., Medina, V., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Capell, T., Sandmann, G., Christou, P., & Zhu, C. (2014). An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. *Plant Journal*, *77*(3), 464–475. https://doi.org/10.1111/tpj.12384

Basavalingaiah, K., Ramesha, Y. M., Paramesh, V., Rajanna, G. A., Lal Jat, S., Misra, S. D., Gaddi, A. K., Girisha, H. C., Yogesh, G. S., Raveesha, S., Roopa, T. K., Shashidhar, K. S., Kumar, B., El-Ansary, D. O., & Elansary, H. O. (2020). Energy budgeting, data envelopment analysis and greenhouse gas emission from rice production system: A case study from puddled transplanted rice and direct-seeded rice system of Karnataka, India. *Sustainability (Switzerland)*, *12*(16). https://doi.org/10.3390/SU12166439

Bzour, M. I., Zuki, F. M., & Mispan, M. S. (2018). Introduction of imidazolinone herbicide and Clearfield® rice between weedy rice-control efficiency and environmental concerns. In *Environmental Reviews* (Vol. 26, Issue 2). https://doi.org/10.1139/er-2017-0096

Camacho, J. R., Linscombe, S. D., Sanabria, Y., Mosquera, P. A., & Oard, J. H. (2019). Inheritance of Provisia™ rice resistance to quizalofop-p-ethyl under laboratory and greenhouse environments. *Euphytica*, *215*(4). https://doi.org/10.1007/s10681-019-2407-4

Chakrabarty, D., Trivedi, P. K., Shri, M., Misra, P., Asif, M. H., Dubey, S., Kumar, S., Rai, A., Tiwari, M., Shukla, D., Pandey, A., Nigam, D., Tripathi, R. D., & Tuli, R. (2010). Differential

transcriptional expression following thidiazuron-induced callus differentiation developmental shifts in rice. *Plant Biology*, *12*(1), 46–59. https://doi.org/10.1111/j.1438-8677.2009.00213.x

Chen, E., Huang, X., Tian, Z., Wing, R. A., & Han, B. (2019). The Genomics of Oryza Species Provides Insights into Rice Domestication and Heterosis. *Annual Review of Plant Biology*, *70*, 639–665. https://doi.org/10.1146/annurev-arplant-050718-100320

CONARROZ, C. A. N. (2021). Informe anual estadístico 2020/2021. *Control Estadístico Arrocero*, 45.

de Andrade, A., Tulmann-Neto, A., Tcacenco, F. A., Marschalek, R., Pereira, A., de Oliveira Neto, A. M., Scheuermann, K. K., Wickert, E., & Noldin, J. A. (2018). Development of rice (Oryza sativa) lines resistant to aryloxyphenoxypropionate herbicides through induced mutation with gamma rays. *Plant Breeding*, *137*(3), 364–369. https://doi.org/10.1111/pbr.12592

Fan, Y., & Li, Y. (2019). Molecular, cellular and Yin-Yang regulation of grain size and number in rice. *Molecular Breeding*, *39*(12). https://doi.org/10.1007/s11032-019-1078-0

Fichtner, F., & Lunn, J. E. (2021). The Role of Trehalose 6-Phosphate (Tre6P) in Plant Metabolism and Development. *Annual Review of Plant Biology*, *72*, 737–760. https://doi.org/10.1146/annurev-arplant-050718-095929

Gutaker, R. M., Groen, S. C., Bellis, E. S., Choi, J. Y., Pires, I. S., Bocinsky, R. K., Slayton, E. R., Wilkins, O., Castillo, C. C., Negrão, S., Oliveira, M. M., Fuller, D. Q., Guedes, J. A. d'Alpoim, Lasky, J. R., & Purugganan, M. D. (2020). Genomic history and ecology of the geographic spread of rice. *Nature Plants*, *6*(5), 492–502. https://doi.org/10.1038/s41477-020-0659-6

Hernández Soto, A., Pérez, J., Fait Zúñiga, R., Rojas Vásquez, R., Gatica Arias, A., Vargas Segura, W., & Abdelnour Esquivel, A. (2022). A Temporary Immersion System Improves Regeneration of In Vitro Irradiated Recalcitrant Indica Rice (*Oryza sativa* L.) Embryogenic Calli. *Plants*, *11*(3), 375. https://doi.org/10.3390/plants11030375

Hernández-Soto, A., Delgado-Navarro, J. P., Benavides-Acevedo, M., Paniagua, S. A., & Gatica-Arias, A. (2022). NTH2 1271_1272delTA Gene Disruption Results in Salt Tolerance in *Saccharomyces cerevisiae*. *Fermentation 2022, Vol. 8, Page 166*, *8*(4), 166. https://doi.org/10.3390/FERMENTATION8040166

Hernández-Soto, A., Echeverría-Beirute, F., Abdelnour-Esquivel, A., Valdez-Melara, M., Boch, J., & Gatica-Arias, A. (2021). Rice breeding in the new era: Comparison of useful agronomic traits. *Current Plant Biology*, *27*(100211), 100211. https://doi.org/10.1016/j.cpb.2021.100211

Hernández-Soto, A.; Méndez-Navarro, D.; Pérez, J.; Rojas-Vásquez, R.; Gatica-Arias, A.; Vargas, W.; Echeverria-Beirute,F; Abdelnour-Esquivel, A. Tolerance to aryloxy-phenoxy-

propionate (APP) as a model for Lazarroz FL rice *in vitro* gamma irradiation variability selection. (2022). *Research Square.* https://doi.org/10.21203/rs.3.rs-1950230/v1

Hu, W., Zhou, T., Hu, G., Wu, H., Han, Z., Xiao, J., Li, X., & Xing, Y. (2020). An ethyl methanesulfonate-induced neutral mutant-bridging method efficiently identifies spontaneously mutated genes in rice. *Plant Journal*, *104*(4), 1129–1141. https://doi.org/10.1111/tpj.14969

Indoliya, Y., Tiwari, P., Chauhan, A. S., Goel, R., Shri, M., Bag, S. K., & Chakrabarty, D. (2016). Decoding regulatory landscape of somatic embryogenesis reveals differential regulatory networks between japonica and indica rice subspecies. *Scientific Reports*, *6*(October 2015), 1–15. https://doi.org/10.1038/srep23050

Karmakar, S., Das, P., Panda, D., Xie, K., Baig, M. J., & Molla, K. A. (2022). Plant Science A detailed landscape of CRISPR-Cas-mediated plant disease and pest management. *Plant Science*, *323*(April), 111376. https://doi.org/10.1016/j.plantsci.2022.111376

Lancaster, Z. D., Norsworthy, J. K., & Scott, R. C. (2018). Residual Activity of ACCase-Inhibiting Herbicides on Monocot Crops and Weeds. *Weed Technology*, *32*(4), 364–370. https://doi.org/10.1017/wet.2018.13

Li, F., Shimizu, A., Nishio, T., Tsutsumi, N., & Kato, H. (2019). Comparison and characterization of mutations induced by gamma-ray and carbon-ion irradiation in rice (Oryza sativa L.) using whole-genome resequencing. *G3: Genes, Genomes, Genetics*, *9*(11), 3743–3751. https://doi.org/10.1534/g3.119.400555

Li, H., Li, J., Chen, J., Yan, L., & Xia, L. (2020). Precise Modifications of Both Exogenous and Endogenous Genes in Rice by Prime Editing. *Molecular Plant*, *13*(5), 671–674. https://doi.org/10.1016/j.molp.2020.03.011

Li, J., Meng, X., Zong, Y., Chen, K., Zhang, H., Liu, J., Li, J., & Gao, C. (2016). Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. *Nature Plants*, *2*(10). https://doi.org/10.1038/nplants.2016.139

Li, M., Li, X., Zhou, Z., Wu, P., Fang, M., Pan, X., Lin, Q., Luo, W., Wu, G., & Li, H. (2016). Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. *Frontiers in Plant Science*, *7*(MAR2016), 1–13. https://doi.org/10.3389/fpls.2016.00377

Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., Zhu, Z., Wang, Y., Anzalone, A. v., Raguram, A., Doman, J. L., Liu, D. R., & Gao, C. (2020). Prime genome editing in rice and wheat. *Nature Biotechnology*, *38*(5), 582–585. https://doi.org/10.1038/s41587-020-0455-x

Liu, L., Kuang, Y., Yan, F., Li, S., Ren, B., Gosavi, G., Spetz, C., Li, X., Wang, X., Zhou, X., & Zhou, H. (2021). Developing a novel artificial rice germplasm for dinitroaniline herbicide resistance by base editing of OsTubA2. *Plant Biotechnology Journal*, *19*(1), 5–7. https://doi.org/10.1111/pbi.13430

Liu, X., Qin, R., Li, J., Liao, S., Shan, T., Xu, R., Wu, D., & Wei, P. (2020). A CRISPR-Cas9-mediated domain-specific base-editing screen enables functional assessment of ACCase variants in rice. *Plant Biotechnology Journal*, *18*(9), 1845–1847. https://doi.org/10.1111/pbi.13348

Lovarelli, D., Bacenetti, J., & Fiala, M. (2016). Water Footprint of crop productions: A review. *Science of the Total Environment*, *548–549*, 236–251. https://doi.org/10.1016/j.scitotenv.2016.01.022

McCusker, K., & Gunaydin, S. (2015). Research using qualitative, quantitative or mixed methods and choice based on the research. *Perfusion (United Kingdom)*, *30*(7), 537–542. https://doi.org/10.1177/0267659114559116

Mohd Din, A. R. J., Iliyas Ahmad, F., Wagiran, A., Abd Samad, A., Rahmat, Z., & Sarmidi, M. R. (2016). Improvement of efficient in vitro regeneration potential of mature callus induced from Malaysian upland rice seed (Oryza sativa cv. Panderas). *Saudi Journal of Biological Sciences*, *23*(1), S69–S77. https://doi.org/10.1016/j.sjbs.2015.10.022

Mohd Saad, N. S., Neik, T. X., Thomas, W. J. W., Amas, J. C., Cantila, A. Y., Craig, R. J., Edwards, D., & Batley, J. (2022). Advancing designer crops for climate resilience through an integrated genomics approach. *Current Opinion in Plant Biology*, *67*(MI), 102220. https://doi.org/10.1016/j.pbi.2022.102220

Morales, M., Vargas, A., Vignola, R., & Watler, W. (2018). Prácticas efectivas para la reducción de impactos por eventos climáticos en costa rica. *Instituto Nacional de Tecnología Agropecuaria*, 1–85. http://www.mag.go.cr/bibliotecavirtual/reduccion-impacto-por-eventos-climaticos/Informe-final-Arroz.pdf

Muller, H. J. (1927). Artificial transmutation of the gene. *Science*, *66*(1699), 84–87. https://doi.org/10.1126/science.66.1699.84

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 474–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Murphy, B. P., & Tranel, P. J. (2019). Target-site mutations conferring herbicide resistance. *Plants*, *8*(10), 1–16. https://doi.org/10.3390/plants8100382

Nadir, S., Xiong, H. B., Zhu, Q., Zhang, X. L., Xu, H. Y., Li, J., Dongchen, W., Henry, D., Guo, X. Q., Khan, S., Suh, H. S., Lee, D. S., & Chen, L. J. (2017). Weedy rice in sustainable rice production. A review. *Agronomy for Sustainable Development*, *37*(5). https://doi.org/10.1007/s13593-017-0456-4

Nakagawa, H., & Kato, H. (2017). Induced Mutations for Food and Energy Security : Challenge of Inducing Unique Mutants for New Cultivars and Molecular Research. *Bull. NARO, Crop Sci.*, *1*, 33–124.

Oladosu, Y., Rafii, M. Y., Abdullah, N., Hussin, G., Ramli, A., Rahim, H. A., Miah, G., & Usman, M. (2016). Principle and application of plant mutagenesis in crop improvement: A review. *Biotechnology and Biotechnological Equipment*, *30*(1), 1–16. https://doi.org/10.1080/13102818.2015.1087333

Oladosu, Y., Rafii, M. Y., Samuel, C., Fatai, A., Magaji, U., Kareem, I., Kamarudin, Z. S., Muhammad, I., & Kolapo, K. (2019). Drought Resistance in Rice from Conventional to Molecular Breeding: A Review. *International Journal of Molecular Sciences*, *20*(14). https://doi.org/10.3390/ijms20143519

Orozco, A. Z., & Carrodeguas, A. (2022). Pacífico Central, Costa Rica Morpho-agronomic variability in rice genotypes in the Central Pacific, Costa Rica. *Ciencia y Agricultura*, *19*(1), 1–15. https://doi.org/https://doi.org/10.19053/01228420.v19.n1.2022.12567

Ostlie, M., Haley, S. D., Anderson, V., Shaner, D., Manmathan, H., Beil, C., & Westra, P. (2014). Development and characterization of mutant winter wheat (Triticum aestivum L.) accessions resistant to the herbicide quizalofop. *Theoretical and Applied Genetics*, *128*(2), 343–351. https://doi.org/10.1007/s00122-014-2434-4

Pawar, B., Kale, P., Bahurupe, J., Jadhav, A., Kale, A., & Pawar, S. (2015). Proline and Glutamine Improve in vitro Callus Induction and Subsequent Shooting in Rice. *Rice Science*, *22*(6), 283–289. https://doi.org/10.1016/j.rsci.2015.11.001

Pereira, A., Tcacenco, F. A., Klabunde, G. H. F., & de Andrade, A. (2019). Detecting acetyl-coenzyme a carboxylase resistance gene in rice (*Oryza sativa* L.). *Molecular Biology Reports*, *46*(6), 6271–6276. https://doi.org/10.1007/s11033-019-05068-z

Powles, S. B., & Yu, Q. (2010). Evolution in Action: Plants Resistant to Herbicides. In *Annual Review of Plant Biology* (Vol. 61, Issue 1). https://doi.org/10.1146/annurev-arplant-042809-112119

Rapoport, A., Golovina, E. A., Gervais, P., Dupont, S., & Beney, L. (2019). Anhydrobiosis: Inside yeast cells. *Biotechnology Advances*, *37*(1), 51–67. https://doi.org/10.1016/j.biotechadv.2018.11.003

Sagare, D. B., Abbai, R., Jain, A., Jayadevappa, P. K., Dixit, S., Singh, A. K., Challa, V., Alam, S., Singh, U. M., Yadav, S., Sandhu, N., Kabade, P. G., Singh, V. K., & Kumar, A. (2020). More and more of less and less: Is genomics-based breeding of dry direct-seeded rice (DDSR) varieties the need of hour? *Plant Biotechnology Journal*, 2173–2186. https://doi.org/10.1111/pbi.13454

Shen, L., Hua, Y., Fu, Y., Li, J., Liu, Q., Jiao, X., Xin, G., Wang, J., Wang, X., Yan, C., & Wang, K. (2017). Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice. *Science China Life Sciences*, *60*(5), 506–515. https://doi.org/10.1007/s11427-017-9008-8

Shimatani, Z., Kashojiya, S., Takayama, M., Terada, R., Arazoe, T., Ishii, H., Teramura, H., Yamamoto, T., Komatsu, H., Miura, K., Ezura, H., Nishida, K., Ariizumi, T., & Kondo, A. (2017). Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nature Biotechnology*, *35*(5), 441–443. https://doi.org/10.1038/nbt.3833

Singer, S. D., Laurie, J. D., Bilichak, A., Kumar, S., & Singh, J. (2021). Genetic Variation and Unintended Risk in the Context of Old and New Breeding Techniques. *Critical Reviews in Plant Sciences*, *40*(1), 68–108. https://doi.org/10.1080/07352689.2021.1883826

Singh, V., Singh, S., Black, H., Boyett, V., Basu, S., Gealy, D., Gbur, E., Pereira, A., Scott, R. C., Caicedo, A., & Burgos, N. R. (2017). Introgression of Clearfield™ rice crop traits into weedy red rice outcrosses. *Field Crops Research*, *207*, 13–23. https://doi.org/10.1016/j.fcr.2017.03.004

Song, X., Meng, X., Guo, H., Cheng, Q., Jing, Y., Chen, M., Liu, G., Wang, B., Wang, Y., Li, J., & Yu, H. (2022). Targeting a gene regulatory element enhances rice grain yield by decoupling panicle number and size. *Nature Biotechnology*. https://doi.org/10.1038/s41587-022-01281-7

Smyth, S. J. (2022). Contributions of Genome Editing Technologies Towards Improved Nutrition, Environmental Sustainability and Poverty Reduction. *Frontiers in Genome Editing*, *4*(March), 1–9. https://doi.org/10.3389/fgeed.2022.863193

Stadler, L. J. (1928). Genetic Effects of X-Rays in Maize. *Genetics*, *14*(1), 69–75. https://doi.org/10.1073/pnas.14.1.69

Sudhakar, D., Duc, L. T., Bong, B. B., Tinjuangjun, P., Maqbool, S. B., Valdez, M., Jefferson, R., & Christou, P. (1998). An efficient rice transformation system utilizing mature seed-derived explants and a portable, inexpensive particle bombardment device. *Transgenic Research*, *7*(4), 289–294. https://doi.org/10.1023/A:1008870012568

Takano, H. K., Ovejero, R. F. L., Belchior, G. G., Maymone, G. P. L., & Dayan, F. E. (2020). ACCase-inhibiting herbicides: Mechanism of action, resistance evolution and stewardship. *Scientia Agricola*, *78*(1), 1–11. https://doi.org/10.1590/1678-992x-2019-0102

Tan, Y., & Li, S. (2022). *Generation of Mutants by Combined Treatment of Physical and Chemical Mutagens in RiceRice BT- Plant Gametogenesis: Methods and Protocols* (C. Lambing, Ed.; pp. 137–142). Springer US. https://doi.org/10.1007/978-1-0716-2253-7_11

Tanaka, N., Shenton, M., Kawahara, Y., Kumagai, M., Sakai, H., Kanamori, H., Yonemaru, J., Fukuoka, S., Sugimoto, K., Ishimoto, M., Wu, J., & Ebana, K. (2020). Whole-Genome Sequencing of the NARO World Rice Core Collection (WRC) as the Basis for Diversity and Association Studies. *Plant and Cell Physiology*, *61*(5), 922–932. https://doi.org/10.1093/pcp/pcaa019

Viana, V. E., Pegoraro, C., Busanello, C., & Costa de Oliveira, A. (2019). Mutagenesis in Rice: The Basis for Breeding a New Super Plant. *Frontiers in Plant Science*, *10*(November), 1–28. https://doi.org/10.3389/fpls.2019.01326

Wang, W., Peng, S., Liu, H., Tao, Y., Huang, J., Cui, K., & Nie, L. (2017). The possibility of replacing puddled transplanted flooded rice with dry seeded rice in central China: A review. *Field Crops Research*, *214*(August), 310–320. https://doi.org/10.1016/j.fcr.2017.09.028

Wei, S., Li, X., Lu, Z., Zhang, H., Ye, X., Zhou, Y., Li, J., Yan, Y., Pei, H., Duan, F., Wang, D., Chen, S., Wang, P., Zhang, C., Shang, L., Zhou, Y., Yan, P., Zhao, M., Huang, J., … Zhou, W. (2022). A transcriptional regulator that boosts grain yields and shortens the growth duration of rice. *Science*, *377*(6604), eabi8455. https://doi.org/10.1126/science.abi8455

Xu, W., Zhang, C., Yang, Y., Zhao, S., Kang, G., He, X., Song, J., & Yang, J. (2020). Versatile Nucleotides Substitution in Plant Using an Improved Prime Editing System. *Molecular Plant*, *13*(5), 675–678. https://doi.org/10.1016/j.molp.2020.03.012

Yan, W., Deng, X. W., Yang, C., & Tang, X. (2021). The Genome-Wide EMS Mutagenesis Bias Correlates With Sequence Context and Chromatin Structure in Rice. *Frontiers in Plant Science*, *12*(March), 1–16. https://doi.org/10.3389/fpls.2021.579675

Yang, L., Yueying, W., Jahan, N., Haitao, H., Ping, C., Lianguang, S., Haiyan, L., Guojun, D., Jiang, H., Zhenyu, G., Qian, Q., Yu, Z., & Longbiao, G. (2019). Genome-Wide Association

Analysis and Allelic Mining of Grain Shape-Related Traits in Rice. *Rice Science*, *26*(6), 384–392. https://doi.org/10.1016/j.rsci.2018.09.002

Zhang, A., Liu, Y., Wang, F., Li, T., Chen, Z., Kong, D., Bi, J., Zhang, F., Luo, X., Wang, J., Tang, J., Yu, X., Liu, G., & Luo, L. (2019). Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the OsRR22 gene. *Molecular Breeding*, *39*(3). https://doi.org/10.1007/s11032-019-0954-y

Zhang, R., Liu, J., Chai, Z., Chen, S., Bai, Y., Zong, Y., Chen, K., Li, J., Jiang, L., & Gao, C. (2019). Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. *Nature Plants*, *5*(5), 480–485. https://doi.org/10.1038/s41477-019-0405-0

Zhang, X., Zhang, Y., & Li, H. (2020). Regulation of trehalose, a typical stress protectant, on central metabolisms, cell growth and division of Saccharomyces cerevisiae CEN.PK113-7D. *Food Microbiology*, *89*(January), 103459. https://doi.org/10.1016/j.fm.2020.103459

10. Anexo 1. Otros artículos publicados durante el Doctorado (4)

10.1 Rojas-Vásquez, R., Zuñiga-Umaña, J. M., Abdelnour-Esquivel, A., **Hernández-Soto, A.,** & Gatica-Arias, A. (2022). Development of synthetic seeds in Arabica coffee embryos under aseptic and non-aseptic conditions. Vegetos, 1-11. (0123456789). https://doi.org/10.1007/s42535-022-00364-9

10.2. Hernández-Soto, Alejandro, and Randall Chacón-Cerdas. 2021. "RNAi Crop Protection Advances." *International Journal of Molecular Sciences* 22(22): 12148. https://www.mdpi.com/1422-0067/22/22/12148.

10.3 Hernández-Soto, Alejandro, Fabián Echeverría-Beirute, and Tomás Guzmán-Hernández. 2021. "The RNAi as a Tool to Control Tropical Pathogens." *Agronomía Mesoamericana* 32(1): 326–37.https://doi.org/10.15517/am.v32i1.40896

10.4 Macall, Diego Maximiliano, Carlos Rogelio Trabanino, **Alejandro Hernández Soto**, and Stuart J. Smyth. 2020. "Genetically Modified Maize Impacts in Honduras: Production and Social Issues." *Transgenic Research* 29(5–6): 575–86. https://doi.org/10.1007/s11248-020-00221-y

Vegetos
https://doi.org/10.1007/s42535-022-00364-9

SHORT COMMUNICATIONS

Development of synthetic seeds in Arabica coffee embryos under aseptic and non-aseptic conditions

Randall Rojas-Vásquez[1,2] · Juan M. Zuñiga-Umaña[1] · Ana Abdelnour-Esquivel[3] · Alejandro Hernández-Soto[3] · Andres Gatica-Arias[1]

Received: 20 April 2021 / Revised: 4 February 2022 / Accepted: 7 February 2022

Abstract
In many tropical countries, *Coffea arabica* L. is a crop of great commercial and social importance. Synthetic seeds are an effective technique to propagate elite, hybrids, and genetically modified plant material. However, they are highly susceptible to microbial contamination when cultivated in non-aseptic conditions which results in limiting their applications. Here we aimed to evaluate different strategies under both aseptic and non-aseptic conditions to culture encapsulated coffee zygotic embryos with and without organic compounds (vitamins and sucrose). For both methods, encapsulated zygotic embryos were cultured in peat moss, soil with rice husk, and germination paper under greenhouse or plant growth culture room conditions. Growth room conditions resulted in better germination and capsules conservation compared with the greenhouse. The capsules with organic compounds cultivated in germination paper, under growth room conditions and previously sprayed with 1.5 mgl^{-1} carbendazim, allowed better germination percentages (93%) after 1 month of culture. On the other hand, zygotic embryos encapsulated without organic compounds, under the same conditions, stopped their development and died with the course of the days until obtaining 100% of their mortality.

Keywords Calcium alginate · Synthetic seeds · Hydrogels technique · Greenhouse · Growth culture room

Introduction

The taste, fragrance, and stimulating properties of caffeine make the non-alcoholic coffee as one of the most appreciated beverage by consumers worldwide (Ivamoto et al. 2017). In Costa Rica, the production and export of coffee plays an essential role in the economy. According to ICAFE (2021) data, the national production of coffee (fruit) was 1,769,152 bushels between 2020 and 2021.

Synthetic seeds have tremendous potential for producing and propagating elite, hybrid, or genetically modified plants (Ravi and Anand 2012). An artificial or synthetic seed is an artificially encapsulated embryo or tissue that can form an entire plant, under both in-vitro and in-vivo conditions (Islam and Bari 2012). Several gelling agents help in synthetic seed preparation, such as ethylene glycol, DMSO, Polyox, agar, and agarose. Nevertheless, the most accepted agent is calcium alginate due to its low toxicity, low cost, efficient gelation, and compatibility feature (Chandrasekhara et al. 2012).

Although synthetic seeds provide significant advantages, such as facilitating the handling of small explants and facilitate cryopreservation, synthetic seeds are highly susceptible to microbial contamination when cultivated in non-aseptic conditions. Therefore, synthetic seed technology involves cultivation in presence of aseptic conditions and substrates. However, it is possible to grow these artificial seeds in non-sterile substrates and/or in vivo. Currently, there are few studies demonstrating the culture and germination of capsules under non-sterile conditions. For example, this method

✉ Andres Gatica-Arias
andres.gatica@ucr.ac.cr

[1] Escuela de Biología, Laboratorio de Biotecnología de Plantas, San Jose Costa Rica, Universidad de Costa Rica, P.O. Box 11501-2060, San José, Costa Rica

[2] Programa de Posgrado en Ciencias Agrícolas y Recursos Naturales (PPCARN), Escuela de Agronomía, Universidad de Costa Rica, San José, Costa Rica

[3] Centro de Investigación en Biotecnología (CIB), Instituto Tecnológico de Costa Rica, P.O Box 159-7050, Cartago, Costa Rica

Published online: 19 March 2022

International Journal of
Molecular Sciences

MDPI

Review

RNAi Crop Protection Advances

Alejandro Hernández-Soto [1,2,*] and Randall Chacón-Cerdas [2]

[1] Doctorado en Ciencia Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia, Cartago P.O. Box 159-7050, Costa Rica
[2] Costa Rica Institute of Technology, Biology School, Biotechnology Research Center, Cartago P.O. Box 159-7050, Costa Rica; rchacon@tec.ac.cr
* Correspondence: alhernandez@itcr.ac.cr

Abstract: RNAi technology is a versatile, effective, safe, and eco-friendly alternative for crop protection. There is plenty of evidence of its use through host-induced gene silencing (HIGS) and emerging evidence that spray-induced gene silencing (SIGS) techniques can work as well to control viruses, bacteria, fungi, insects, and nematodes. For SIGS, its most significant challenge is achieving stability and avoiding premature degradation of RNAi in the environment or during its absorption by the target organism. One alternative is encapsulation in liposomes, virus-like particles, polyplex nanoparticles, and bioclay, which can be obtained through the recombinant production of RNAi in vectors, transgenesis, and micro/nanoencapsulation. The materials must be safe, biodegradable, and stable in multiple chemical environments, favoring the controlled release of RNAi. Most of the current research on encapsulated RNAi focuses primarily on oral delivery to control insects by silencing essential genes. The regulation of RNAi technology focuses on risk assessment using different approaches; however, this technology has positive economic, environmental, and human health implications for its use in agriculture. The emergence of alternatives combining RNAi gene silencing with the induction of resistance in crops by elicitation and metabolic control is expected, as well as multiple silencing and biotechnological optimization of its large-scale production.

Keywords: RNAi; dsRNA; silencing; encapsulation; liposomes; virus-like particles; polyplex nanoparticles; bioclay; regulatory

Citation: Hernández-Soto, A.; Chacón-Cerdas, R. RNAi Crop Protection Advances. *Int. J. Mol. Sci.* **2021**, *22*, 12148. https://doi.org/10.3390/ijms222212148

Academic Editor: Tomer Ventura

Received: 12 October 2021
Accepted: 4 November 2021
Published: 10 November 2021

1. Introduction

The world is moving toward a more sustainable crop production system that requires specific and efficient tools to battle plant pathogens. RNAi can be used for such purposes. The molecule is used in nature, degrades quickly, can disrupt the pathogen at a genetically specific level, and can complement the current agronomic crop protection practices used for organic, conventional, ecological, or technological production [1]. The reader may be familiar with the concept of DNA and genes located in the nucleus of eukaryote cells, containing the instructions to create organic molecules, mainly proteins. RNA messenger works as an intermediator, carrying the nucleus's message to the cytoplasm to be read by the ribosomes to assemble the protein. The RNAi eukaryotic machinery is a complex system for virus defense and gene expression control, sometimes called post transcriptional gene silencing (PTGS). The system can be triggered by external specific dsRNA, resulting in its RNA messenger being blocked before it gets to the ribosome, leaving the organism, such as a pathogen, disarmed [2]. The extravesical delivery of dsRNA to disarm the expression system was proven to be natural and bidirectional from plant to fungal pathogens and vice versa cross-kingdom communication [3–8].

Consequently, RNAi represents an opportunity to emulate or improve the natural plant pathogen control system by providing well-designed external dsRNA [9]. Here, we aimed to present advantages in crop protection mediated by RNAi. There are two RNAi plant-based technologies: host-induced gene silencing (HIGS) since the 1990s and

Agronomía Mesoamericana

UNIVERSIDAD DE COSTA RICA

Literature review
Volumen 32(1):326-337. Enero-abril, 2021
e-ISSN 2215-3608, doi:10.15517/am.v32i1.40896
http://www.revistas.ucr.ac.cr/index.php/agromeso

The RNAi as a tool to control tropical pathogens[1]

El ARNi como una herramienta para el control de patógenos tropicales

Alejandro Hernández-Soto[2], Fabián Echeverría-Beirute[3], Tomás Guzmán-Hernández[3]

[1] Reception: 4 de marzo, 2020. Acceptance: 20 de julio, 2020. This work was part of the Doctoral Thesis of the first author, Doctorado en Ciencia Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica, Universidad Nacional, Universidad Estatal a Distancia**,** Cartago, Costa Rica**,** and financed by the Project "Producción de mutantes de arroz (*Oryza sativa*) tolerantes a herbicidas utilizando rayos gamma para contribuir con el manejo sostenible del cultivo". Vice-Rectory for Research of thel Instituto Tecnológico de Costa Rica.
[2] Instituto Tecnológico de Costa Rica, Biology School, Biotechnology Research Center. Costa Rica. alhernandez@itcr.ac.cr (corresponding author, https://orcid.org/0000-0001-9435-5117).
[3] Instituto Tecnológico de Costa Rica, sede San Carlos. Apartado postal 159-7050, Florencia, Alajuela, Costa Rica. Tel: (506) 2475-5310. fecheverria@itcr.ac.cr (http://orcid.org/0000-0002-7238-220X); tjguzman@itcr.ac.cr (https://orcid.org/0000-0002-2719-8550).

Abstract

Introduction. Sustainable farming requires new tools for the control of pathogens, since there is a constant evolution to overcome the current biological and chemical strategies. The information provided by the transcriptomics allows creating new possibilities to tackle the pathogens. It is possible to interrupt the genetic expression of a pathogen and disable it using RNA interference (RNAi). **Objective.** To perform an analysis of an emerging technology useful for pest control, based on RNA interference. Development. Sustainable farming is measured based through social, economic, and environmental indicators. A key indicator of agriculture is the decrease in inputs for the control of pathogens and the increase in their specificity. Pest control mechanisms based on RNA interference meet both parameters. RNAi is known to have at least two functions, first for gene expression regulation, and secondly as a defense mechanism against pathogens. Consequently, RNAi can be used to protect crops from pathogens by developing genetically modified plants, or by the external application form of an aerosol. The RNAi aerosol is a tool that relies on inactivating the pathogen genes and can complement other agronomic tools available for this purpose. It is possible to design RNAi against tropical pests based on published transcriptomes, although it is necessary to overcome limitations regarding design, degradation, and stability. **Conclusion.** Interference RNA methods have the potential to be useful tools to control tropical pathogens as an alternative to achieve sustainable farming.

Keywords: sustainable farming, biotechnology, aerosol RNAi.

Resumen

Introducción. La agricultura sostenible requiere de nuevas herramientas para el control de patógenos, dado que existe una constante evolución para sobrepasar las estrategias biológicas y químicas que se usan actualmente. La información derivada de los transcriptomas permite crear nuevas posibilidades para controlar a los patógenos. Es posible interrumpir la expresión genética de un patógeno e inhabilitarlo mediante ARN de interferencia (ARNi).

Transgenic Res (2020) 29:575–586
https://doi.org/10.1007/s11248-020-00221-y

ORIGINAL PAPER

Genetically modified maize impacts in Honduras: production and social issues

Diego Maximiliano Macall · Carlos Rogelio Trabanino · Alejandro Hernández Soto · Stuart J. Smyth

Received: 20 June 2020 / Accepted: 27 October 2020 / Published online: 11 November 2020

Abstract Maize production is one of the most important activities for the Honduran economy, both in terms of area cultivated and food security provided. This article reports the results of a survey undertaken to gauge knowledge, perceptions, opinions, and attitudes of Honduran farmers towards genetically modified (GM) maize. Data were collected from 32 maize producers in 2018–19, of both conventional and GM, in five different departments (regions) of Honduras. Results show that over 75% of interviewed farmers have significant knowledge of basic biotechnology concepts and GM maize. Overall, producers have a positive opinion about GM maize because yields are higher than conventional maize, and adopting farmers have higher incomes. A significant finding was the reduction in the number of necessary pesticide applications, 84% of interviewees who used GM maize did not apply any pesticides. Farmers indicate the two main reasons for using GM maize are higher incomes (48%) and ease of use of the crop (33%). Overall, GM maize impacts in Honduras could be greater if the federal government took on a more proactive role in knowledge dissemination and facilitation of credit access.

Keywords Adoption benefits · Chemical use · Economic impacts · Farm-level evidence · Yield increases

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11248-020-00221-y) contains supplementary material, which is available to authorised users.

D. M. Macall (✉) · S. J. Smyth
Department of Agricultural and Resource Economics, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK S7N 5A8, Canada
e-mail: diego.macall@usask.ca

S. J. Smyth
e-mail: stuart.smyth@usask.ca

C. R. Trabanino
Panamerican Agricultural School (Zamorano University), Zamorano, Honduras
e-mail: rtrabanino@zamorano.edu

A. H. Soto
Instituto Tecnológico de Costa Rica (ITCR), Cartago, Costa Rica
e-mail: alhernandez@itcr.ac.cr

Introduction

In 2002, Honduras became the first Latin American country to authorize the commercial cultivation of genetically modified (GM) maize. Production of maize is an important activity for the Honduran economy (Hintze 2003); it is also responsible for providing 26% of the calories consumed by urban dwelling Hondurans, and 48% of the calories consumed by those residing in rural areas (Cruz 2013).