UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Engineering viral vectors for CRISPR-Cas mediated genome editing in plants

PhD Thesis

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Advisor: Dr. José Antonio Daròs Arnau

Valencia, March 2022

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D. José Antonio Daròs Arnau, Doctor en Ciencias Biológicas por la Universitat de València y Profesor de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Biología Molecular y Celular de Plantas (IBMCP), centro mixto del CSIC y Universitat Politècnica de València,

CERTIFICA:

Que Dña. Mireia Uranga Ruiz de Eguino, Graduada en Bioquímica y Biología Molecular por la Universidad del País Vasco – Euskal Herriko Unibertsitatea (UPV-EHU), ha realizado bajo su dirección el trabajo con título "Engineering viral vectors for CRISPR-Cas mediated genome editing in plants" que presenta para optar al grado de Doctora en Biotecnología por la Universitat Politècnica de València.

Y para que así conste a los efectos oportunos, firma el presente certificado en Valencia a 8 de marzo de 2022.

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D. José Antonio Daròs Arnau

Eta ez galdetu inoiz zer galdu genuen
Gure ametsaren bidean
Izan garelako
Oraindik ere bagara
Eta beti izango gara

— Ken Zazpi, *Itsasoa gara*

Summary

Innovative breeding technologies are urgently needed to ensure food supply to a rapidly growing population in the face of climate change. The recent emergence of tools based on the clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins has revolutionized targeted genome editing, thus holding great promise to both basic plant science and precision crop breeding. Most common CRISPR-Cas arrangements include a Cas endonuclease and a single guide RNA (sgRNA) that determines the specific target sequence to edit in the genome. The delivery of CRISPR-Cas reaction components within a plant cell is a crucial step that greatly influences editing speed and efficiency. Conventional approaches rely on supplying editing reaction components by transformation technologies or transient delivery to protoplasts, both of which are laborious processes that can raise legal concerns. Alternatively, recent studies have highlighted the potential of plant RNA viruses as transient delivery vectors of CRISPR-Cas reaction components, following the so-called virus-induced genome editing (VIGE). Since the applicability of each viral vector is limited to its molecular biology properties and a specific host range, the main objective of this Thesis has been to expand and improve the available toolbox for VIGE.

First, we engineered a vector derived from *Potato virus X* (PVX; genus *Potexvirus*; family *Alphaflexiviridae*) to deliver multiple sgRNAs in a *Nicotiana benthamiana* transformed line constitutively expressing *Streptococcus pyogenes* Cas9. Using the PVX-derived vector, host endogenous genes were efficiently targeted, producing nearly 80% indels in the tissues of adult plants. Interestingly, PVX allowed the simultaneous expression of unspaced sgRNA arrays, achieving highly efficient multiplex editing in a few days. We obtained edited progeny with a high rate of heritable bi-allelic mutations either from plants regenerated from infected tissue or infected plant seeds; in the latter

case, the sgRNA was previously fused to a mobile RNA module. Hence, since PVX is not seed-transmitted, all edited seedlings were virus-free.

Aiming to expand the virus-based toolbox for transformation-free editing, we next developed a two-compatible virus vector system for the simultaneous delivery of all CRISPR-Cas reaction components in the plant. *Tobacco etch virus* (TEV; genus *Potyvirus*; family *Potyviridae*) was engineered to express a Cas12a nuclease, and in combination with PVX-assisted sgRNA delivery, we achieved successful transformation-free genome editing in a *N. benthamiana* line constitutively expressing potyviral NIb. Moreover, we demonstrated that a single PVX vector can supply the potyviral NIb activity as well as perform sgRNA delivery for genome editing in wild-type plants.

Altogether, the work performed in this Thesis contributes to the enrichment of the current VIGE toolbox. The wide host range that both PVX and TEV possess, particularly among solanaceous species, postulates them as promising candidates for future applications in VIGE-mediated functional genomics and precision breeding.

Resumen

En el contexto actual de cambio climático, resulta urgente desarrollar nuevas tecnologías de fitomejoramiento que garanticen el suministro de alimentos a una población en rápido crecimiento. La reciente aparición de herramientas basadas en las repeticiones palindrómicas cortas agrupadas y regularmente interespaciadas (del acrónimo CRISPR en inglés) y sus proteínas asociadas (Cas) ha revolucionado la edición genómica dirigida, resultando muy prometedora tanto para la biología vegetal básica como para la mejora de cultivos. Los sistemas CRISPR-Cas más comunes incluyen una endonucleasa Cas y un ARN guía que determina específicamente la secuencia diana a editar en el genoma. El suministro de los componentes de reacción CRISPR-Cas a una célula vegetal es un paso crucial que influye notablemente en la velocidad y la eficiencia de edición. Los enfoques convencionales se basan en el suministro de dichos componentes mediante tecnologías de transformación o la expresión transitoria en protoplastos, siendo ambos procesos laboriosos que pueden acarrear problemas legales. Alternativamente, estudios recientes han destacado el potencial de virus de ARN para ser utilizados como vectores de expresión transitoria de los componentes de reacción CRISPR-Cas en plantas, también conocido como edición genómica inducida por virus (VIGE en inglés). Puesto que la aplicabilidad de cada vector viral se encuentra limitada por sus propiedades moleculares y un rango específico de plantas huésped, esta Tesis ha tenido como objetivo principal expandir y mejorar las herramientas disponibles para el VIGE.

En primer lugar, diseñamos un vector derivado del *Virus X de la patata* (PVX; género *Potexvirus*; familia *Alphaflexiviridae*) para suministrar múltiples ARNs guía a una línea transformada de *Nicotiana benthamiana* que expresaba constitutivamente la nucleasa Cas9 de *Streptococcus pyogenes*. Mediante el vector derivado de PVX, conseguimos editar genes endógenos de la planta huésped de manera eficiente,

produciendo casi un 80% de modificaciones en tejidos de plantas adultas. Curiosamente, PVX permitió la expresión simultánea de matrices de ARNs guía no espaciados, lo cual resultó en la edición de múltiples genes en pocos días. Obtuvimos progenies editadas con una alta tasa de mutaciones bialélicas hereditarias tanto de plantas regeneradas a partir de tejido infectado como de semillas de plantas infectadas; en este último caso, el ARN guía fue previamente fusionado a un módulo de ARN móvil. Dado que PVX no se transmite por semillas, todas las plántulas editadas estaban libres de virus.

A fin de expandir las estrategias basadas en virus de plantas para una edición genómica sin transformación, seguidamente desarrollamos un sistema de dos vectores virales compatibles para el suministro simultáneo de todos los componentes de reacción CRISPR-Cas en la planta. Modificamos el *Virus del grabado del tabaco* (TEV; género *Potyvirus*; familia *Potyviridae*) para que expresase una nucleasa Cas12a y, en combinación con el envío de ARN guía mediante PVX, logramos la edición sin transformación de una línea de *N. benthamiana* que expresaba constitutivamente la NIb del potyvirus. Además, demostramos que un único vector PVX era capaz de proporcionar la actividad de NIb, así como de suministrar el ARN guía para la edición genómica en plantas silvestres.

En conjunto, el trabajo realizado en esta Tesis contribuye a la expansión de las herramientas actuales para VIGE. La amplia gama de huéspedes que poseen tanto PVX como TEV, particularmente entre solanáceas, postula a ambos virus como candidatos muy prometedores para futuras aplicaciones en genómica funcional y mejora de cultivos.

Resum

En el context actual de canvi climàtic, resulta urgent desenvolupar noves tecnologies de fitomilloramentque garantisquen el subministrament d'aliments a una població en ràpid creixement. La recent aparició d'eines basades en les repeticions palindròmiques curtes agrupades i regularment interespaiades (de l'acrònim CRISPR en anglés) i les seues proteïnes associades (Cas) ha revolucionat l'edició genòmica dirigida, resultant molt prometedora tant per a la biologia vegetal bàsica com per a la millora de cultius. Els sistemes CRISPR-Cas més comuns inclouen una endonucleasa Cas i un ARN guia que determina específicament la seqüència diana a editar en el genoma. El subministrament dels components de reacció CRISPR-Cas a una cèl·lula vegetal és un pas crucial que influeix notablement en la velocitat i l'eficiència d'edició. Els enfocaments convencionals es basen en el subministrament d'aquests components mitjançant tecnologies de transformació o l'expressió transitòria en protoplasts, sent tots dos processos laboriosos que poden implicar problemes legals. Alternativament, estudis recents han destacat el potencial de virus d'ARN per a ser utilitzats com a vectors d'expressió transitòria dels components de reacció CRISPR-Cas en plantes, també conegut com a edició genòmica induïda per virus (VIGE en anglés). Com que l'aplicabilitat de cada vector viral es troba limitada per les seues propietats mol·leculars i un rang específic de plantes hoste, aquesta Tesi ha tingut com a objectiu principal expandir i millorar les eines disponibles per al VIGE.

En primer lloc, dissenyem un vector derivat del *Virus X de la creïlla* (PVX; gènere *Potexvirus*; família *Alphaflexiviridae*) per a subministrar múltiples ARNs guia a una línia transformada de *Nicotiana benthamiana* que expressava constitutivament la nucleasa Cas9 de *Streptococcus pyogenes*. Mitjançant el vector derivat de PVX, aconseguim editar gens endògens de la planta hoste de manera eficient, produint quasi un 80% de modificacions en teixits de plantes adultes. Curiosament, PVX va permetre l'expressió

simultània de matrius d'ARNs guia no espaiats, la qual cosa va resultar en l'edició de múltiples gens en pocs dies. Vam obtindre progènies editades amb una alta taxa de mutacions bial·lèliques hereditàries tant de plantes regenerades a partir de teixit infectat com de llavors de plantes infectades; en aquest últim cas, l'ARN guia va ser prèviament fusionat a un mòdul d'ARN mòbil. Atés que PVX no es transmet per llavors, totes les plàntules editades estaven lliures de virus.

A fi d'expandir les estratègies basades en virus de plantes per a una edició genòmica sense transformació, seguidament desenvolupem un sistema de dos vectors virals compatibles per al subministrament simultani de tots els components de reacció CRISPR-Cas en la planta. Modifiquem el *Virus del gravat del tabac* (TEV; gènere *Potyvirus;* família *Potyviridae*) perquè expressara una nucleasa Cas12a i, en combinació amb l'enviament d'ARN guia mitjançant PVX, aconseguim l'edició sense transformació d'una línia de *N. benthamiana* que expressava constitutivament la NIb del potyvirus. A més, vam demostrar que un únic vector PVX era capaç de proporcionar l'activitat de NIb, així com de subministrar l'ARN guia per a l'edició genòmica en plantes silvestres.

En conjunt, el treball realitzat en aquesta Tesi contribueix a l'expansió de les eines actuals per a VIGE. L'àmplia gamma d'hostes que posseeixen tant PVX com TEV, particularment entre solanàcies, postula a tots dos virus com a candidats molt prometedors per a futures aplicacions en genòmica funcional i millora de cultius.

"The power to control our species' genetic future is awesome and terrifying. Deciding how to handle it may be the biggest challenge we have ever faced."

— Jennifer A. Doudna

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"But you look at science (or at least talk of it) as some sort of demoralizing invention of man, something apart from real life, and which must be cautiously guarded and kept separate from everyday existence. (...) Science, for me, gives a partial explanation of life."

— Rosalind E. Franklin

No recuerdo con exactitud en qué momento germinó la semilla de la curiosidad científica en mí. Tan sólo viene a mi mente una niña en diversos escenarios: fijándose en toda clase de vegetación mientras recorría los húmedos bosques guipuzcoanos, para a su vez preguntarle a su aita cómo se llamaban y para qué servían; ayudando a recoger hortalizas en el huerto de los aitonas; admirando y cuidar intentando cuidar la inmensa variedad de flores del balcón de casa. Una niña que se maravillaba ante libros ilustrados de ciencias naturales y, aunque no lo comprendiese del todo, leyó sobre términos como átomo, célula o fotosíntesis mucho antes de que un profesor se los explicara. Aquella misma niña creció con los años, pero jamás perdió el interés por entender cómo funciona la vida. Tras haber recorrido un camino de vivencias y aprendizajes, hoy me detengo por un instante para mirar hacia atrás y escribir unas líneas de agradecimiento que espero no sean demasiado extensas. Comencemos pues con ello.

A mi director José Antonio, por haber confiado en mí en un momento en el que tomé una decisión arriesgada, la cual considero como una de las más acertadas de mi vida profesional. Me has formado y orientado durante la etapa predoctoral, aconsejándome y proponiendo soluciones a los problemas rutinarios a los que me enfrentaba en el laboratorio. Para mí, no ha tenido precio el volver a sentirme escuchada y valorada. Nuestras conversaciones han contribuido a reconducir el proyecto cuando éste tomaba una dirección incierta, así como a desarrollar un pensamiento crítico que seguro me será de gran ayuda en el futuro.

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más en el intuitivo. Em complementes tan bé que al teu costat he començat un procés d'evolució com a persona a la qual espere que li falte molt camí per recorrer.

A mi familia, por haber seguido mis progresos y haber confiado en mí en todas las etapas académicas. Desde un punto de vista externo al de la investigación científica, asumo que no es fácil comprender aspectos como las largas jornadas de trabajo, la inestabilidad laboral o la necesidad de desplazarse a nuevos lugares. Desde que comencé mis estudios universitarios he ido marcando puntos en el mapa cada vez más lejanos de casa. Ello ha contribuido enormemente a mi desarrollo tanto profesional como personal. que El apoyo familiar adquiere un valor distinto en la distancia, pero no quiero que se confunda con desarraigo, ya que cada vez que vuelvo a contemplar mi tierra me siento como en casa. Bide luze honen amaierara iritsi banaiz, neurri handi batean zuen pazientzia eta laguntzari esker izan da. Doktoretza tesi honen zati bat ere zuena da. Eskerrik asko nigan sinesteagatik.

Y por último, a ti, CIENCIA. Me has enseñado a comprender el mundo que me rodea, a respetarlo y a intentar contribuir a aportar mi conocimiento para hacerlo un poco mejor. Sigo manteniendo la chispa de curiosidad por esclarecer las nuevas preguntas que me hago tras cada respuesta que surge en mi trabajo, por difícil que pueda resultar el proceso. No obstante, las condiciones laborales del sector de la investigación pública en España, empezando por los investigadores predoctorales, se encuentran hoy en día muy lejos de ser ideales. A pesar de que adquirimos una formación excelente, la alta apreciación que se tiene de nosotros en el extranjero contrasta visiblemente con la falta de derechos y oportunidades en nuestro país. Nos apasiona lo que hacemos, sí, pero también nos merecemos ser valorados dignamente. Desconozco cómo será mi yo dentro de unos años, pero lo que sí sé es que lucharé porque ningún factor externo apague mi ilusión por hacer de mi pasión mi profesión. De tu mano, ciencia, siempre será más fácil hacer las maletas en busca de nuevas oportunidades, pero seguiré haciendo lo que esté en mi mano para que la investigación en España camine hacia un futuro más próspero y

prometedor en el que podamos desarrollar nuestras ideas al calor del hogar donde dimos nuestros primeros pasos.

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Abbreviations

Viruses

BaMV, *Bamboo mosaic virus*

BeYDV, *Bean yellow dwarf virus*

BNYVV, *Beet necrotic yellow vein virus*

BSMV, *Barley stripe mosaic virus*

BYSMV, *Barley yellow striate mosaic virus*

CaLCuV, *Cabbage leaf curl virus*

CaMV, *Cauliflower mosaic virus*

CLCrV, *Cotton leaf crumple virus*

FoMV, *Foxtail mosaic virus*

PEBV, *Pea early browning virus*

PVX, *Potato mosaic virus*

SYNV, *Sonchus yellow net virus*

TEV, *Tobacco etch virus*

TMV, *Tobacco mosaic virus*

TRV, *Tobacco rattle virus*

WDV, *Wheat dwarf virus*

Others

bp, base pair
cDNA, complementary DNA

CP, coat protein

CRISPR, clustered, regularly interspaced, short palindromic repeat

Cas, CRISPR–associated protein

crRNA, CRISPR RNA

crtB, *Pantoea ananatis* phytoene synthase

DNA, deoxyribonucleic acid

dpi, days post-inoculation

DSB, double-strand break

dsDNA, double-stranded DNA

FT, Flowering Locus T

GMO, genetically modified organism

HDR, homology-directed repair

LbCas12a, *Lachnospiraceae bacterium* ND2006 Cas12a

Nb, *Nicotiana benthamiana*

NHEJ, non-homologous end-joining

NIb, nuclear inclusion *b*

ΔNIb, deleted nuclear inclusion *b*

nt, nucleotide

PAM, protospacer adjacent motif

PDS, phytoene desaturase

RNA, ribonucleic acid

RNP, ribonucleoprotein

RT, reverse transcription

sgRNA, single guide RNA

SpCas9, *Streptococcus pyogenes* Cas9

ssDNA, single-stranded DNA

SSN, site-specific nuclease

ssRNA, single-stranded RNA

TALEN, transcription activator-like effector nuclease

tFT, truncated FT

tracrRNA, trans-activating crRNA

tRNA or tR, transfer RNA

VIGE, virus-induced genome editing

XT, UDP-xylosyltransferase

ZFN, zinc-finger nuclease

wpi, weeks post-inoculation

GENERAL INTRODUCTION

A brief retrospective on strategies for targeted genome editing in plants

Plant breeding for the improvement of crops essential for human nutrition dates back to the very beginnings of agriculture (Hartung and Schiemann, 2014). For a long time, farmers have relied on **traditional breeding** for the obtention of new varieties by crossing major edible crops with wild plants harboring the desired traits (Scheben *et al.*, 2017). Although genetic recombination increases variability, thousands of years of directed evolution have fixed large parts of crop genomes, thus limiting the potential for improving many traits. After genes were identified as the underlying elements controlling qualitative and quantitative traits in plants, **mutation breeding** was used for increasing genetic diversity by the introduction of random modifications through physical irradiation or chemical mutagens (Pacher and Puchta, 2017). However, these procedures are characterized by their stochastic nature, so that large numbers of mutants need to be screened to identify the desired modifications. Such non-specific and time-consuming breeding programs cannot fulfill the demands for increased crop production, even when marker-assisted breeding is used to enhance selection efficiency. In the mid-1990s, recombinant DNA technology was adopted to break the bottleneck of reproductive isolation. In **transgenic breeding**, exogenous genes are transferred into elite varieties to generate the desired traits (Prado *et al.*, 2014). Transgenesis greatly increases genetic variability beyond conventional techniques, but at the same time raises concerns on the potential impact of genetically modified organisms (GMOs) on human health and the environment. Transgene insertion occurs at random positions into the genome and along with recombinant sequences that drive its expression, which may also have unintended effects on the targeted organism. Consequently, the commercialization of GMOs is strictly limited in many countries by long and costly regulatory processes (Hartung and Schiemann, 2014; O'Connell *et al.*, 2014).

The remarkable progress in functional genomics over the last decades raised the opportunity to modify genomes predictably, boosting the transition to precision plant breeding (Osakabe *et al.*, 2010; Sikora *et al.*, 2011). This was possible due to the discovery of **site-specific nucleases (SSNs)** that promote deletions, insertions, or replacements of a DNA sequence in the target genome (Voytas, 2013).

Plants are exposed to diverse environmental (e.g. UV radiation, natural radioactivity, pathogen infection) and biological (i.e. DNA replication, metabolic processes) conditions that cause DNA damages, being double-strand breaks (DSBs) the most mutagenic lesions. To maintain genome stability, DSBs are repaired by one of the two major host repair mechanisms (Que *et al.*, 2019) (**Figure 1**). **Non-homologous end-joining (NHEJ)** is by far the preferred pathway in somatic cells. It involves minimal end processing, thus generating small insertions or deletions (indels) at the junction point. These modifications can cause a frameshift mutation or alter key amino acids of the gene product, leading to gene disruption (Chang *et al.*, 2017; Seol *et al.*, 2018) (**Figure 1, left panel**). Due to its high efficiency, NHEJ is broadly used for large-scale knock-out experiments, but it lacks enough accuracy for sophisticated engineering. The second repair pathway, known as **homology-directed repair (HDR)**, is activated when a template with large homology to the DSB is present, such as the sister chromatid or exogenous DNA (Ceccaldi *et al.*, 2016) (**Figure 1, right panel**). The exact re-joining of the broken ends can be used to precisely introduce specific point mutations, or to insert the sequence of interest into the target gene. However, HDR-based approaches are still challenging due to their low efficiency and limitations of donor DNA delivery in plants.

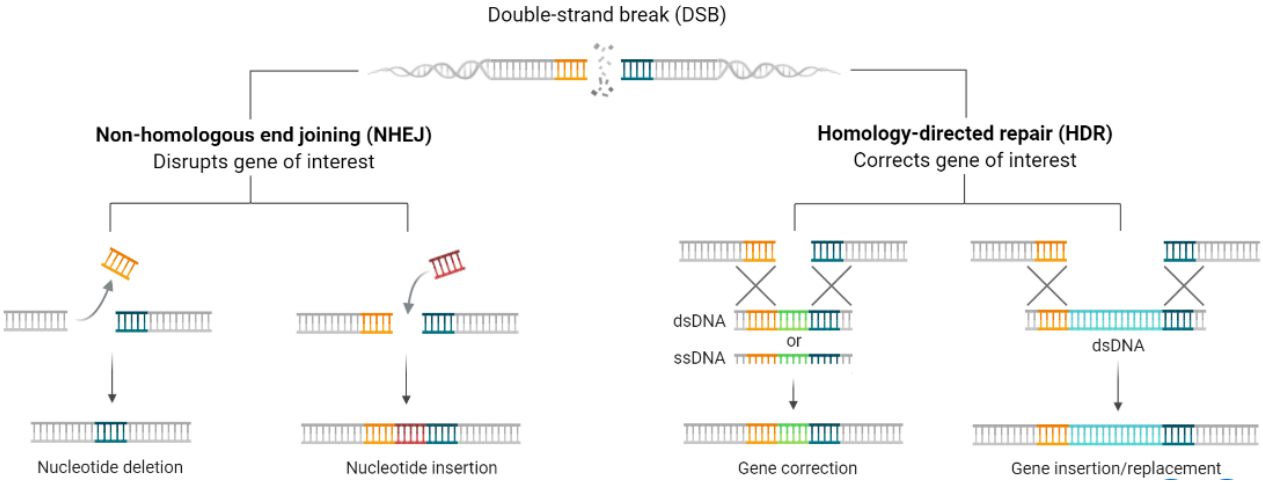

Figure 1. Double-strand break (DSB) repair mechanisms. DSBs that occur in the genome are usually repaired by error-prone non-homologous end joining (NHEJ), generating small insertions or deletions that eventually lead to gene disruption(left). In the presence of a DNA template with homology to the DSB, homology-directed repair (HDR) enables an accurate gene correction or insertion (right). Adapted from Chen *et al.* (2019). Figures in this Introduction were created with Biorender (https://biorender.com/).

Decades of progress in genome editing have culminated with the development of four major classes of SSNs that can specifically bind to the user-selected genomic region and induce precise modifications in the target gene: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effectors (TALENs), and CRISPR-Cas systems (**Figure 2**). The state-of-the-art of this field, with a special focus on the programmability and editing specificity of each SSN, will be further explained below.

Meganucleases

Meganucleases derive from naturally occurring enzymes encoded by mobile introns. They are natural mediators of DNA targeting and were the first SSNs to be used for genome editing in plants, being yeast I-SceI meganucleases the best-characterized (Puchta and Fauser, 2014; Voytas, 2013). These proteins recognize large DNA sequences (20-40 bp) and typically act as a dimer of two identical subunits (**Figure 2a**). Despite their small size (~165 amino acids for a meganuclease monomer), difficulties arise for

engineering such a large recognition sequence by protein redesign; so, meganucleases are nowadays rarely used in comparison to other SSNs.

Zinc-finger nucleases

Zinc-finger nucleases (ZFNs) are artificial bipartite enzymes (~310 amino acids in size) consisting of a specific zinc-finger protein (ZFP) domain that binds to the DNA and is linked to the catalytic domain of *Fok*I endonuclease (Urnov *et al.*, 2010) (**Figure 2b**). The ZFP module comprises three to six Cys2-His2 arrays derived from a human transcription factor and recognizes a 3-bp region in the DNA. Subsequently, *Fok*I dimerization is required to leave an 18-24 bp target sequence and induce DSBs with staggered cut ends. ZFNs have been successfully employed in genome modification of various plant species (Curtin *et al.*, 2011; Ainley *et al.*, 2013). Nevertheless, the construction of modular enzymes is time-consuming and expensive, which together with low specificity and high rate of off-target mutations, have limited their application.

Transcription activator-like effector nucleases

Transcription activator-like effector nucleases (TALENs) comprise a customizable array of TALEs artificially fused to the *Fok*I cleavage domain (Christian *et al.*, 2010) (**Figure 2c**). The DNA-binding domain contains 13-28 copies of a tandemly arrayed 34-aminoacid sequence known as TALE repeat, which is highly identical except for positions 12 and 13. These two residues specifically recognize a single base within the target sequence and thus define the DNA binding specificity. TALENs possess a high editing efficiency and are considered the most specific of all SSNs since their target sequence is 50-60 bp long. As for drawbacks, a new TALE array needs to be designed for each target sequence (Joung and Sander, 2013).

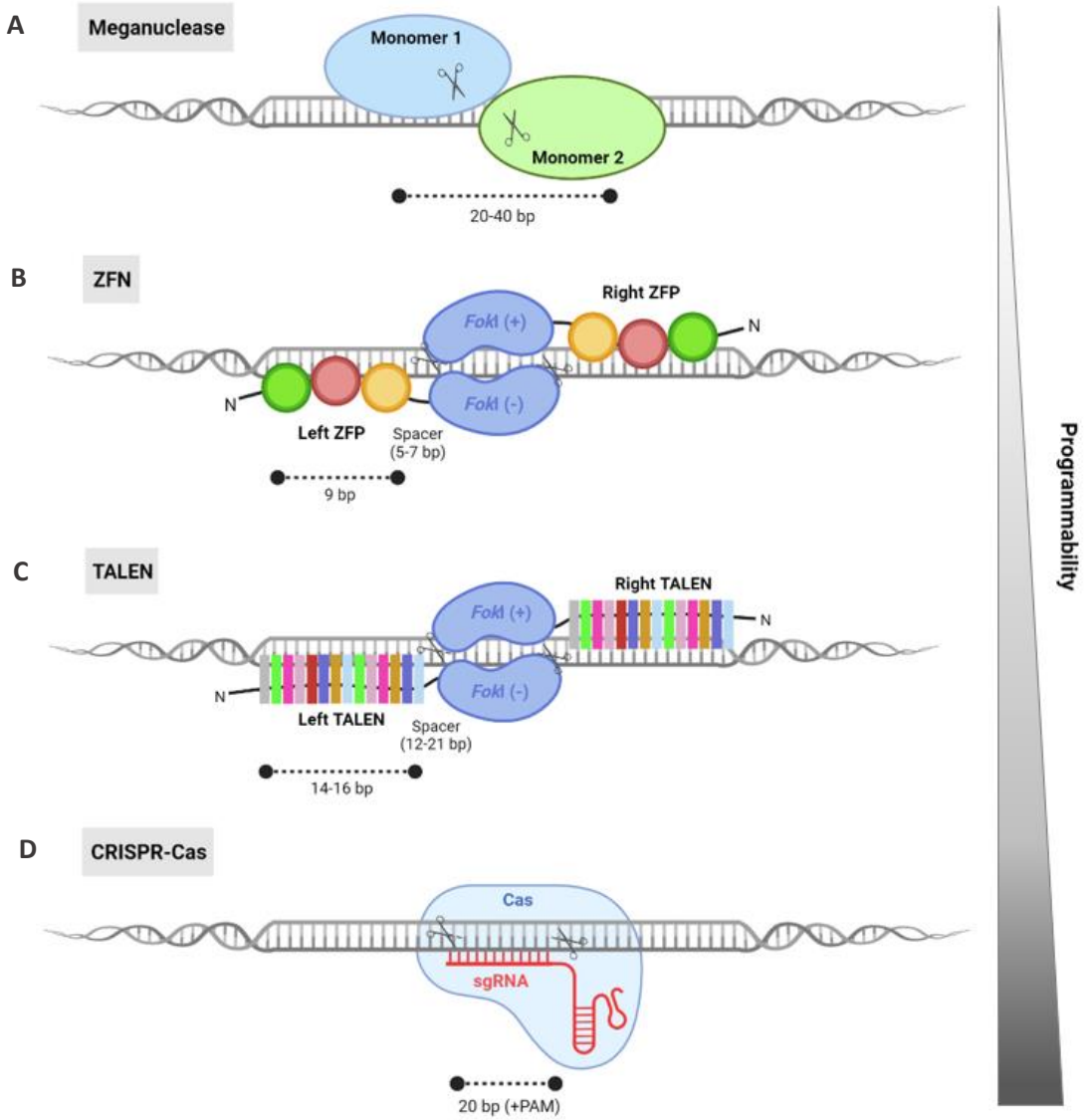

Figure 2. Site-specific nucleases (SSNs) used in genome editing. (A) Meganucleases are homodimeric proteins that recognize target DNA sequences 20-40 bp in length. (B) Zinc-finger nucleases (ZFNs) act as dimers, where each ZFN is composed of a zinc finger protein (ZFP) DNA binding domain at the N-terminal and a *Fok*I nuclease domain at the C-terminal. The linker between both domains is represented by a black line (spacer). ZFNs typically recognize target DNA sequences 18-36 bp in length, excluding spacers. (C) Transcription activator-like effector nucleases (TALENs) are dimeric enzymes SSNs similar to ZFNs. Each TALEN consists of a TALE DNA binding domain (highly conserved 34-aminoacid sequence, except for positions 12 and 13) at the N-terminal and the *Fok*I domain at the C-terminal. TALENs typically recognize target DNA sequences 30-40 bp in length, excluding spacers. (D) CRISPR-Cas system is composed of the monomeric Cas endonuclease and a single guide RNA (sgRNA), which is complementary to a 20-bp target DNA sequence upstream of the protospacer adjacent motif (PAM). Adapted from Arora and Narula (2017) and Langner *et al.* (2018). SSNs and their corresponding target DNA sequences are not in scale.

CRISPR-Cas systems: the revolution of genome editing

All the protein-dependent DNA cleavage systems described above rely on repetitive chimeric proteins that must be customized for each target sequence through an expensive, time-consuming process. In 2013, the field of genome editing was revolutionized by the emergence of the **clustered, regularly interspaced, short palindromic repeat (CRISPR)–associated protein (Cas) systems** (Mojica *et al.*, 2005; Gasiunas *et al.*, 2012; Jinek *et al.*, 2012; Cong *et al.*, 2013; Mali *et al.*, 2013). CRISPR-Cas is a two-component system consisting of the monomeric Cas endonuclease and a customizable single guide RNA (sgRNA) that combines the functions of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) (**Figure 2d**). Unlike previously developed SSNs, sequence specificity is achieved due to a complementary sgRNA that can be easily engineered to specifically target any DNA sequence. Moreover, Cas itself possesses intrinsic nuclease activity, obviating the need to fuse it to an additional protein (Wang *et al.*, 2017). The design flexibility of this system favored its rapid adoption at laboratories with no expertise in protein engineering. Consequently, applications of CRISPR-Cas systems were promptly proven to be effective for genome engineering in eukaryotic cells, including plant model species and economically important crops (Nekrasov *et al.*, 2013; Zhang *et al.*, 2016; Li *et al.*, 2017).

CRISPR-Cas is an RNA-mediated adaptive immune system in bacteria and archaea that provides defense against phage infection (Mojica *et al.*, 2005; Barrangou *et al.*, 2007; Brouns *et al.*, 2008). A CRISPR locus consists of clusters of Cas genes and CRISPR arrays, where series of 21-40 bp direct repeats (i.e. scaffolds) are interspaced by 25-40 bp variable sequences (i.e. protospacers). These protospacers are traces of past invasions of foreign DNA and share a common end sequence known as the protospacer adjacent motif (PAM). At every infection, new fragments of phage DNA are incorporated into the CRISPR array, which can be transcribed into small RNAs that guide Cas proteins to

cleave the invader's genetic material. According to Cas genes and the interference complex, CRISPR-Cas systems are divided into two classes that can be further subdivided into six types (Shmakov *et al.*, 2015). Class 1 systems (types I, II, and IV) employ multiple Cas effectors for pre-crRNA processing, protospacer loading, and target cleavage, while in class 2 systems (types II, V, and VI) all steps are performed by a single protein.

Class 2 type II CRISPR-Cas from *Streptococcus pyogenes* was the first system shown to specifically cleave DNA both in vitro and in eukaryotic cells (Barrangou *et al.*, 2007; Jinek *et al.*, 2012; Mali *et al.*, 2013). The system was repurposed for gene editing by the creation of an artificial sgRNA consisting of the fusion between a crRNA and a fixed tracrRNA by a short loop (**Figure 3a**). First, the CRISPR-associated nuclease (**SpCas9**, hereinafter referred as Cas9) binds to the scaffold region of the sgRNA to create a catalytically active complex. Then, 20 nucleotides at the 5' end of the crRNA direct the complex to a specific target DNA site adjacent to 5'-NGG-3' PAM. Upon genome binding, Cas9 catalyzes the formation of a DSB through its two nuclease domains (i.e. RuvC and HNH) that cleave the sgRNA-bound complementary and PAM-containing non-complementary DNA strands, respectively. The DSBs created by Cas9 activate the host DNA repair mechanisms, thus leading to targeted genomic modifications (see **Figure 1**). Since Cas9 remains bound to the target sequence for a long time, it tends to produce considerably smaller indels compared to the large deletions generated by ZFNs and TALENs (Richardson *et al.*, 2016). Moreover, multiple sites can be targeted simultaneously using several sgRNAs while expressing a single Cas9 (i.e. multiplexing), a property difficult to achieve with other SSNs.

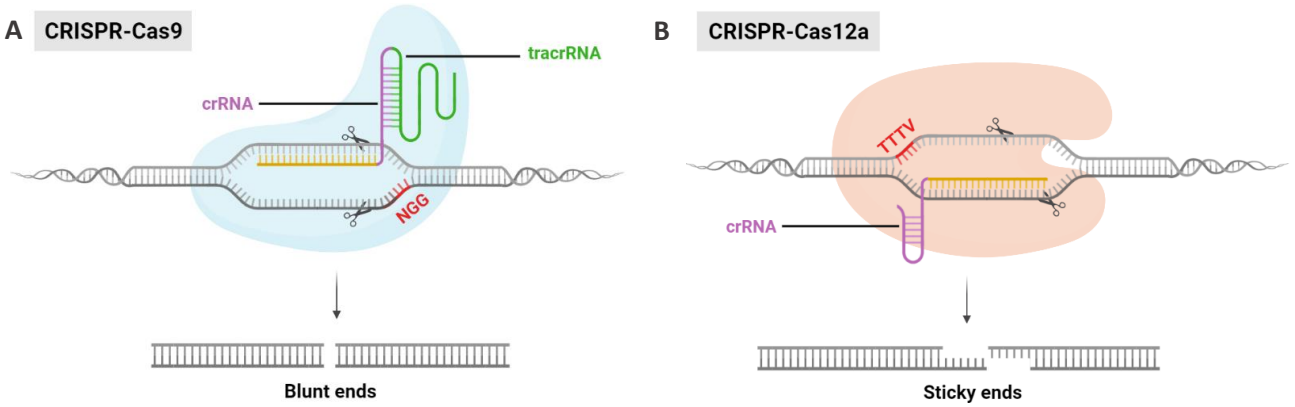

Figure 3. Schematic overview of the main class 2 CRISPR-Cas systems for genome editing. (A) In the Cas9-sgRNA system, the crRNA (light purple) and the tracrRNA (green) are fused by a short loop to form a synthetic single guide RNA (sgRNA). When Cas9 recognizes a 5'-NGG-3' PAM in the target DNA, the 20-bp protospacer of the crRNA (yellow) base pairs with its complementary DNA site and the endonucleolytic cleavage generates a PAM-proximal blunt DSB. (B) In the Cas12a-crRNA system, the mature crRNA (light purple) is generated by intrinsic RNase activity. When Cas12a recognizes a 5'-TTTV-3' PAM in the target DNA, the 23-bp protospacer of the crRNA (yellow) base pairs with its complementary DNA site and the endonucleolytic cleavage generates a PAM-distal DSB with 5' overhangs. Adapted from Chen *et al.* (2019).

Exploring the diversity of bacterial CRISPR-Cas systems

The potential applications of the CRISPR-Cas9 system in gene editing have motivated research in the field at an accelerating rate. In this context, Cas9 variants including SpCas9-VQR, SpCas9-EQR, Cas9-NG, and xCas9 3.7 with PAM requirements of 5'-NGA-3', 5'-NGAG-3', 5'-NG-3', and 5'-NG/GAA/GAT-3', respectively, have been proven in several plant species (Kleinstiver *et al.*, 2016; Zhang *et al.*, 2019). Additionally, studies on CRISPR-Cas9 diversity in bacteria led to the identification of orthologs from *Neisseria meningitidis* (NmCas9) (Hou *et al.*, 2013), *Staphylococcus aureus* (SaCas9) (Ran *et al.*, 2015), *Streptococcus thermophilus* (St1Cas9) (Xu *et al.*, 2015) and *Campylobacter jejunii* (CjCas9) (E., Kim *et al.*, 2017). Each orthogonal Cas9 system holds unique characteristics regarding protein size, PAM requirements, and sgRNA scaffolds for performing gene editing (Anders *et al.*, 2016). Both SaCas9 and StCas9 showed to be

successful in diverse plant species with relatively high editing efficiency (Steinert *et al.*, 2015; Kaya *et al.*, 2016; Jia *et al.*, 2017; Veillet *et al.*, 2020). However, the use of orthologues is restricted mainly due to their PAM complexity (i.e. 5'-NNGRRT-3' and 5'-NNAGAAW-3' for SaCas9 and St1Cas9, respectively), making SpCas9 the SSN of choice for most genome engineering approaches.

The applicability of the CRISPR-Cas9 system mostly relies on a convenient location of the 5'-NGG-3' PAM within the target gene, hence restricting the editing machinery to G-rich regions in the genome. This limitation can be counteracted with an alternative class 2 type V CRISPR system encoding **Cas12a** as the effector protein (formerly Cpf1) (Zetsche *et al.*, 2015) (**Figure 3b**). Several shreds of evidence support that type II and V CRISPR systems evolved through independent but remarkably similar pathways (Koonin *et al.*, 2017; Shmakov *et al.*, 2017). This provides Cas12a with unique features from that of Cas9 (Zaidi *et al.*, 2017): (i) a single crRNA uncoupled to the tracrRNA directs the cleavage of target DNA, which is notably shorter than the Cas9 sgRNA (42 nt vs. 100 nt); (ii) target recognition is based on a T-rich PAM sequence (5'-TTTV-3'); (iii) a staggered cleavage with 4-5-bp 5' overhangs is generated as opposed to the blunt ends induced by Cas9, which might facilitate HDR-mediated knock-in of target genes; (iv) the intrinsic RNase activity that Cas12a uses for processing the pre-crRNA can also be exploited to target multiple genes at once; and (v) the smaller size of Cas12a nuclease might facilitate its cellular delivery. Targeted mutagenesis has already been achieved in plants using Cas12a orthologs from *Francisella novicida U112* (FnCas12a), *Acidaminococcus sp. BV3L6* (AsCas12a) and *Lachnospiraceae bacterium ND2006* (LbCas12a) (Endo et al., 2016; Tang et al., 2017; Wang et al., 2017; Xu et al., 2017). Direct comparisons among these orthologues showed that LbCas12a possesses the highest efficiency of all (Tang et al., 2017). Deep sequencing analysis also showed that LbCas12a mostly induces 6-13 bp deletions, in contrast to the editing pattern of Cas9 characterized by 1-3 bp deletions (Tang *et al.*, 2017).

The large size of Cas proteins currently used for genome editing often restricts delivery into cells, since, for example, vectors have limited packaging capacity. Consequently, researchers are now focusing on engineering compact and versatile CRISPR-Cas systems that could facilitate the next generation of genome editing applications. The first step towards this direction was the discovery of **CasΦ**, a hyper compact type V system from huge bacteriophages composed of a single Cas protein half the size of Cas9 or Cas12a (700-800 aa) (Pausch *et al.*, 2020). CasΦ recognizes the 5'-TBN-3' PAM (where B= G, T, or C) and generates staggered cut ends similarly to that of Cas12a. This new system was found to be active when delivered as ribonucleoproteins (RNPs) into *A. thaliana* protoplasts, albeit with a very low editing efficiency (0.85%). More recently, Xu *et al.*, (2021) subjected the exceptionally compact type V-F CRISPR-Cas12f (formerly Cas14) system from uncultivated archaea to RNA and protein engineering. When fused to a transcriptional activator, the miniature system named **CasMINI** (only 529 aa in size) could drive high levels of gene activation comparable to that of Cas12a in mammalian cells. The work also demonstrated that CasMINI allows robust base editing and gene editing, with a unique indel pattern. In another study, Wu *et al.* (2021) reported the effectivity of Cas12f effector protein from *Acidibacillus sulforoxidans* (AsCas12f, 422 amino acids) for genome editing in bacteria and human cells. Overall, the compact nature of CRISPR-Cas12f systems opens new possibilities for the development of genome editing technologies in the future.

CRISPR specifications in plants

Successful CRISPR-mediated editing in plants requires specific vectors, efficient target sites, and suitable delivery methods to be used in each plant species. A crucial factor is the choice of **optimal promoters** for regulating the expression of the editing machinery (Arora and Narula, 2017). sgRNAs are usually regulated by tissue-specific

RNA polymerase III promoters driving the expression of small RNAs, for instance, U6 promoter from *A. thaliana* (AtU6) in dicots or maize (TaU6) in monocots. Likewise, Cas9 is placed downstream RNase II promoters such as that of ubiquitin and tagged with a nuclear localization signal (NLS) to target the nuclease to the host cell nucleus. Most work in eukaryotic cells has been done using codon-optimized versions of Cas9. This requirement seems not to be of special relevance in plants, since either human (Li *et al.*, 2013; Miao *et al.*, 2013) or plant codon-optimized versions of Cas9 (Nekrasov *et al.*, 2013; Feng *et al.*, 2013; Xie and Yang, 2013) have been validated for gene editing in several plant species.

An additional factor of outstanding importance in gene editing approaches is the selection of a **specific target site** harboring a short PAM sequence on its 3' end. Target site should be determined according to two main selection criteria: (i) on-target activity, which is defined by the cleavage specificity and predicted editing efficiency of the sgRNA; and (ii) minimum or no off-target effects, that prevent unintended cuts in the genome. Many bioinformatic tools can help researchers in designing highly specific sgRNAs such as COSMID (Cradick *et al.*, 2014) or CRISPR-PLANT (Xie *et al.*, 2014), but it is unclear to which extent their predictions agree with actual measurements. Another web tool named **CRISPOR** ranks potential sgRNAs for an input sequence according to their predicted on-target activity and potential off-targets in the genome (http://crispor.org) (Concordet and Haeussler, 2018). Since its creation, the popularity of CRISPOR within the scientific community has greatly increased, as it not only provides an accurate scoring model but also helps with cloning, expressing, and validating sgRNAs. Off-target effects associated with gene editing may not cause serious issues, because ethical issues related to this phenomenon are almost negligible in plants as compared to other organisms. Several studies have reported no off-target cleavage in CRISPR-Cas9-induced mutant plants subjected to whole-genome sequencing (Jacobs *et al.*, 2015; Nekrasov *et al.*, 2017). These findings suggest that off-targeting seems to be particularly low in plants.

However, attention needs to be paid to gene paralogs with almost identical sequences to the targets, which may also be cleaved thus producing undesired modifications.

Delivery of CRISPR-Cas components to plants

The presence of a functional Cas-sgRNA complex in the cell nucleus is crucial for efficient genome editing. Direct delivery of the editing complex might seem the simplest option but transferring such a large protein across the cell membrane is exceptionally difficult. Moreover, plants pose unique challenges such as the presence of a rigid cell wall, the common occurrence of polyploidy, and recalcitrant regeneration in many species (Varanda *et al.*, 2021). Therefore, the delivery and expression of CRISPR-Cas components within a plant cell are considered crucial steps in the genome editing process.

From GMO approaches to DNA-free genome editing

Current CRISPR-Cas approaches in plants have mainly focused on the delivery of the editing machinery by transformation technologies (Cong *et al.*, 2013; Nekrasov *et al.*, 2013) (**Figure 4a**). By far, the standard method to obtain transgenic plants is based on ***Agrobacterium tumefaciens***. This plant pathogen possesses the ability to transfer its T-DNA to the host cell nucleus, where it becomes randomly integrated (Nester, 2015). The *A. tumefaciens*-based approach has been widely used for the delivery of CRISPR-Cas components due to its simplicity and low economic cost. Several studies have confirmed that the stable integration of CRISPR-Cas DNA leads to high editing efficiencies in diverse plant species such as *A. thaliana*, rice, tomato, maize, and grapevine (Miao *et al.*, 2013; Feng *et al.*, 2014; Pan *et al.*, 2016; Char *et al.*, 2017; Tian *et al.*, 2018). The other method commonly used for genetic transformation, particularly in monocot species, is

particle bombardment. It consists of coating metallic microprojectiles (generally gold, silver, or tungsten) with DNA constructs that are subsequently fired into plant cells with high pressure. By using gold microparticles carrying CRISPR-Cas components that stably integrate into the host genome, targeted mutations have been successfully produced in several plant species (Wang *et al.*, 2014; Li *et al.*, 2015).

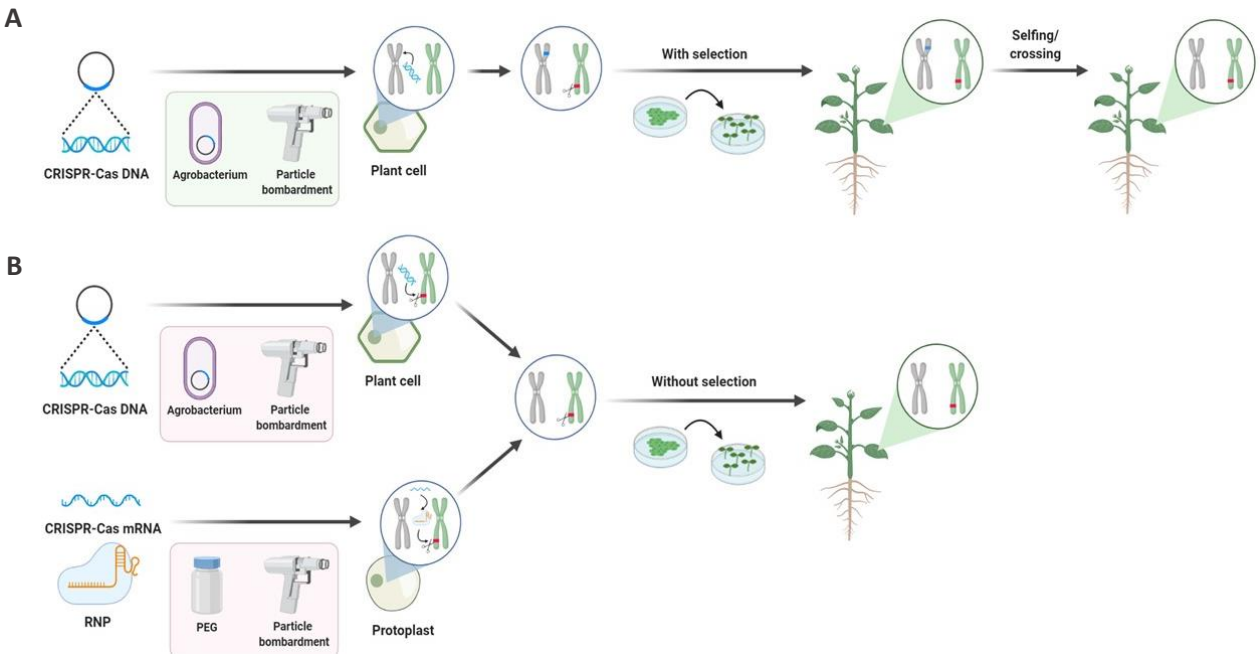

Figure 4. Delivery strategies of CRISPR-Cas components to plants. (A) Traditional transformation technologies for the stable expression of CRISPR-Cas DNA combined with herbicide or antibiotic selection. Breeding techniques such as selfing and crossing are required for the genetic segregation of the transgene before edited plants can be commercialized. Integration of CRISPR-Cas DNA and genome editing are represented as a blue and a red band within the chromosomes, respectively. (B) Transient expression strategies to obtain transgene-free, edited plants bypassing the selection steps. CRISPR-Cas components can be delivered as DNA in plant cells (top) and as mRNA or RNP in protoplasts (bottom). Details are as in the legend in **Figure 4a**. mRNA, messenger RNA; RNP, ribonucleoprotein; PEG, polyethylene glycol. Adapted from Chen *et al.* (2019).

Despite their high efficiency and multiplexing capacity, the main disadvantage of transformation technologies relies on random integration of foreign DNA, which is likely to produce undesirable off-target mutations. Moreover, crop plants carrying coding regions for Cas9 and sgRNAs are predisposed to GMO regulation (Gao *et al.*, 2015) and

require traditional breeding techniques to remove unwanted DNA before their commercialization (Pyott *et al.*, 2016; Peng *et al.*, 2017). Alternatively, transient expression of CRISPR-Cas components together with the elimination of the canonical selection steps can result in the regeneration of foreign DNA-free plants (Gleba *et al.*, 2007; Nekrasov *et al.*, 2013) (**Figure 4b, top panel**). This strategy significantly reduces transgene integration, but it does not completely eradicate it since degraded DNA fragments may still get incorporated into the host genome.

The use of **ribonucleoproteins (RNPs)** for the delivery of preassembled Cas9-sgRNA into protoplasts constitutes a promising approach to achieve DNA-free editing in plants (Woo *et al.*, 2015; Andersson *et al.*, 2018; González *et al.*, 2020) (**Figure 4b, bottom panel**). Protoplasts are uncovered plant cells that result from the enzymatic digestion of the polysaccharide-rich cell wall. They resemble very much animal cells and can be transfected via electroporation or polyethylene glycol (PEG) treatment. In the RNP strategy, the gene-editing complex can cleave target DNA immediately upon delivery, without requiring the cellular transcription and translational machinery, and is rapidly degraded afterward. Consequently, off-target cleavage is considerably reduced as compared to DNA-based expression of CRISPR-Cas. When this strategy is used for gene knock-out, DNA-free edited plants can be obtained which may not be subjected to GMO regulatory issues. More recently, the RNP editing toolbox in plants has also expanded to Cas12a by delivering both LbCas12a- and AsCas12a-crRNA RNPs into soybean and tobacco protoplasts (Kim *et al.*, 2017). However, regenerating a whole plant from single-celled protoplasts requires tissue culture procedures that frequently generate undesired somaclonal mutations.

Plant viruses as delivery vehicles of CRISPR-Cas components

Plant viruses were implemented as heterologous gene expression vectors from the beginning of genetic engineering more than three decades ago. The advent of molecular biology and reverse genetics, as well as the discovery of RNA silencing and the development of high-throughput sequencing technologies, enabled the manipulation of viral genomes to express heterologous proteins and RNAs in plants (Scholthof *et al.*, 1996). Since then, a variety of *A. tumefaciens*-based viral vectors has been developed to load them into plant cells by simple inoculation methods. Recent studies highlight the potential use of viral vectors as transient delivery vehicles for CRISPR-Cas components in many biological systems, including plants (Platt et al., 2014; Senís et al., 2014; Lau and Suh, 2017; Xu et al., 2019). This strategy is known as **virus-induced genome editing (VIGE)** and postulates as a game-changer in CRISPR-based genome editing. VIGE possesses several advantages in comparison to conventional delivery methods (Cody and Scholthof, 2019). First, owing to prolific viral replication CRISPR-Cas components can accumulate to high levels within the host cell, leading to efficient (up to 60-70%) and fast (3-7 days) genome editing. This transient expression platform provides an ideal screening tool to assess the effectivity and specificity of sgRNA designs. Second, when using RNA viruses, targeted modifications can be obtained without the integration of any foreign material into the plant genome, which should avoid raising additional regulatory and ethical issues. Last, viral vectors can be deployed even if editing the target gene negatively affects plant fitness, because they are directly delivered into adult plants and editing may occur rapidly, before the onset of any severe physiological effects. Nevertheless, it should be noted that the application of each viral vector encounters its challenges related to molecular biology and specific host range. Since the first VIGE reports, researchers have done great progress in engineering viral vectors that enable efficient delivery of CRISPR-Cas components into plants, which has helped to expand the VIGE toolbox to different plant species and purposes. In the following lines, we will

discuss all the studies carried out so far on plant viral-assisted CRISPR-Cas editing (**Table 1**).

Table 1. Plant virus vectors developed for the delivery of CRISPR-Cas components in VIGE strategies. +ssDNA viruses are indicated in blue, +ssRNA viruses in green, and -ssRNA viruses in yellow. Adapted from Oh *et al.* (2021).

Viral vector	Virus family/ genus	Nuclease	sgRNA type	Plant species	Systemic expression	Heritability of gene editing	References
BeYDV	*Geminiviridae/ Mastrevirus*	ZFN, TALEN, SpCas9	AtU6-sgRNA	Tobacco (*N. tabacum*)	No	Not determined	(Baltes *et al.*, 2014)
		TALEN, SpCas9	AtU6-sgRNA	Tomato (*Solanum lucopersicum*)	No	Not determined	(Čermák *et al.*, 2015)
				Potato (*Solanum tuberosum*)	No	Not determined	(Butler *et al.*, 2016)
		SpCas9	AtU6-sgRNA	Potato (*S. tuberosum*)	No	Not determined	(Butler *et al.*, 2015)
				Tomato (*S. lucopersicum*)	No	Not determined	(Dahan-Meir *et al.*, 2018)
WDV	*Geminiviridae/ Mastrevirus*	SpCas9	OsU6-sgRNA	Rice (*Oryza sativa*)	No	Not determined	(Wang *et al.*, 2017)
			TaU6-sgRNA	Wheat (*Triticum aestivum*)	No	Not determined	(Gil-Humanes *et al.*, 2017)
CaLCuV	*Geminiviridae/ Begomovirus*	-	AtU6-sgRNA	*N. benthamiana*	Yes	Not determined	(Yin *et al.*, 2015)
CLCrV	*Geminiviridae/ Begomovirus*	-	AtU6-sgRNAt (+/- FT)	*A. thaliana*	Yes	Low frequency (progeny of infected plants)	(Lei *et al.*, 2021)
TRV	*Virgaviridae/ Tobravirus*	Mega-nuclease	-	*N. alata*	Yes	Low frequency	(Honig *et al.*, 2015)
		ZFN, TALEN	-	Tobacco (*N. tabacum*), petunia (*P. hybrida*)	Yes	Not determined	(Marton *et al.*, 2010)
		-	PEBV-sgRNA	*N. benthamiana*	Yes	Low frequency (progeny of infected plants)	(Ali *et al.*, 2015)
		-	PEBV-sgRNA	*A. thaliana*, *N. benthamiana*	Yes	Not determined	(Ali *et al.*, 2018)
		-	PEBV-sgRNA (+/- FT)	*N. benthamiana*	Yes	High frequency (progeny of infected plants)	(Ellison *et al.*, 2020)
TMV	*Virgaviridae/ Tobamovirus*	-	TMV-sgRNA-ribozyme	*N. benthamiana*	No	Not determined	(Cody *et al.*, 2017)

PEBV	*Virgaviridae/ Tobravirus*	-	PEBV-sgRNA	*A. thaliana*, *N. benthamiana*	Yes	Not determined	(Ali *et al.*, 2018)
BSMV	*Virgaviridae/ Hordeivirus*	-	BSMV-sgRNA	*N. benthamiana*, wheat, maize (*Zea mays*)	Yes	*N. benthamiana:* yes (via tissue regeneration)	(Hu *et al.*, 2019)
			BSMV-sgRNA (+/- FT)	Wheat (*Triticum aestivum*)	Yes	Yes (progeny of infected plants)	(Li *et al.*, 2021)
BNYVV	*Benyviridae/ Benyvirus*	-	p31-sgRNA	*N. benthamiana*	Yes	Not determined	(Jiang *et al.*, 2019)
FoMV	*Alphaflexiviridae/ Potexvirus*	-	FoMV-sgRNA	*N. benthamiana*, maize (*Zea mays*), foxtail (*Setaria viridis*)	Yes	Not determined	(Mei *et al.*, 2019)
		SpCas9	AtU6-sgRNA	*N. benthamiana*	Yes	Not determined	(Zhang *et al.*, 2020)
PVX	*Alphaflexiviridae/ Potexvirus*	SpCas9	AtU6-sgRNA	*N. benthamiana*	Yes	High frequency (via tissue regenerationd	(Ariga *et al.*, 2020)
BYSMV	*Rhabdoviridae/ Cytorhabdovirus*	SpCas9	BYSMV-sgRNA	*N. benthamiana*	No	Not determined	(Gao *et al.*, 2019)
SYNV	*Rhabdoviridae/ Betanucleo-rhabdovirus*	SpCas9	SYNV-sgRNA-tRNA	*N. benthamiana*	Yes	Yes (via tissue regeneration)	(Ma *et al.*, 2020)

DNA viruses as a "proof-of-concept" of VIGE

The first VIGE reports were based on the use of **geminiviruses** for the generation of plant knock-out lines. Geminiviruses are a widespread group of plant DNA viruses characterized by single-stranded, circular DNA with monopartite or bipartite genomes (Lozano-Durán, 2016). Their small size allows easy manipulation of the viral genome, but on the other hand, it physically limits their cargo capacity. The coat protein (CP) of some bipartite geminiviruses can be replaced by a heterologous DNA of up to 800-1000 bp while maintaining most of the features required for viral movement and replication. Although this modification enables the virus to produce high amounts of sgRNA, it is still insufficient to carry long DNA fragments such as genes encoding Cas nucleases.

Aiming to additionally increase geminivirus cargo capacity, further removal of CP and movement protein (MP) coding sequences result in non-infectious replicons. Since deconstructed viruses are unable to move systemically, they must be delivered into plants by *A. tumefaciens*-mediated transformation, in contrast to other techniques such as agroinfiltration or mechanical inoculation where infectious replicons are required. ***Bean yellow dwarf virus*** (**BeYDV**; genus *Mastrevirus*) was the first to be adapted for the expression of ZFNs, TALENs as well as Cas9 nuclease and the sgRNA in plants (Baltes *et al.*, 2014; Čermák *et al.*, 2015; Butler *et al.*, 2016), showing a considerable cargo capacity and notably higher editing efficiency than conventional *A. tumefaciens* T-DNA delivery. Another application of geminiviruses in VIGE is related to gene knock-in. As replication of DNA viruses occurs in the host cell nucleus with high-copy numbers, it leads to the production of large amounts of repair templates that are required for HDR to outcompete NHEJ. As a result, an increase in the frequency of HDR was documented in several works with BeYDV (Dahan-Meir *et al.*, 2018), ***Cabbage leaf curly virus*** (**CaLCuV**; genus *Begomovirus*) (Yin *et al.*, 2015), and ***Wheat dwarf virus*** (**WDV**; genus *Mastrevirus*) (Wang *et al.*, 2017; Gil-Humanes *et al.*, 2017).

sgRNA delivery with positive-strand RNA viruses

The use of DNA viruses for gene editing inevitably leads to the possibility of accidental integration of foreign genetic material into the host genome. Conversely, RNA viruses possess the advantage of developing their infectious cycle exclusively in the cytoplasm, so resulting in foreign DNA-free plants that should avoid raising regulatory and ethical issues.

Several plant RNA virus-based vectors have been tested as vectors for the delivery of sgRNAs in plants. ***Tobacco rattle virus*** (**TRV**; genus *Tobravirus*) is a bipartite positive-strand RNA virus composed of TRV1 and TRV2 genomes (Varanda *et al.*, 2021). TRV1 is

essential for viral replication and movement, whereas TRV2 encodes the CP and other nonstructural proteins. This nonessential region has been engineered for the expression of heterologous proteins, as well as fragments of host plant genes for virus-induced gene silencing (VIGS) (Senthil-Kumar and Mysore, 2014). As compared to geminivirus, TRV has various advantages for VIGE including a broad host range (more than 400 plant species) and the ability to migrate to growing plant tissues (Cody and Scholthof, 2019).

TRV-based vectors were first used in plant genome engineering to deliver ZFNs and TALENs in tobacco and petunia (Marton *et al.*, 2010). In this system, targeted editing enabled the recovery of a reporter gene, and most importantly, modifications were transmitted to the next generation. A similar approach was used for the expression of meganucleases in *N. alata*, yielding mutant seeds from infected plants (Honig *et al.*, 2015). Regarding CRISPR-Cas technology, Ali *et al.* (2015) developed a TRV-based vector as a sgRNA delivery vehicle to successfully edit *A. thaliana* and *N. benthamiana* genomes. The detection of targeted modifications in the progeny of infected plants confirmed the ability of TRV to infect germline cells. However, the same authors demonstrated in a later work that ***Pea early browning virus*** (**PEBV**; genus *Tobravirus*) delivered sgRNAs in the plant more efficiently than TRV being also able to infect meristematic tissue, which allowed to recover seeds with the desired modifications, although with a low rate (Ali *et al.*, 2018).

Tobacco mosaic virus (**TMV**; genus *Tobamovirus*; family *Virgaviridae*) is a monopartite positive-strand RNA virus that expresses large amounts of CP from a viral subgenomic promoter. It can be easily manipulated by partial substitution of CP with heterologous genes, which in turn allows a high-level gene expression in several hosts and prolonged integrity of its derived vectors (Gleba *et al.*, 2007). Based on this potential, a TMV-based vector was developed for sgRNA delivery (Cody *et al.*, 2017). Despite CP deletion impairs systemic movement of the virus, high concentrations of sgRNA were

delivered that led to efficient editing in *N. benthamiana* plants previously infiltrated with a plasmid expressing Cas9.

Transgenic plants constitutively expressing Cas9 are a useful tool to explore the feasibility for VIGE of plant viral vectors. For instance, ***Beet necrotic yellow vein virus*** (**BNYVV**; genus *Benyvirus*) replicons previously engineered for simultaneous expression of foreign proteins were shown to deliver sgRNAs for efficient genome editing in *N, benthamiana* (Jiang *et al.*, 2019). This approach has also expanded for targeted mutagenesis in non-model plant species using ***Barley stripe mosaic virus*** (**BSMV**; genus *Hordeivirus*) in wheat and maize (Hu *et al.*, 2019) or ***Foxtail mosaic virus*** (**FoMV**; genus *Potexvirus*; family *Alphaflexiviridae*) in *N. benthamiana*, maize, and foxtail (*Setaria viridis*) (Mei *et al.*, 2019).

Virus-mediated expression of Cas proteins

All the previously mentioned VIGE attempts were based on plant virus-mediated expression of sgRNAs in transgenic plants constitutively expressing Cas9 nuclease. For a long time, researchers failed in their efforts to develop a plant virus-derived vector able to deliver the entire CRISPR-Cas system and move systemically, since the expression of large heterologous genes with positive-strand RNA viruses is known to affect vector stability and cell-to-cell movement (Avesani *et al.*, 2007). This limitation was overcome when two independent reports demonstrated the use of negative-strand RNA viruses to deliver both Cas9 nuclease and sgRNAs at the whole plant level.

Rhabdoviruses form a diverse family of negative-strand RNA viruses that show a stable expression of foreign genes up to 6 kb in vertebrates (Jackson and Li, 2016). Such a property opens the possibility to deliver Cas9 proteins into plant cells, even though plant rhabdoviruses have been poorly exploited due to difficulties in genetic manipulation

(Zhou *et al.*, 2019). In a first report, Gao *et al.* (2019) validated targeted mutagenesis in *N. benthamiana* using ***Barley yellow striate mosaic virus*** (**BYSMV**; genus *Cytorhabdovirus*) to express both Cas9 and the sgRNA. Moreover, work by Ma *et al.* (2020) showed that ***Sonchus yellow net virus*** (**SYNV**; genus *Betanucleorhabdovirus*)-mediated expression of all CRISPR-Cas9 components led to an effective DNA-free genome editing either in single or multiple genes. Unlike BYSMV, SYNV vector generated modifications at the whole plant level that were inherited by subsequent generations of the edited plants. However, since rhabdoviruses rarely infect germline cells, tissue culture is still necessary to obtain individual edited plants.

More recently, two positive-strand RNA viruses in the genus *Potexvirus* have been developed to express Cas9 proteins with the help of RNAi suppressor *p19* derived from *Tomato bushy stunt virus*. Due to their filamentous flexible structure, gene insert size might not be physically limited in potexviruses as it happens to small viruses (Kendall *et al.*, 2008). A first approach based on **FoMV** confirmed that *N. benthamiana* was edited at the whole plant level when viral vectors harboring both Cas9 and the sgRNA were delivered together (Zhang *et al.*, 2020). Nonetheless, no mutations were transmitted to germline cells and it is unclear how two FoMV replicons could infect the same cell. Alternatively, ***Potato virus X*** (PVX; genus *Potyvirus*; family *Alphaflexiviridae*) was engineered to deliver the entire CRISPR-Cas9 system in plants, and the recombinant vector was both agroinfiltrated and mechanically inoculated in *N. benthamiana* (Ariga *et al.*, 2020). Most cells were infected with PVX-Cas9 RNA and showed high levels of Cas9 expression, while the integration of T-DNA into the host genome was scarce. Targeted modifications were inherited in the next generation with no PVX transmission through seeds, hence resulting in DNA-free edited progeny. This simple and efficient delivery system of CRISPR-Cas components is a great improvement of the VIGE toolbox for achieving transgene-free genome editing in plants.

Toward tissue culture-free editing of plants

Except for *A. thaliana* and its close relatives suitable for floral dip transformation, tissue culture is required in most plant species to regenerate whole plants from somatic cells. Besides being a time-consuming and labor-intensive process, this strategy can generate undesired genetic or epigenetic variations. In tissue culture, hormones are added to the medium to stimulate cell division and maintain the resulting callus at a similar stage as meristematic cells, but their levels vary between species and need to be optimized individually. The finding that transient expression of developmental regulators including *Wuschel* (WUS), *Shoot meristemless* (STM), and *Isopentenyl transferase* (IPT) induces somatic embryogenesis in plants was a breakthrough advance in the field (Lowe *et al.*, 2016). Very lately, a novel approach explored the simultaneous delivery of developmental regulators and sgRNAs in Cas9-expressing *N. benthamiana* plants, either by co-culturing seedlings germinated in liquid culture with *A. tumefaciens* or by agroinfiltration of soil-grown plants (Maher *et al.*, 2020) (**Figure 5a**). Ectopic expression of WUS, STM, and IPT induced *de novo* meristem formation and edited shoots could be regenerated bypassing tissue culture. In a different study, the simultaneous expression of *Growth-regulating factor 4* (GFR4) *and GRF-interacting factor 1* (GIF1) with CRISPR-Cas9 notably increased the frequency of genome editing (Debernardi *et al.*, 2020). These results postulate the combined delivery of CRISPR-Cas components and developmental regulators as a promising future method for DNA manipulation in a broad range of recalcitrant species.

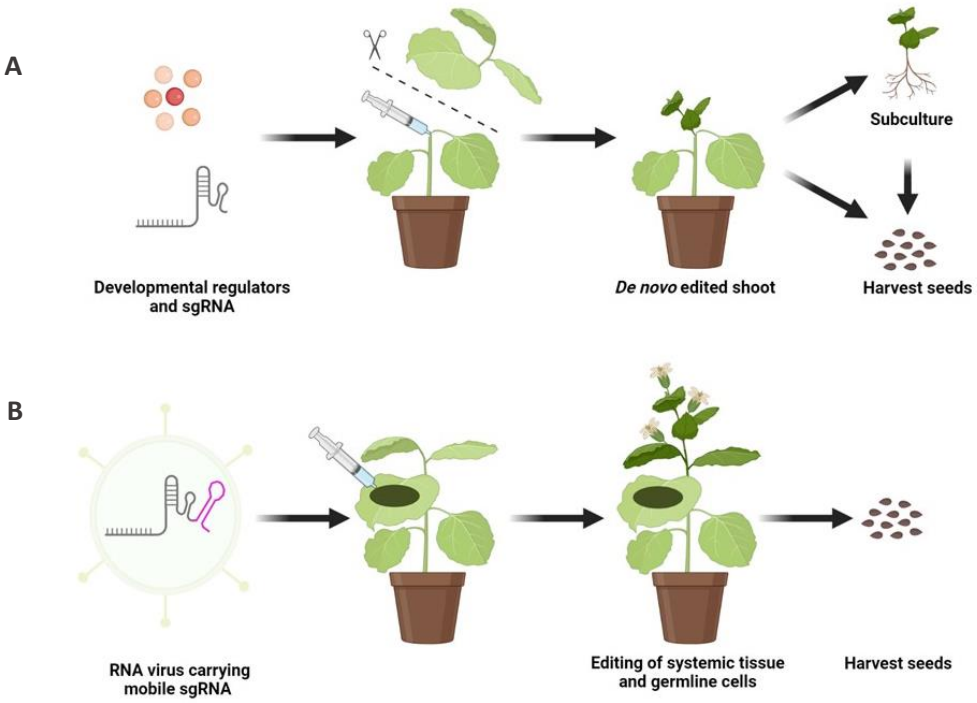

Figure 5. Tissue culture-free strategies for plant genome editing. (A) Overexpression of developmental regulators can induce de novo meristem formation that carries targeted genome modifications. These shoots can either be propagated for regeneration or might set flowers directly for the collection of edited seeds. (B) In Cas9-expressing plants, systemic infection with a recombinant RNA virus carrying mobile sgRNAs can cause mutations in the germline, thus resulting in a high rate of edited seeds. sgRNA, single guide RNA. Adapted from Huang and Puchta (2021).

Another recent approach aiming to bypass tissue culture has been the use of a positive-strand RNA virus such as TRV to deliver mobile sgRNAs into Cas9-expressing plants (Ellison *et al.*, 2020) (**Figure 5b**). In this case, the viral vector was engineered to produce a sgRNA fused on its 3' end to mobile RNA elements like *Flowering locus T* (*FT*) mRNA, which promoted the mobility of the editing complex to apical meristems. Surprisingly, these apical parts of the plants showed a higher editing efficiency compared to that of the initially infected tissues. Moreover, the induction of mutations in the germline resulted in a high rate of bi-allelic mutations with no evidence of virus transmission in the progeny of infected plants. This strategy is of especial interest when using plants with a single copy of Cas9, as transgene-free edited plants can be easily obtained by the segregation of the edited progeny.

OBJECTIVES

In the context of a fast-growing population and climate change, enhanced agricultural production is urgently needed to ensure food supply worldwide. Creating genetic variation in the gene pool is an essential requirement for developing new plant varieties with improved agronomic traits. Recent advances in CRISPR-Cas mediated genome editing off great promise for both basic and applied plant research, as it can rapidly generate stable and heritable modifications without affecting the existing traits. The delivery of CRISPR-Cas components within the plant cell is a crucial step in the genome engineering process that greatly influences the editing speed and efficiency. In addition, all CRISPR-Cas components must be transiently expressed to avoid legal and ethical hurdles associated with transgenic plants.

Due to their comprehensive genetic structure and remarkable biological properties, plant viruses can be manipulated into biotechnological tools for many purposes, including the delivery of CRISPR-Cas reaction components into plant cells. However, the fact that each plant virus is restricted to a specific host range highlights the need to expand and optimize the current toolbox of viral vectors for precise genome editing. To address this general goal, the following specific objectives were defined:

1. To engineer a new RNA virus vector based on *Potato* virus X (PVX) for the delivery of single or multiple sgRNAs, assessing the role of processable spacers in sgRNA viability.

2. To develop a system of two compatible RNA virus vectors for the delivery of all the CRISPR-Cas reaction components into plants.

3. To study the heritability of genome modifications in the progeny of parental plants infected with RNA virus vectors carrying CRISPR-Cas reaction components.

CHAPTER I

Efficient Cas9 multiplex editing using unspaced sgRNA arrays engineering in a *Potato virus X* vector

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All authors participated in work conception and design. MU, VA, MVV and SS performed the experiments. All authors participated in results analyses. MU, DO and JAD wrote the manuscript with input from the rest of the authors.

ABSTRACT

Systems based on the clustered, regularly interspaced, short palindromic repeat (CRISPR) and CRISPR associated proteins (Cas) have revolutionized genome editing in many organisms, including plants. Most CRISPR-Cas strategies in plants rely on genetic transformation using *Agrobacterium tumefaciens* to supply the gene editing reaction components, such as Cas nucleases or the synthetic guide RNA (sgRNA). While Cas nucleases are constant elements in editing approaches, sgRNAs are target-specific and a screening process is usually required to identify those most effective. Plant virus-derived vectors are an alternative for the fast and efficient delivery of sgRNAs into adult plants, due to the virus capacity for genome amplification and systemic movement, a strategy known as virus-induced genome editing. We engineered *Potato virus X* (PVX) to build a vector that easily expresses multiple sgRNAs in adult solanaceous plants. Using the PVX-based vector, *Nicotiana benthamiana* genes were efficiently targeted, producing nearly 80% indels in a transformed line that constitutively expresses *Streptococcus pyogenes* Cas9. Interestingly, results showed that the PVX vector allows the expression of arrays of unspaced sgRNAs, achieving highly efficient multiplex editing in a few days in adult plant tissues. Moreover, virus-free edited progeny can be obtained from plants regenerated from infected tissues or infected plant seeds, which exhibit a high rate of heritable biallelic mutations. In conclusion, this new PVX vector allows easy, fast, and efficient expression of sgRNA arrays for multiplex CRISPR-Cas genome editing and will be a useful tool for functional gene analysis and precision breeding across diverse plant species, particularly in Solanaceae crops.

Keywords: CRISPR-Cas9, sgRNA expression, virus-induced genome editing, multiplexing, *Potato virus X*, solanaceous plants, technical advance.

INTRODUCTION

Targeted genome editing of plant DNA is a valuable tool for basic and applied biology as it facilitates gene function studies and crop improvement (Pennisi, 2010; Chen *et al.*, 2019). This can be performed by sequence-specific nucleases (SSNs) that specifically bind to the user-selected genomic region and induce DNA double-strand breaks (DSBs) (Voytas, 2013), but the recent emergence of tools based on bacterial clustered, regularly interspaced, short palindromic repeat (CRISPR)–associated protein (Cas) systems have revolutionized targeted genome editing (Cong et al., 2013). The most common arrangements comprise the Cas9 endonuclease from *Streptococcus pyogenes* and a synthetic guide RNA (sgRNA), which combines the functions of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The sgRNA directs the Cas9 endonuclease to a target sequence complementary to 20 nucleotides preceding the 5'-NGG-3' protospacer-associated motif (PAM) required for Cas9 activity. The DSBs created by Cas9 activate the native host DNA repair mechanisms of non-homologous end-joining (NHEJ) or homology-directed repair (HDR). In plants, NHEJ occurs more frequently and results in small insertions or deletions (indels) that restore the integrity of the host DNA. These indels cause localized DNA disruption and have been used for sequence-specific knock-out of downstream gene products, such as proteins and long noncoding RNAs (Ran et al., 2013; Schiml et al., 2014). Thus, the specificity and versatility provided by the CRISPR-Cas tools allow for unprecedented, simple genome engineering of plant model species and economically important crops (Nekrasov *et al.*, 2013; Zhang *et al.*, 2016; Li *et al.*, 2017, 2018).

A key advantage of CRISPR genome editing is the possibility to program the cellular pool of Cas9 proteins with several sgRNAs operating in parallel, a feature known as multiplexing. The multiplexing capacity of an editing tool determines the speed at which simultaneous modifications can be introduced in the genome and therefore the ability to

perform comprehensive genome engineering. An efficient way to deliver several sgRNAs acting simultaneously in the cell consists of engineering long RNAs comprising several sgRNA units arrayed in tandem, which are later processed into single functional sgRNAs. Contrary to other CRISPR nucleases as Cas12a (Zetsche *et al.*, 2017), Cas9 itself cannot process sgRNA arrays on its own. Therefore, functional tandem sgRNAs require processable spacers to be included in the array design. Spacers can be engineered involving either self-cleavable ribozymes (Gao and Zhao, 2014; Xu et al., 2017) or other RNA motifs processable by trans-acting RNases. For the second option, two main strategies have been described. One of them makes use of tRNAs as spacers, which are then removed by endogenous tRNA-processing RNases, RNase P, and RNase Z (Xie et al., 2015). In the second approach, spacers are small recognition sequences processed by a trans-acting Csy4 RNase (Nissim et al, 2014; Tsai et al., 2014), which needs to be exogenously supplied, usually as a transgene.

CRISPR-Cas approaches in plants have mainly focused on the delivery of the nucleases and sgRNAs by transformation technologies or transient delivery to protoplasts (Cong et al., 2013; Nekrasov et al., 2013). However, recent studies indicate that viral vectors may be most useful to express CRISPR-Cas reaction components, as they are in other biological systems (Platt et al., 2014; Senís et al., 2014; Lau and Suh, 2017; Xu et al., 2019), following the so-called virus-induced genome editing (VIGE). Several plant RNA virus-based replicons have been tested as vectors for the delivery of sgRNAs to create gene knockouts and insertions, including *Tobacco rattle virus* (TRV) (Ali *et al.*, 2015; Ellison *et al.*, 2020), *Tobacco mosaic virus* (Cody et al., 2017), *Pea early browning virus* (PEBV) (Ali et al., 2018), *Beet necrotic yellow vein virus* (BNYVV) (Jiang et al., 2019) and *Barley stripe mosaic virus* (BSMV) (Hu et al., 2019). Compared to delivery methods via *Agrobacterium tumefaciens*, plant virus-mediated sgRNA delivery systems possess some advantages: (i) the sgRNAs can accumulate to high levels owing to viral replication, which usually contributes to a higher genome editing efficiency; (ii) if the viral

vector moves systemically, phenotypic alterations may appear in infected plants in a relatively short period after virus inoculation; and (iii) in the case of RNA viruses, they can produce a high rate of edited cells without the risk of integration of heterologous material into the plant genome, which avoids raising additional regulatory and ethical issues. As for drawbacks, each viral vector has its own particularities based on virus molecular biology and is restricted to a specific host range. Consequently, there is a necessity to enlarge and improve the available toolbox for VIGE.

Potato virus X (PVX) is a member of the genus *Potexvirus* (family *Alphaflexiviridae*) that infects 62 plant species of 27 families (Edwardson and Christie, 1997), including important crops in the family Solanaceae, such as potato, tomato, pepper, or tobacco, and is transmitted mechanically from plant to plant (Adams et al., 2004). PVX has a plus (+) single-strand RNA genome 6.4-kb in length, with a 5'-methylguanosine cap, a polyadenylated 3' tail, and five open reading frames (ORFs) (Loebenstein and Gaba, 2012). In this study, we describe a novel PVX-based sgRNA delivery vector for CRISPR-Cas genome editing in plants, which produces targeted indels with high efficiency (nearly 80%) in *N. benthamiana* plants previously transformed to constitutively express Cas9 nuclease. We also demonstrate that PVX is a suitable vector for the simultaneous delivery of multiple sgRNAs per viral genome. Unexpectedly, we found that, in the absence of spacers and processing signals, sgRNAs engineered in tandem in the PVX genome can induce efficient gene editing. Efficiency of editing on unspaced sgRNA arrays is apparently influenced by the relative position of each sgRNA in the arrays, with positions close to the 3’ end of the viral RNA precursor transcript showing higher efficiency. Finally, we demonstrate that virus-free progeny efficiently carrying biallelic indels at the target genes are obtained from plants regenerated from infected tissue or from seeds of infected plants, strategies that could facilitate high-throughput screenings.

RESULTS

Engineering a PVX-based sgRNA delivery system for genome editing in plants

CRISPR-Cas9 is a two-component system that requires both Cas9 nuclease and a small sgRNA to perform gene editing. We aimed to develop a sgRNA delivery system based on PVX, an RNA virus that infects many plant species, particularly important crops in the family Solanaceae. To set up this system, we used an *N. benthamiana* transformed line that constitutively expresses a version of *Streptococcus pyogenes Cas9* (SpCas9) under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and *A. tumefaciens Nopaline synthase* (NoS) terminator (Bernabé-Orts et al., 2019). More specifically, this line overexpresses a human codon-optimized version of the SpCas9 coding sequence fused to nuclear localization signal (NLS) at the carboxyl terminus, which has been shown to improve Cas9 activity in plants (Yin et al., 2015). Our PVX-based sgRNA delivery system (PVX::sgRNA) was designed using as a template a PVX mutant where the 29 initial codons of the coat protein (CP) are deleted and heterologous RNA expression is controlled by the subgenomic CP promoter. In turn, PVX CP is expressed from a heterologous promoter derived from that of CP of *Bamboo mosaic virus* (BaMV; genus *Potexvirus*). These features significantly reduce sequence redundancy within PVX and provide high vector stability for the expression of heterologous genes (Dickmeis *et al.*, 2014). The PVX::sgRNA system was constructed by cloning a cDNA corresponding to the 96-nt sgRNA downstream the PVX CP promoter. The sgRNA consisted of a 20-nt protospacer sequence specific to the target gene and a 76-nt scaffold highly conserved in the CRISPR-Cas9 system, also known as direct repeat.

Xie et al. (2015) showed that the host endogenous tRNA-processing system cleaves tandemly arrayed tRNA-sgRNA constructs into sgRNAs, thus improving the efficiency of genome editing. Aiming to explore whether this strategy also applies to

splice out the sgRNA from the viral subgenomic RNA that is transcribed from the BaMV CP promoter, four different constructs were designed: (i) sgRNA flanked with tRNAs at both 5' and 3' ends (tR-sgRNA-tR); (ii) sgRNA flanked with tRNA only at 5' end (tR-sgRNA); (iii) sgRNA flanked with tRNA only at 3' end (sgRNA-tR); and (iv) sgRNA without any flanking tRNAs (sgRNA) (**Figure 1**).

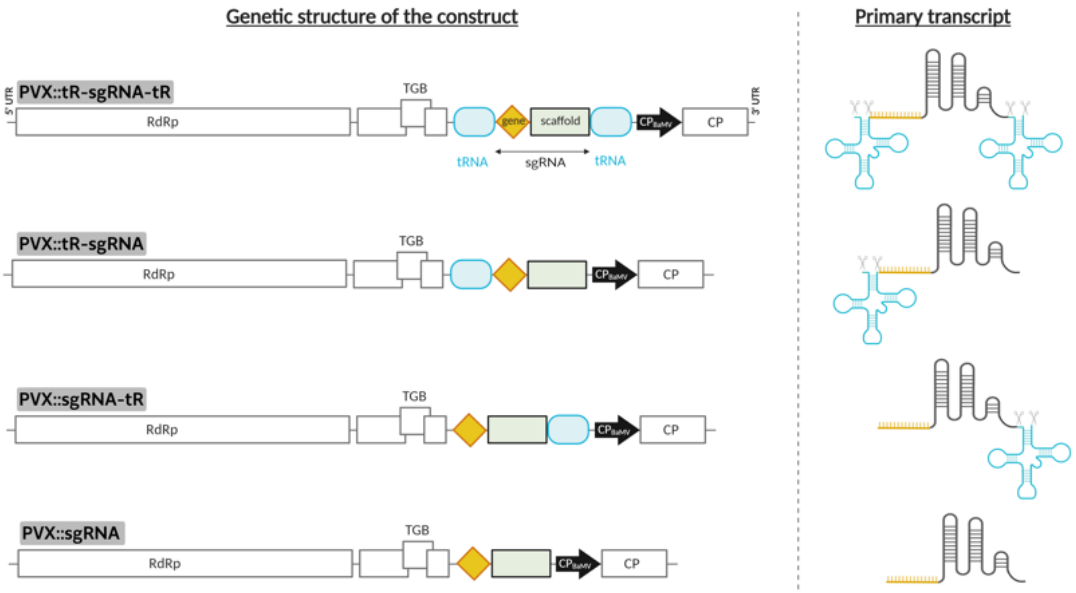

Figure 1. PVX vector to express sgRNAs for CRISPR-Cas9-based gene editing in *N. benthamiana*. Left panel, schematic representation of recombinant clones PVX::tR-sgRNA-tR, PVX::tR-sgRNA, PVX::sgRNA-tR, and PVX::sgRNA, which express different versions of a sgRNA flanked or not with tRNAs to promote RNA processing by endogenous RNases. Right panel, structure of the subgenomic primary transcripts of each PVX recombinant clone. RdRp, RNA-dependent RNA polymerase; TGB, triple gene block; and CP, coat protein, are represented by open boxes. Heterologous *Bamboo mosaic virus* CP promoter (CP_{BaMV}) is represented by a black arrow. 5' and 3' untranslated regions (UTRs) are represented by lines. tRNA sequences are represented by round, light blue boxes. sgRNA consists of a gene-specific 20-nt protospacer (orange diamond) and a conserved 76-nt scaffold (grey box). CP_{BaMV}, tRNAs, protospacer and scaffold, not at scale.

PVX::sgRNA system can efficiently target host genes without tRNA-mediated processing of the sgRNA

As a proof-of-concept of the PVX::sgRNA system, xylosyl transferase 2B (XT2B) was selected as the target gene to be edited. Loss of function of XT2B alters the glycosylation pattern in endogenous and recombinant proteins (Cavalier and Keegstra, 2006). Previous work assessed the efficiency of Cas9-mediated gene editing of several *N. benthamiana* loci, including this gene, with different sgRNAs (Bernabé-Orts et al., 2019). Thus, *NbXT2B*-specific sgRNA was cloned into the four different tRNA-sgRNA constructs mentioned above to generate the PVX::tR-sgXT2B-tR, PVX::tR-sgXT2B, PVX::sgXT2B-tR and PVX::sgXT2B derivatives (**Figure 2a**). *A. tumefaciens* carrying the corresponding plasmids were then inoculated into leaves of Cas9 *N. benthamiana*. As a control for gene editing, additional plants were inoculated with *A. tumefaciens* carrying PVX::crtB. This is a visual tracker of PVX infection and movement previously developed, which results in a bright yellow pigmentation of infected tissue (Majer et al., 2017).

At 7 days post-inoculation (dpi), symptoms of PVX infection characterized by the appearance of vein banding, ring spots and leaf atrophy were observed in the upper non-inoculated leaves of all PVX::sgRNA infiltrated *N. benthamiana* plants. These symptoms persisted and became more noticeable over time. However, symptoms of PVX::sgXT2B were slightly more intense than those of PVX::tR-sgXT2B, PVX::sgXT2B-tR, and at the same time, symptoms of these two last recombinant clones were slightly more intense than those of PVX::tR-sgXT2B-tR. Samples from the first systemically infected upper leaf were collected at 14 and 21 dpi. On the one hand, RNA was extracted and reverse transcription (RT)-polymerase chain reaction (PCR) analysis confirmed the presence of PVX in all inoculated plants. The insertion of the tRNA-sgRNA region in the PVX progeny was also confirmed by RT-PCR. These results indicated that, although the addition of one or two tRNA sequences into the genome may be affecting virus fitness, they do not abolish PVX infectivity and systemic spread, and that the heterologous tRNA-sgRNA region is conserved in PVX progeny over time. On the other hand, DNA was extracted

from the leaf samples collected at 14 and 21 dpi, and a 750-bp fragment of the *NbXT2B* gene covering the Cas9 target site was amplified by PCR. Sanger sequencing of the PCR products and inference of CRISPR edits (ICE) analysis revealed an efficient genome editing in systemic leaves of all PVX::sgRNA inoculated plants (**Figure 2b**). No statistically significant differences were observed in *NbXT2B* gene editing among the four different PVX::sgRNA derivatives, with average indel percentage ranging from 37% to 85%. These results suggest that all PVX::sgRNA derivatives are suitable for targeted editing of *N. benthamiana* genes in the presence of Cas9, regardless of the addition of a tRNA sequence at 5' and/or 3' ends of the sgRNA.

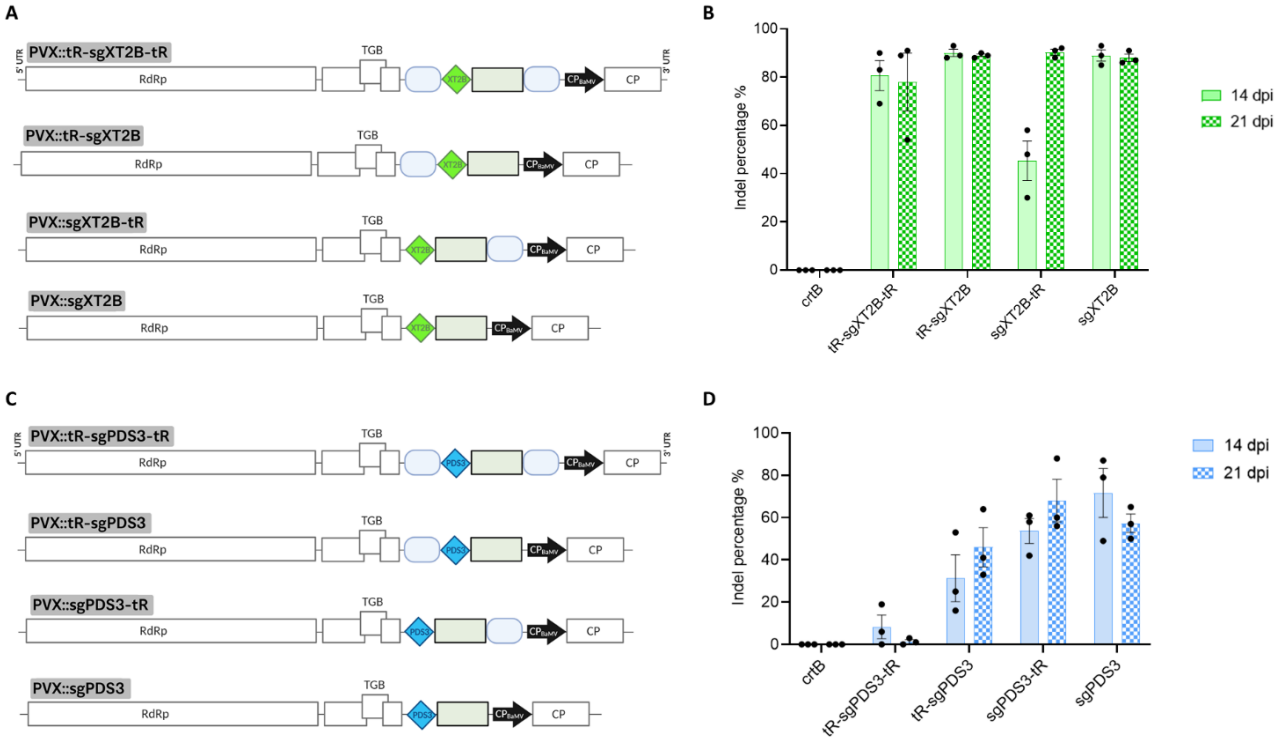

Figure 2. Single gene editing, with or without flanking tRNAs, using the PVX-based system in *N. benthamiana*. (A) Schematic representation of recombinant clones PVX::tR-sgXT2B-tR, PVX::tR-sgXT2B, PVX::sgXT2B-tR and PVX::sgXT2B. *NbXT2B* protospacer is represented by a green diamond. Other details as in the legend to Figure 1. (B) Inference of CRISPR edits (ICE) analysis of the first systemically infected upper leaf of *N. benthamiana* plants inoculated with PVX::sgXT2B derivatives at 14 (n=3) and 21 dpi (n=3). PVX::crtB was used as a negative editing control. (C) Schematic representation of recombinant clones PVX::tR-sgPDS3-tR, PVX::tR-sgPDS3, PVX:: sgPDS3-tR, PVX::sgPDS3 and PVX::sgPDS3. *NbPDS3* protospacer is represented

by a blue diamond. Other details as in the legend to Figure 1. (d) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants inoculated with PVX::PDS3 derivatives at 14 (n=3) and 21 dpi (n=3). (b and d) Columns and error bars represent average indels (%) and standard error of the mean, respectively.

To confirm this finding, the experiment with the four PVX::sgRNA derivatives was repeated following the same procedure, but this time selecting *NbPDS3* as the target gene (**Figure 2c**). Phytoene desaturase (PDS) is a key enzyme of the carotenoid biosynthetic pathway in plants (Burch-Smith et al., 2006). At 14 and 21 dpi, the first systemically infected upper leaf was sampled, following extraction of genomic DNA and PCR amplification of a 650-bp fragment covering the Cas9 target site of *NbPDS3*. For this gene, ICE analysis displayed no editing for PVX::tR-sgPDS3-tR, a relatively low efficiency (25%) for PVX::tR-sgPDS3, and high efficiency (68-73%) for PVX::sgPDS3-tR and PVX::sgPDS3 (**Figure 2d**). Owing to its high efficiency and design simplicity, PVX::sgRNA constructs lacking tRNA sequences were used for subsequent experiments.

Efficient and easy editing of multiple genes from a single PVX construct

After the successful editing of endogenous genes in *N. benthamiana*, we wondered whether our PVX-based vector could be feasible for the delivery of multiple sgRNA molecules from a single construct (i.e. multiplexing). The capacity to edit several genes at once using transient sgRNA delivery strategies remains of great interest for generating plants with multiple gene knockouts. In addition to the *N. benthamiana* genes tested through the single sgRNA strategy (*NbXT2B* and *NbPDS3*), we decided to include an additional target gene for the multiplexing. Based on the results on editing efficiency obtained by Bernabé-Orts et al. (2019), we selected *NbFT*. Flowering locus T (FT) promotes the development of inflorescences from vegetative meristem, and loss of function of this gene results in late flowering (Wigge et al., 2005). Considering the finding that our PVX::sgRNA system can efficiently produce indels on target genes without any sgRNA processing, we hypothesized that multiple sgRNAs could be

tandemly delivered within a single PVX construct. We further proposed to position the three sgRNA sequences next to each other, without any spacer sequence and under the control of the same CP promoter. Thus, using the PVX vector as a template, sgPDS3 and sgXT2B were positioned on the 5' and 3' ends of the sgRNA construct, respectively, and sgFT was included between them, creating the PVX::sgPDS3:sgFT:sgXT2B construct (**Figure 3a**). Additionally, a PVX::sgFT construct harboring the single sgRNA for *NbFT* was designed to compare the editing efficiency between single and multiplex sgRNA strategies (**Figure 3a**). *A. tumefaciens* carrying either PVX::sgPDS3:sgFT:sgXT2B or PVX::sgFT were inoculated into Cas9 *N. benthamiana* leaves, and inoculation with PVX::crtB was used as a control for gene editing. Again, at 7 dpi symptoms of PVX infection were observed in the upper non-inoculated leaves of all PVX::sgRNA infiltrated *N. benthamiana* plants. Samples from the first systemically infected upper leaf were collected at 7, 14, 21 and 28 dpi.

Following DNA extraction and PCR amplification of the target sites, *NbPDS3*, *NbFT* and *NbXT2B* amplicons were subjected to ICE analysis (**Figure 3b**). For PVX::sgPDS3:sgFT:XT2B infected leaves, at 7 dpi indels were detected only in the *NbXT2B* gene but at a low percentage (9%). Remarkably, at 14 dpi gene editing was boosted as indels were detected in all three genes. However, efficiencies appeared to be determined by sgRNA positioning on the heterologous construct: the lowest average indel percentage (21%) corresponded to sgPDS3 located in the 5' side of the sgRNA precursor; sgFT, located in the middle of the precursor, showed 36% indels; whereas the highest indel percentage (75%) corresponded to sgXT2B, located in the 3' side. A slight increase in gene editing was observed over time for *NbPDS3* and *NbFT* (**Figure 3b**, right panel). Time-course comparison with the single sgRNA construct, in the case of sgFT, showed that multiplexing lowers the editing efficiency (**Figure 3b**, compare left and right graphs). These results indicate that various sgRNAs can be delivered simultaneously using the PVX-based vector, although indel production is lower compared to single

sgRNA delivery. Moreover, results suggest that editing efficiency could be influenced by sgRNA positioning on the heterologous construct, as in our case the sgRNA proximal to the 3' end was the most efficient among all. However, additional experiments assessing sgRNA efficiency on multiplexing are necessary to confirm these findings.

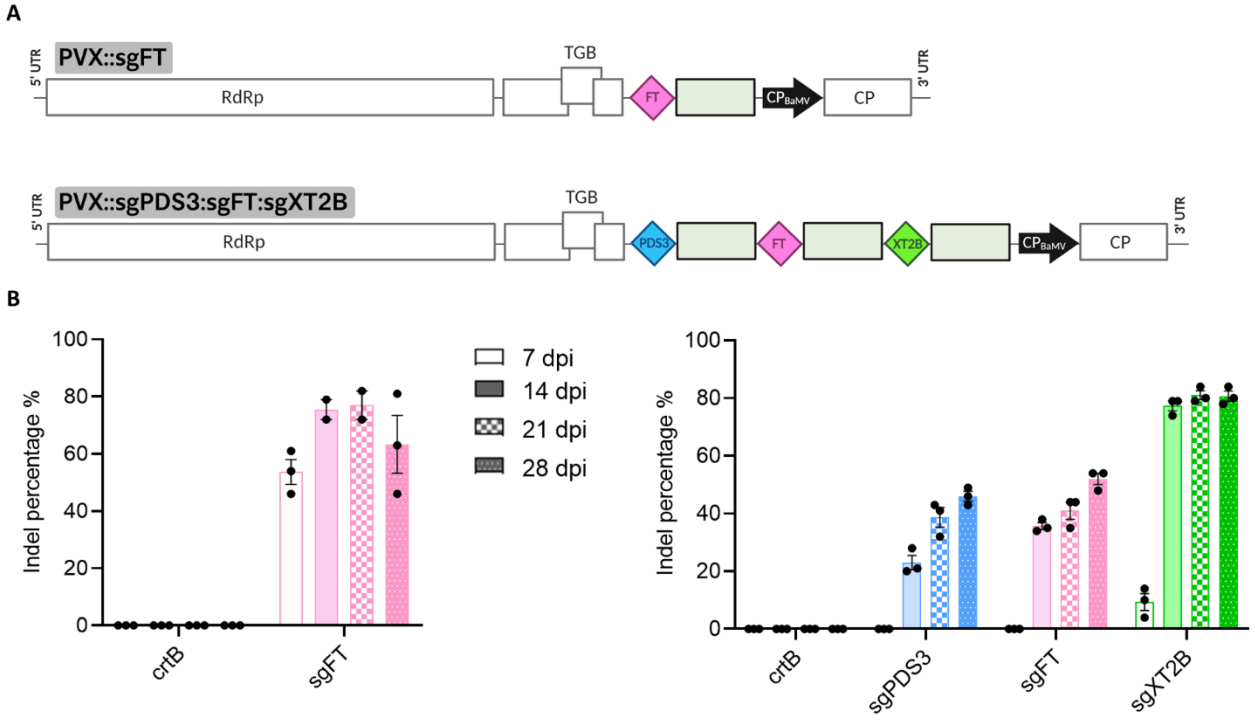

Figure 3. Single versus multiplex gene editing using the PVX-based system in *N. benthamiana*. (A) Schematic representation of recombinant clones PVX::sgFT (single sgRNA strategy) and PVX::sgPDS3:sgFT:sgXT2B (multiplex sgRNA strategy). Protospacers for *NbPDS3*, *NbFT* and *NbXT2B* are represented by blue, pink and green diamonds, respectively. Other details as in the legend to Figure 1. (B) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants (n=3) inoculated with PVX::sgFT (left) and PVX::sgPDS3:sgFT:sgXT2B (right). PVX::crtB was used as a negative control. dpi, days post-inoculation. Columns and error bars represent average indels (%) and standard error of the mean, respectively.

Heritable gene editing in the progeny of plants regenerated from tissue infected with PVX::sgRNA vector

The above-explained results demonstrate that the PVX-based vector is suitable for the delivery of either single or multiple sgRNAs leading to efficient editing of target

genes. It is well known that PVX is transmitted mainly by mechanical contact between infected and healthy plants, although transmission by zoospores of the fungus *Synchytrium endobioticum* has also been reported (Loebenstein and Gaba, 2012). Moreover, it is well established that this virus is not transmitted through seed or pollen. As *NbXT2B* appeared to be the most efficiently edited gene, seeds from PVX::sgXT2B infected plants were recovered. The progeny was then screened for the presence of PVX and the heritability of editing in *NbXT2B*. All plants were virus-free, but none of them carried indels at the target site of *NbXT2B*. These results indicate that PVX is unable to infect germline cells and therefore the progeny of transiently modified plants cannot inherit sequence modifications in the target genes.

We proposed that leaf tissue from plants infected with PVX::sgRNA could be regenerated into whole plants and screened for the presence of gene editing. We decided to test this possibility for plants modified at either single or multiple target genes. Thus, leaf discs from plants inoculated with PVX::sgXT2B or PVX::sgPDS3:sgFT:sgXT2B were collected at 14 and 21 dpi and regenerated following tissue culture. We next studied whether the regenerated plants, which still showed symptoms of virus infection, carried modifications in the target genes. ICE analysis of PCR products indicated a strong presence of indels in plants regenerated from leaf tissue that had been edited at either single or multiple genes, regardless of the sampling time (**Figure 4**). Interestingly, editing efficiency in regenerated plants was higher compared to that of parental tissues: from 77% to 95.8% for *NbXT2B* in single sgRNA delivery strategy (**Figure 4**, left panel); and from 46% to 74% for *NbPDS3*, from 52% to 78.5% for *NbFT*, and from 52% to 94.5% for *NbXT2B* in multiple sgRNA delivery strategy, respectively (**Figure 4**, right panel).

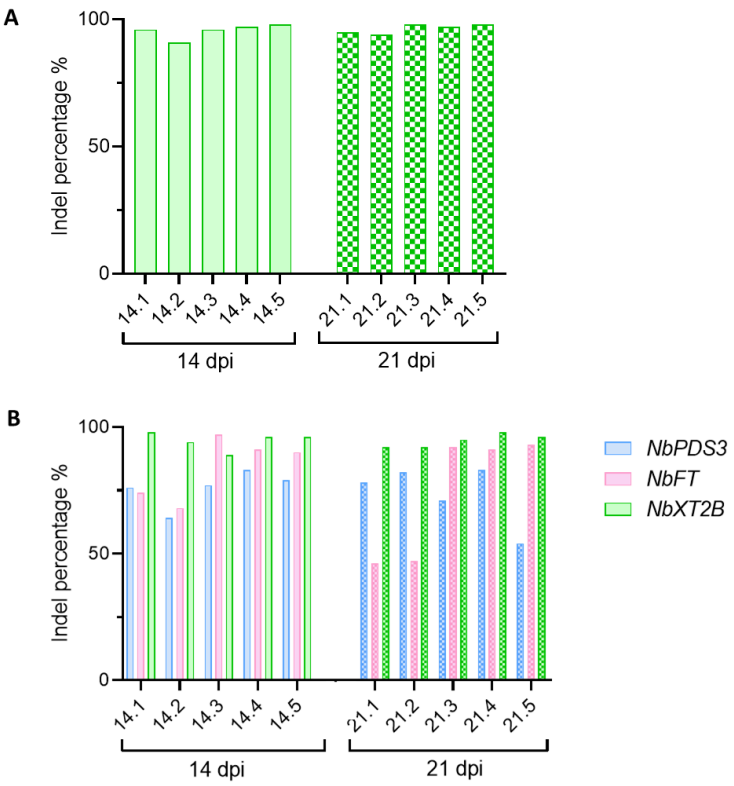

Figure 4. Gene editing in *N. benthamiana* plants regenerated from infected tissue. ICE analysis of plants regenerated from leaf discs infected with (A) PVX::sgXT2B or (B) PVX::sgPDS3:sgFT:sgXT2B. Individual plants regenerated from leaf discs collected at 14 dpi (n=5) or 21 dpi (n=5) are represented as 14.1-.5 or 21.1-.5, respectively. Columns represent average indels (%).

Subsequently, we wondered whether the gene editing observed in regenerated plants resulted in germline modifications that were transmitted to seedlings. Seeds were collected from plants 14.3 and 14.1, which had been regenerated from leaf discs infected with PVX::sgXT2B and PVX::sgPDS3:sgFT:sgXT2B, respectively (**Figure 4**). The absence of symptoms of PVX infection indicated that all progeny was virus-free. ICE analysis of the target genes confirmed the inheritance of genome modifications (**Figure 5a**). Progeny derived from the single-edited plant 14.3 contained mutations in both *NbXT2B* alleles (i.e. biallelic) (**Figure 5b**, left panel). In turn, genotyping of progeny derived from plant 14.1, which carried modifications in all target genes, indicated that both *NbXT2B* alleles were edited in all cases, while 20% and 30% of seedlings contained biallelic mutations for *NbPDS3* and *NbFT*, respectively (**Figure 5b**, right panel). Only 40% of the progeny showed no modifications in neither of the two *NbFT* alleles (**Figure 5b**, right panel). Remarkably, with this small screening of only ten plants from the progeny, we were able to find individuals with 100% biallelic mutations of all three target genes (**Figure 5a**, right panel).

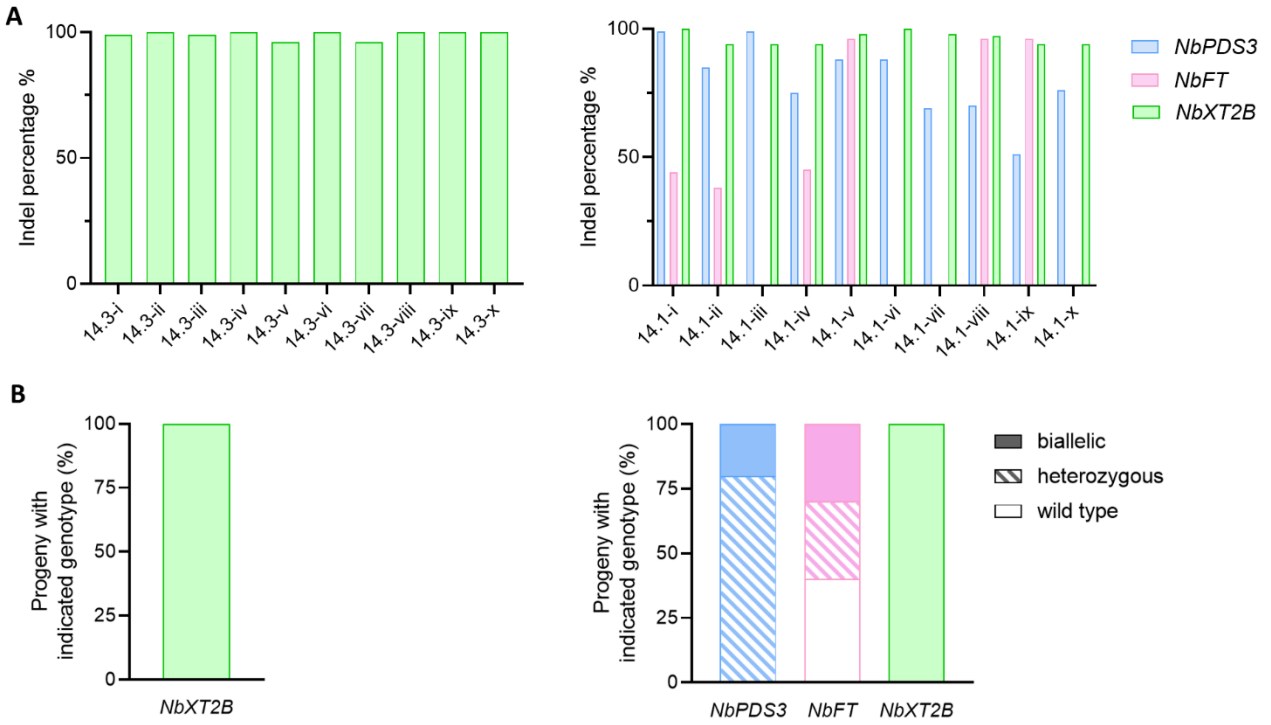

Figure 5. Heritable gene editing using the PVX-based system in *N. benthamiana*. (A) ICE analysis of the progeny of plants 14.3 (left) and 14.1 (right), regenerated from leaf discs infected with PVX::sgXT2B or PVX::sgPDS3:sgFT:sgXT2B, respectively. Individual plants of the progeny (n=10) are numbered from i to x. Columns represent average indels (%). (B) Genotype analysis of the same progeny (n=10) used to determine heritable gene editing in Figure 5a. Heterozygous progeny contains one wild-type allele and one edited allele; biallelic progeny contains both alleles edited. The percentage of each genotype is represented in stacked columns and was determined by the fraction of progeny containing that genotype divided by total progeny assessed.

Recently, Ellison *et al.* (2020) reported a TRV-based system where the fusion of the 3' end of the sgRNA to RNA sequences that promote cell-to-cell mobility resulted in germline modifications that were transmitted to progeny of infected plants. Aiming to explore whether this strategy can be expanded to other VIGE vectors, we proposed to add the coding sequence of the first 102 bp of *A. thaliana* FT (truncated FT, tFT) to the 3' end of the *NbXT2B*-specific sgRNA or to a novel sgRNA targeting both *NbPDS3* homoelogs (i.e. PDS3ii), creating the PVX::sgXT2B-tFT and PVX::sgPDS3ii-tFT constructs, respectively (**Figure 6a**). tFT RNA has been demonstrated to promote mobility

of GFP-encoding RNAs to the meristem (Li *et al.*, 2009), and its small size should avoid cargo constraints within our PVX::sgRNA system. Approximately 3 weeks after inoculation, white spots started to appear in the upper non-inoculated leaves of plants infected with PVX::sgPDS3ii-tFT, clearly indicating PDS3 loss-of-function, and leaf bleaching increased notably in the following weeks as these plants matured (**Figure 6b**). Seeds were collected from parental plants inoculated with PVX clones carrying mobile sgRNAs and genotyping of the progeny indicated that up to 22% and 30% of seedlings contained biallelic mutations for *NbXT2B* or modifications in at least one *NbPDS3* homoelog, respectively (**Figure 6c**). Interestingly, all the *NbXT2B*-edited individuals shared the same 1-bp insertion (**Figure S3**). In addition, in the case of *NbPDS3*, we identified an individual carrying biallelic mutations for both homeologs that didn't have an albino phenotype, presumably due to the 3-bp deletion that preserved the gene's reading frame (**Figure S4**).

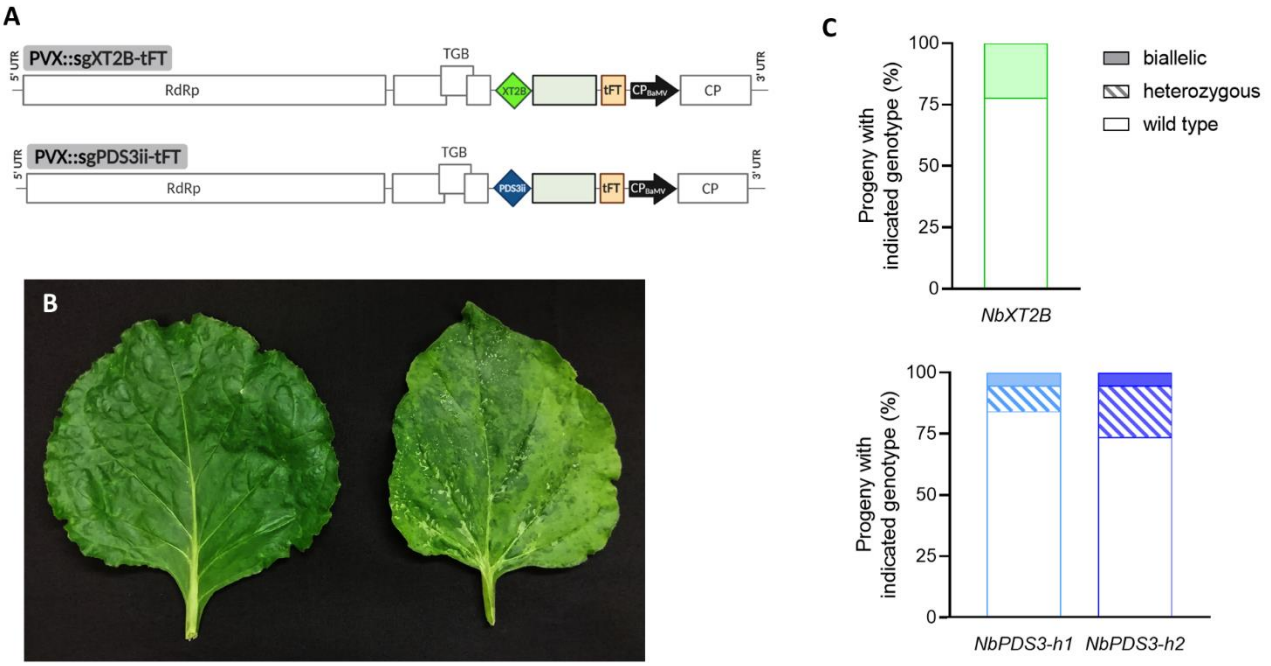

Figure 6. Heritable gene editing with a PVX vector expressing mobile sgRNAs. (A) Schematic representation of recombinant clones PVX::sgXT2B-tFT and PVX::sgPDS3ii-tFT, in which the sgRNA was modified by the 3' addition of the truncated FT motif (tFT) to enhance mobility. tFT and *NbPDS3* protospacer targeting both homeologs are represented by a light orange box and a

dark blue diamond, respectively. Other details as in the legend to Figure 1. (B) Phenotype of systemic leaves of Cas9 *N. benthamiana* plants infected with PVX::sgXT2B-tFT (left) or PVX::sgPDS3ii-tFT (right) approximately 5 weeks after infiltration. White spots are indicative of loss of PDS3 function. The observed phenotype appears approximately 3 weeks after infiltration and becomes more noticeable as the plant matures. (C) Genotype analysis of the progeny of plants inoculated with PVX::sgXT2B-tFT (top, n=36) or with PVX::sgPDS3ii-tFT (bottom, n=20). Seeds were collected from the entire plant and pooled. The percentage of each genotype is represented in stacked columns and was determined as in Figure 5b. *NbPDS3-h1*, homoelog 1 (); *NbPDS3-h2*, homoelog 2.

DISCUSSION

Targeted editing of plant genomes by CRISPR-Cas9 technology is expected to play a crucial role in both basic genetic studies and crop improvement for 21st-century agriculture. In this context, the use of plant viral vectors for the transient delivery of CRISPR-Cas9 components has emerged as a promising strategy over conventional transformation technologies. The first VIGE reports focused on the generation of gene knockout plant lines using sgRNA delivery systems based on geminiviruses (Baltes et al., 2014; Yin et al., 2015). Subsequent studies led to the development of several plant RNA virus-based vectors with the same purpose (Ali et al., 2015, 2018; Cody et al., 2017; Ellison et al., 2020; Jiang et al., 2019; Ma et al., 2020). Here, we describe a PVX-based sgRNA delivery vector that allows easy multiplexing for efficient targeted editing of the model species *N. benthamiana* as a novel approach to expand the current VIGE toolbox.

First, we explored the potential of PVX for the transient delivery of sgRNA molecules into host plants. Several studies have documented that nucleotide overhangs on either 5' or 3' ends of the sgRNA negate Cas9 activity *in vitro*, which leads to a reduction of DSBs (Jinek et al., 2012; Mali et al., 2013; Dahlman et al., 2015; Zalatan et al., 2015). Considering that the host endogenous tRNA-processing system can splice out tandemly arrayed tRNA-sgRNA constructs into mature sgRNAs (Xie et al., 2015), we designed different PVX::sgRNA derivatives where the sgRNA was flanked or not with

tRNAs (**Figure 1**). We hypothesized that, although host plant tRNA processing machinery cleaves some genomes, enough virus will survive to maintain infection. Of note, pre-tRNA processing occurs in the nucleus and PVX replication in the cytoplasm. Certainly, the presence of one or two tRNA sequences in the viral genome had a minor effect on PVX infectivity and the ability to move systemically. Surprisingly, we also found that long sgRNA overhangs at both ends did not affect Cas9 catalytic activity *in planta*, as there were no substantial differences in editing efficiency of target genes among the PVX::sgRNA derivatives with no tRNA and those with tRNAs at either 5' or 3' side (**Figures 2b** and **2d**). The capacity to produce biologically active sgRNAs without tRNA-mediated processing follows findings from previous VIGE reports in which sgRNAs contained long 3' extensions (Cody et al., 2017; Ali et al., 2018). Even more surprising is the observed ability of unspaced tandem sgRNA arrays to drive efficient editing, a feature earlier observed by Cody et al. (2017) in TMV-based VIGE experiments using a smaller tandem comprising only two sgRNAs, and very recently also showed in a TRV-based system (Ellison *et al.*, 2020). To our knowledge, no other biological system has been described where Cas9 shows this level of permissiveness to the presence of 5′ prime extensions, neither are we aware of other systems where spacer-less sgRNA arrays are fully functional for genome editing. These findings suggest that one or more of the following processes may be occurring *in vivo*: (i) Cas9 tolerates large, non-complementary overhangs within the sgRNA protospacer (5' end) *in planta*; (ii) Cas9 is able to precisely cleave sgRNA overhangs *in planta*; (iii) endogenous RNases cleave sgRNA overhangs *in planta;* or (iv) the virus, as result of imperfect replication or partial RNA degradation, produces a relatively large population of subgenomic fragments some of which, by chance, contain the correct 5′ end. Since the functionality of unspaced sgRNAs in Cas9 multiplexing has been only reported associated with viral vectors, we favor the last explanation, perhaps in combination with other mechanisms. For instance, direct repeats may protect the sgRNA scaffold from degradation by unspecific plant RNases, favoring unspecific cleavage to take place in and around the protospacer and

therefore increasing the relative concentration of correctly cleaved (functional) sgRNA species. Regardless of the precise mechanism underlying native sgRNA processing, our results reinforce the fact that sgRNAs can be launched from viral vectors without the need for processing spacer elements, which rather simplifies construct design.

One of the main advantages of VIGE is that high sgRNA levels are accumulated into host plants due to virus replication which leads to a fast and efficient editing process not achievable with conventional delivery methods (Cody and Scholthof, 2019; Schmitz et al., 2020). In fact, we observed a high editing efficiency when single sgRNAs were delivered through PVX, reaching nearly 80% of indels (**Figures 2b**, **2d** and **Figure 3b**, left panel). Remarkably, when multiple sgRNAs were delivered within the same PVX vector with no processing spacers between sgRNAs, some bias was observed related to sgRNA positioning on the construct. sgRNA proximity to the 5' end of the viral RNA apparently caused a decrease in indel production, while when sgRNA was located on the 3' end, the indel rates were comparable to those observed for single sgRNA delivery. This finding may be related to the mechanism of sgRNA processing in viral vectors discussed above and will require further analysis.

The capacity to transmit genome modifications to the next generation is considered a desired feature of any VIGE system. Starting from infected tissue where single or multiple sgRNA had been delivered, we regenerated whole plants and screened them for the presence of modifications. The regenerated plants showed an increase in indel percentages compared to those observed on infected tissue, and most importantly, all of them carried biallelic mutations for the *NbXT2B* gene (**Figure 4**). However, regenerated plants developed symptoms of viral infection, indicating that PVX was still present. It is well documented that PVX is unable to infect germline cells and therefore is not transmitted through true seed or pollen (Loebenstein and Gaba, 2012). Next, we collected seeds from regenerated plants edited in single or multiple target genes. We

confirmed that virus-free plants with heritable gene editing can be obtained from these seeds (**Figure 5a**). Heritable biallelic mutations were observed in the single sgRNA strategy in 100% of the progeny (**Figure 5b**, left panel). For the multiplexing strategy, the percentage of biallelic mutations ranged between 100% *NbXT2B* and 20% and 30% for *NbPDS3* and *NbFT*, respectively (**Figure 5b**, right panel). Remarkably, 100% biallelic mutations in all three genes were observed in individuals from the progeny (i.e. plant 14.1-v, **Figure 5a**, right panel).

Progeny from regenerated plants is also the stage where Cas9 is usually segregated in traditional transgenesis approaches. Hence, PVX-based VIGE offers a similar speed as transgenesis to obtain transgene-free edited plants, but higher efficiency and now also multiplexing capacity. Importantly, a recently published VIGE strategy, based on the fusion of RNA sequences that promote cell-to-cell mobility, has been shown to accelerate the recovery of mutant progeny (Ellison *et al.*, 2020). We confirmed that this strategy can also be expanded to our PVX::sgRNA system since the addition of a truncated FT sequence to single sgRNAs resulted in a high rate of biallelic mutations in the progeny derived from infected parentals (**Figure 6c**). The detection of the same insertion or deletion in all the edited individuals of a certain target gene remarks the usefulness of PVX-sgRNAs when combined with transgenesis to enhance multigene editing efficiency. Often, multiplex editing with a transgenic construct misses some of the intended targets due to the limited capacity to predict sgRNAs efficiency. Provided that Cas9 is already present in parental lines, if missing targets are early genotyped in these lines, they could then be infected with multiplex PVX-sgRNAs designed specifically to fill the editing gaps. This approach can be faster and more efficient than re-transformation, and most importantly, it circumvents the need for a second selection marker.

In summary, the virus-mediated genome editing system described here postulates PVX as a superior vector for the delivery of one or more sgRNAs without the need for

processing elements, thus leading to a highly efficient genome editing without the risk of integration of viral sequences into the plant genome. These modifications on the target genes are inherited to the next generations when plants are regenerated from infected tissue, while the viral vector is lost in the progeny from regenerated plants. Furthermore, the wide range of host species that PVX can infect, including important crops in the family Solanaceae, highlights its potential for CRISPR-Cas9-based modification of economically relevant species. In addition, PVX is a preferred vector in plant biotechnology (Röder et al., 2019). Our findings expand the current knowledge about the VIGE toolbox and will contribute to future applications in plant functional genomics and agricultural biotechnology.

MATERIALS AND METHODS

Design of sgRNAs and vector construction

Three *N. benthamiana* genes were chosen as targets for CRISPR-Cas9 mediated gene editing: *NbFT*, *NbPDS3* and *NbXT2B*. The design of sgRNAs was performed using the CRISPR-P online tool as described in Bernabé-Orts et al. (2019). Target sequences for each gene are described in **Table S1.** Plasmid pPVX contains a full-length PVX cDNA (GenBank accession number MT799816) flanked by the *Cauliflower mosaic virus* CaMV 35S promoter and *A. tumefaciens Nopaline synthase* NoS terminator. In all PVX-based recombinant clones, heterologous genes were expressed from PVX CP promoter, and PVX CP was expressed from a heterologous promoter derived from *Bamboo mosaic virus*. The 29 initial codons of PVX CP were deleted (Dickmeis et al., 2014). Plasmids to express recombinant viruses PVX::tR-sgXT2B-tR, PVX::tR-sgXT2B, PVX::sgXT2B-tR, PVX::sgXT2B, PVX::tR-sgPDS3-tR, PVX::tR-sgPDS3, PVX::sgPDS3-tR, PVX::sgPDS3, PVX::sgFT; PVX::sgPDS3:sgFT:sgXT2B, PVX::sgXT2B-tFT and PVX::sgPDS3ii-tFT were built by standard molecular biology techniques, including PCR amplification of target

sgRNAs with high-fidelity Phusion DNA polymerase (Thermo Scientific, Waltham, MA, USA) and Gibson DNA assembly with the NEBuilder HiFi DNA assembly master mix (New England Biolabs, Ipswich, MA, USA). Primers used for vector construction are listed in **Tables S2** and **S3.** Recombinant virus PVX::crtB that expresses *Pantoea ananatis* phytoene synthase (crtB), which induces a distinctive yellow pigmentation in infected tissue (Majer et al., 2017), was used as a control for the experiments. The sequences of all the recombinant PVX-derived clones were confirmed by standard DNA sequencing techniques and are included in **Figure S1**.

Plant growth conditions and inoculation

Transgenic Cas9-overexpressing *N. benthamiana* plants (**Figure S2**) were grown in growth chambers at 25°C under a 16-h day/8-h night cycle. Fully expanded upper leaves from 4- to 6-week-old plants were used for inoculation of PVX::sgRNA constructs. Electrocompetent *A. tumefaciens* C58C1 containing the helper plasmid pCLEAN-S48 (Thole et al., 2007) were transformed with plasmids containing the different PVX recombinant clones. Transformed cells were spread onto 50 µg/L kanamycin, 50 µg/L rifampicin and 7.5 µg/L tetracycline Luria Bertani agar plates. Single colonies were grown about 24 h at 28°C in 10 ml Luria Bertani containing 50 µg/L kanamycin. At an optical density of 600 nm (OD_{600}) 1-2, cells were pelleted by centrifuging at 7200 *g* for 5 min and resuspended to an OD_{600} of 0.5 in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH, 10 mM $MgCl_2$ and 150 µM acetosyringone, pH 5.6) (Bedoya et al., 2010). Resuspended bacteria were incubated at 28°C for 2 h. Two leaves per plant were agroinfiltrated on the abaxial side using a 1-ml syringe. Control plants were inoculated with PVX::crtB following the same procedure. Immediately following agroinfiltration, plants were watered and transferred to a growth chamber under a 12-h day/12-h night and 25°C cycle. Samples from the first

symptomatic systemic leaf were collected with a 1.2-cm cork borer (approximately 100 mg of tissue), immediately frozen in liquid nitrogen, and stored at -80°C until use.

RT-PCR analysis of PVX progeny

Leaf samples were ground with a VWR Star-Beater for 1 min at 30 sec^{-1}, homogenized in extraction buffer (4 M guanidinium thiocyanate, 0.1 M sodium acetate, 10 mM EDTA and 0.1 M 2-mercaptoethanol, pH 5.5), and centrifuged at 15 000 *g* for 5 min. Next, the collected supernatants were mixed with 0.65 volumes of 96% ethanol and centrifuged at 15 000 *g* for 1 min. The remaining steps for RNA purification were performed using silica gel columns (Zymo Research, Irvine, CA, USA) that were finally eluted with 10 µl of 10 mM Tris-HCl pH 8.5. Aliquots (1 µl) of RNA were subjected to RT with RevertAid reverse transcriptase (Thermo Scientific) using primer D2409 in a 10-µl volume reaction, and 1-µl aliquots of the products were subjected to PCR amplification with *Thermus thermophilus* DNA polymerase (Biotools, Madrid, Spain) using gene-specific primers in 20-µl reactions. Conditions were as previously described (Bedoya et al., 2010). Primers D2410 and D3436 were designed to amplify a 627-nt region of PVX genome corresponding to CP open reading frame. Primers D1789 and D2069 were designed to amplify the heterologous sgRNA region. Primers used for RT and RT-PCR are listed in **Table S4**. RT-PCR products were analyzed by electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, pH 7.2) and staining with ethidium bromide.

Analysis of Cas9-mediated genome editing

To purify DNA from leaf samples, the same procedure explained above for RNA was followed, except for not adding ethanol to the extract before loading the silica gel column. To confirm CRISPR-Cas9-mediated editing of the target genes, products

covering *NbFT*, *NbPDS3* and *NbXT2B* target sites were obtained by PCR amplification of 1 µl genomic DNA using high-fidelity Phusion DNA polymerase and gene-specific primers. The resulting PCR products were purified using silica gel columns after separation by electrophoresis in 1% agarose gels and subjected to Sanger sequencing. The presence of sequence modifications was analyzed using the ICE algorithm (https://www.synthego.com/products/bioinformatics/crispr-analysis). Primers used for amplification of target genes and sequencing are listed in **Table S5**.

In vitro regeneration of edited *Nicotiana benthamiana* plants

Leaves from 4- to 5-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* C58C1 cultures containing plasmids with the PVX recombinant clones PVX::sgXT2B and PVX::sgPDS3:sgFT:sgXT2B. As a control for gene editing, additional plants were infiltrated with PVX::crtB. At 14 and 21 dpi, the first upper leaf showing symptoms of viral infection was removed from each plant and surface-sterilized by submersion in sterilization solution (10% bleach, 0.02% Nonidet P-40) for 10 min. Leaves were then washed three times in sterile water. Sterilized leaves were cut into discs using a 1.2-cm cork borer and plated onto regeneration media (1X Murashige and Skoog with vitamins Gamborg B5, 0.5 g/L MES, 20 g/L sucrose, 8 g/L phytoagar, 1 mg/L 6-benzylaminopurine (6-BAP), 0.1 mg/L 1-naphthaleneacetic acid (NAA) and 50 µg/L kanamycin, pH 5.7). Leaf discs were transferred to fresh plates every 2 weeks until shoots emerged. Shoots were cut and transferred to root induction media (1X Murashige and Skoog with vitamins, 0.5 g/L MES, 10 g/L sucrose, 6 g/L phytoagar, 0.1 mg/L NAA and 50 µg/L kanamycin, pH 5.7). Regenerated plantlets with roots were transferred into soil and covered with plastic cups to keep moisture in. After 2 weeks, plastic cups were removed for plant normal growth.

SUPPORTING INFORMATION

All data relating to Supplementary Figures and Tables can be found at the end of this chapter.

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SUPPORTING INFORMATION

Figure S1. Full sequence of wild-type *Potato virus X* (PVX; GenBank accession number MT799816) and its derived recombinant viruses PVX::crtB, PVX::tR-sgXT2B-tR, PVX::tR-sgXT2B, PVX::sgXT2B-tR, PVX::XT2B, PVX::tR-sgPDS3-tR, PVX::tR-sgPDS3, PVX::sgPDS3-tR, PVX::sgPDS3, PVX::sgFT, PVX::sgPDS3:sgFT:sgXT2B, PVX::sgXT2B-tFT and PVX::sgPDS3ii-tFT. In recombinant PVX clones, heterologous sequences are transcribed from coat protein (CP) promoter and a deleted version of PVX CP, lacking the 29 initial codons, is transcribed from a heterologous promoter derived from the Bamboo mosaic virus (BaMV) CP (Dickmeis et al., 2014). **PVX CP promoter** with a ATG-AGG mutation to abolish start codon is underlined. **BaMV CP promoter** is in blue. cDNA corresponding to *P. ananatis* **phytoene synthase** (crtB) is on yellow background. **tRNA** sequences are bold in light blue. sgRNA sequences corresponding to *NbPDS3*, *NbPDS3-ii*, *NbFT* and *NbXT2B* are on blue, purple and green backgrounds, respectively. **Protospacer** regions are underlined (dotted). cDNA corresponding to the 5' UTR region of *A. thaliana Flowering Locus T* (**truncated FT**, tFT) is on orange background.

>PVX-wt (MT799816)

GAAAACTAAACCATACACCACCAACACAACCAAACCCACCACGCCCAATTGTTACACACCCGCTTGAAAAAGAAAGTTTA
ACAAATGGCCAAGGTGCGCGAGGTTTACCAATCTTTTACAGACTCCACCACACAAAAACTCTCATCCAAGATGAGGCTTATA
GAAACATTCGCCCCATCATGGAAAAACACAAACTAGCTAACCCTTACGCTCAAACGGTTGAAGCGGCTAATGATCTAGAG
GGGTTCGGCATAGCCACCAATCCCTATAGCATTGAATTGCATACACATGCAGCCGCTAAGACCATAGAGAATAAACTTCT
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GAAACCCGCGGATCAAGGACATTTTCCAAAATGTTGCCATTGAACCAAGAGACGTAGCCAGGTACCCCAAGGAAACAATA
ATTGACAAACTCACAGAGATCACAACGGAAACAGCATACATTAGTGACACTCTGCACTTCTTGGATCCGAGCTACATAGT
GGAGACATTCCAAAACTGCCCAAAATTGCAAACATTGTATGCGACCTTAGTTCTCCCCGTTGAGGCAGCCTTTAAAATGG
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GCATACCATCATGAATTCGCTCATCTACAATGGCTCAAAGTGGGAAAGATCAAGTGGAGGGACCCCAAGGATAGCTTTCT
CGGACATCTCAATTACACGACTGAGCAGGTTGAGATGCACACAGTGACAGTACAGTTGCAGGAATCGTTCGCGGCAAACC
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TCAGATTCCACCCCTTGCAAGCGTGGAAAGCCTTCCGACCAAGGGAAGTGTCGGATGTAGAGGAAATGGAAAGTTTGTTC
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AGTGGCACCGAGTCGGTGCATGTCTATAAATATAAGAGACCCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATC
CGTTTAATAGATCAATCACTCTAAAGGTTACTTATGGCCAATAGGGTTTGTTAAGTTTCCCTTTTTACTCGAAAGATG

Figure S2. Sequence of the transgenes inserted into *Nicotiana benthamiana* to generate a line that constitutively expresses a human codon-optimized *Streptococcus pyogenes* Cas9. *Agrobacterium tumefaciens* transfer DNA left and right borders on yellow and red backgrounds, respectively. The kanamycin resistance cassette (in reverse orientation) consists of *A. tumefaciens* NoS promoter (dark blue background), NPTII open reading frame (ORF; gray background) and NoS terminator (light blue background). The SpCas9 cassette consists of *Cauliflower mosaic virus* promoter (light green background), SpCas9 ORF (fuchsia background), including a nuclear localization signal (yellow) and NoS terminator (light blue background).

>SpCas9 transgene

TGGCAGGATATATTGTGGTGTAAACATAACGGATCCGGTCTCAGGAGAGCGATCAGCTTGCATGCCGGTCGATCTAGTAA
CATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATT
GCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTC
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GTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATGGGACCGA
CTCGCGCTGTCATGAGACCGGATCCTGACAGGATATATTGGCGGGTAAAC

Figure S3. Sequencing chromatogram of a biallelic edited plant derived from a parental plant inoculated with PVX::sgXT2B-tFT. The same 1-bp insertion mutation was detected in all the biallelic individuals of the progeny. The complementary reverse strand (3'→5') of the wild-type sequence of *NbXT2B* is shown at the top. PAM is red-dotted and the target site is underlined.

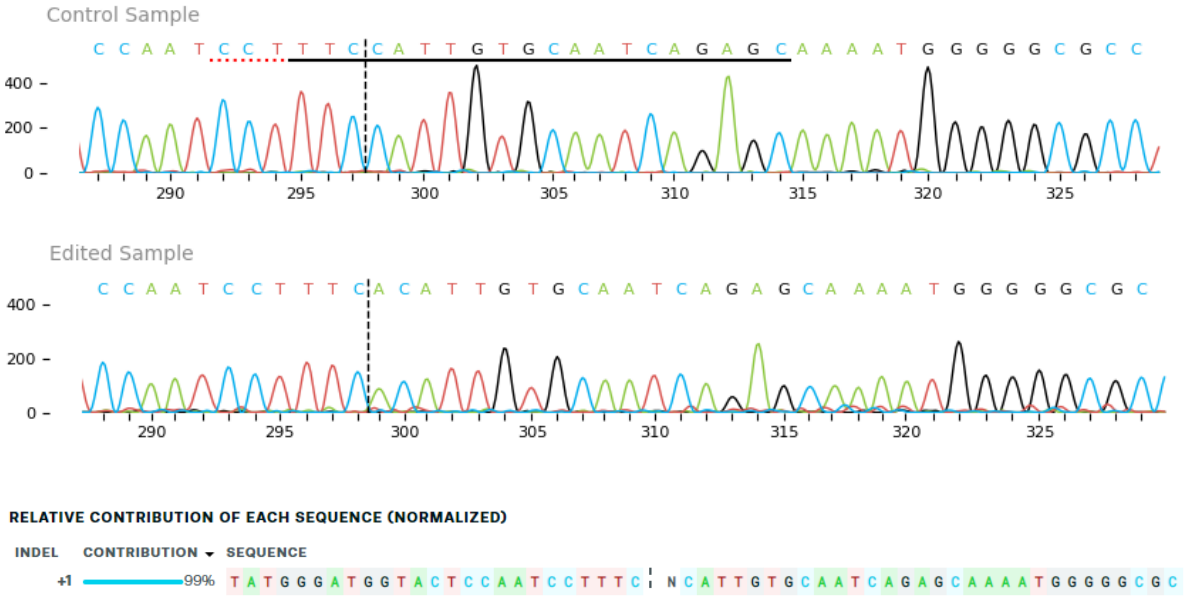
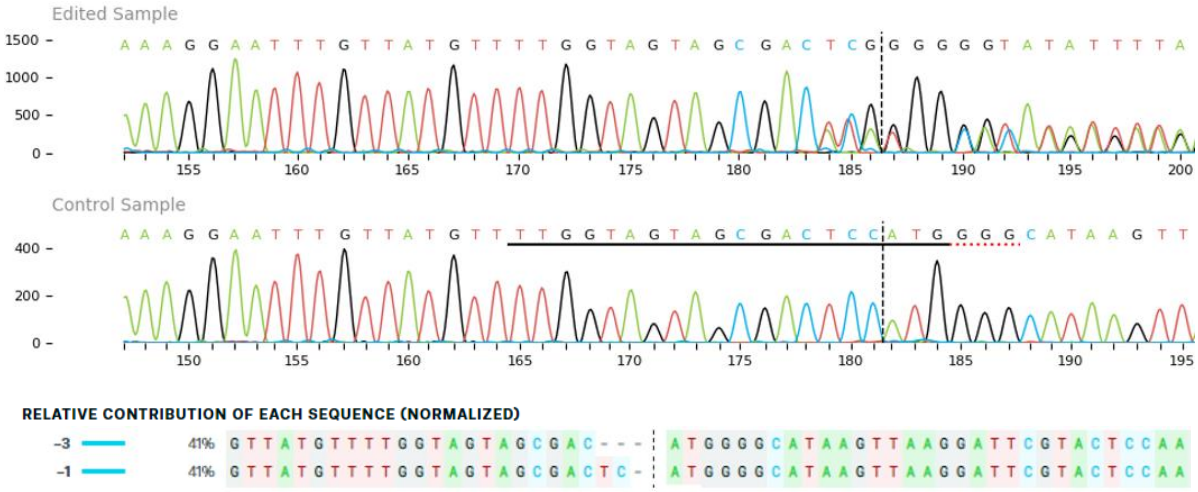


Figure S4. Sequencing chromatogram of a biallelic edited plant derived from a parental plant inoculated with PVX::sgPDS3ii-tFT. The same 3-bp deletion mutation that preserved the gene's reading frame was detected in both *NbPDS3* homoelogs in heterozygosis or homozygosis, respectively all the biallelic individuals of the progeny. A 1-bp deletion was also detected in homoelog 1. The complementary reverse strand (3'→5') of the wild-type sequence of *NbPDS3* is shown at the top. PAM is red-dotted and the target site is underlined.

***NbPDS3*-h1:**



***NbPDS3*-h2:**

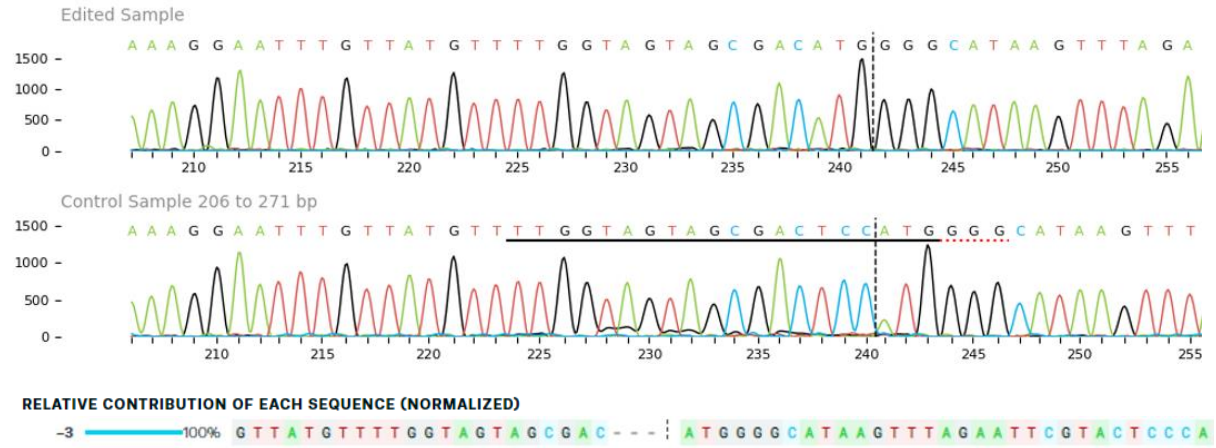


Table S1. *N. benthamiana* genes targeted by the PVX-based CRISPR-Cas9 system. PAMs are highlighted on grey background.

Target gene	SolGenomics accession no.	Target site
NbFT	Niben101Scf01519g10008.1	GGCCAATAGATCTTGTAAAA**GGG**
NbPDS3-h1	Niben101Scf01283g02002.1	sgPDS3: CACGACCCGAAGATTGACAA**AGG** sgPDS3ii: TTGGTAGTAGCGACTCCATG**GGG**
NbPDS3-h2	Niben101Scf14708g00023.1	sgPDS3ii: TTGGTAGTAGCGACTCCATG**GGG**
NbXT2B	Niben101Scf04551g02001.1	GCTCTGATTGCACAATGGAA**AGG**

Table S2. Primers used for the construction of recombinant viruses.

Construct element	Primer*	Primer sequence (5'→3')	Purpose
tRNA	D3450 (F)	gaggtcagcaccagctagcaAACA AAGCACCAGTGGTCTAG	Amplification of 5' tRNA for PVX::tR-sgRNA-tR and PVX::tR-sgRNA constructs
	D3457 (R)	gggaaacttaacaaaccctaTGCA CCAGCCGGGAATCG	Amplification of 3' tRNA for PVX::tR-sgRNA-tR and PVX::sgRNA-tR constructs
scaffold	D3448 (R)	gggaaacttaacaaaccctaGCAC CGACTCGGTGCCAC	Amplification of sgRNA scaffold for PVX::sgRNA and PVX:tR-sgRNA constructs
	D3455 (R)	gtgctttgttGCACCGACTCGGTG CCAC	Amplification of 3' tRNA + scaffold for PVX::tR-sgRNA-tR and PVX:tR-sgRNA
	D3456 (F)	gagtcggtgcAACAAAGCACCAGT GGTCTAG	Amplification of 3' tRNA + XT2B scaffold for PVX::tR-sgRNA-tR and PVX:tR-sgRNA
sgXT2B	D3447 (F)	gaggtcagcaccagctagcaGCTC TGATTGCACAATGGAAGTTTTAG	Amplification of XT2B protospacer for PVX::sgXT2B and PVX:sgXT2B-tR
	D3451 (R)	caatcagagcTGCACCAGCCGGGA ATCG	Amplification of 5' tRNA + XT2B protospacer for PVX::tR-sgXT2B-tR and PVX:tR-sgXT2B
	D3452 (F)	ggctggtgcaGCTCTGATTGCACA ATGGAAGTTTTAG	Amplification of 5' tRNA + XT2B protospacer for PVX::tR-sgXT2B-tR and PVX:tR-sgXT2B
	D3540 (R)	ttccattgtgcaatcagagcGCAC CGACTCGGTGCCAC	Amplification of 5' scaffold+ sgXT2B for PVX::sgPDS3:sgFT:sgXT2B
sgPDS3	D3449 (F)	gaggtcagcaccagctagcaCACG ACCCGAAGATTGACAAGTTTTAGA GCTAGAAATAG	Amplification of sgPDS3 for PVX::sgPDS3, PVX:sgPDS3-tR and PVX::sgPDS3:sgFT:sgXT2B
	D3453 (R)	tcgggtcgtgTGCACCAGCCGGGA ATCG	Amplification of 5' tRNA + PDS3 protospacer for PVX::tR-sgPDS3-tR and PVX:tR-sgPDS3

	D3454 (F)	ggctggtgcaCACGACCCGAAGATTGACAAG	Amplification of 5' tRNA + PDS3 protospacer for PVX::tR-sgPDS3-tR and PVX:tR-sgPDS3
sgPDS3ii	D4166 (F)	gaggtcagcaccagctagcaTTGGTAGTAGCGACTCCATGGTTTTAGAGC	Amplification of pBPVX::sgXT2B for PVX::sgPDSii-tFT
sgFT	D3541 (F)	gaggtcagcaccagctagcaGGCCAATAGATCTTGTAAAAGTTTTAGAGC	Amplification of sgFT for PVX::sgFT
	D3538 (R)	tctattggccGCACCGACTCGGTGCCAC	Amplification of 5' scaffold + sgFT for PVX::sgPDS3:sgFT:sgXT2B
	D3539 (F)	gagtcggtgcGGCCAATAGATCTTGTAAAAGTTTTAGAGC	Amplification of 5' scaffold+ sgFT for PVX::sgPDS3:sgFT:sgXT2B
pBPVX:: sgXT2B	D3536 (R)	TGCTAGCTGGTGCTGACC	Amplification of pBPVX::sgXT2B for PVX::sgPDS3:sgFT:sgXT2B
	D3537 (F)	GCTCTGATTGCACAATGGAAG	Amplification of pBPVX::sgXT2B for PVX::sgPDS3:sgFT:sgXT2B
	D3895 (F)	TAGGGTTTGTTAAGTTTCCC	Amplification of pBPVX::sgXT2B for PVX::sgXT2B-tFT
	D3896 (R)	GCACCGACTCGGTGCCAC	Amplification of pBPVX::sgXT2B for PVX::sgXT2B-tFT
tFT	D3897 (F)	aagtggcaccgagtcggtgcATGTCTATAAATATAAGAGACCC	Amplification of tFT for PVX::sgXT2B-tFT and PVX::sgPDS3ii-tFT
	D3898 (R)	gggaaacttaacaaaccctaTTGGCCATAAGTAACCTTTAG	Amplification of tFT for PVX::sgXT2B-tFT and PVX::sgPDS3ii-tFT

*F, forward primer; R, reverse primer.

Table S3. Primer combinations used for the construction of recombinant viruses.

Recombinant virus	Primers
pPVX::tR-sgXT2B-tR	D3450 + D3455 + D3456 + D3457 (template: pBPVX::tR-sgXT2B)
pPVX::tR-sgXT2B	D3450 + D3451 + D3452 + D3448
pPVX::sgXT2B-tR	D3447 + D3455 + D3456 + D3457
pPVX::sgXT2B	D3447 + D3448
pPVX::tR-sgPDS3-tR	D3450 + D3455 + D3456 + D3457
pPVX::tR-sgPDS3	D3450 + D3453 + D3454 + D3448
pPVX::sgPDS3-tR	D3449 + D3455 + D3456 + D3457 (template: pBPVX::tR-sgPDS3)
pPVX::tR-sgPDS3	D3449 + D3448
pPVX::sgFT	D3541 + D3448
pPVX::sgPDS3:sgFT:sgXT2B	D3449 + D3538 (template: pBPVX::sgPDS3) D3539 + D3540 (template: pBPVX::sgFT) D3536 + D3537 (template: pBPVX::sgXT2B)
pPVX::sgXT2B-tFT	D3895 + D3896 (template: pBPVX::sgXT2B) D3897 + D3898
pPVX::sgPDS3ii-tFT	D4166 + D3898 (template: pBPVX::sgXT2B)

Table S4. Primers used for PVX diagnosis by RT-PCR.

Primer*	Primer sequence (5'→3')	Purpose
D2409 (R)	ATTTATATTATTCATACAATCAAACC	Reverse transcription of PVX mRNA into cDNA
D3436 (F)	ATGTCAGGCCTGTTCACTATCC	Analysis of PVX infection
D2410 (R)	TGGTGGTGGTAGAGTGACAAC	
D1789 (F)	GGGAATCAATCACAGTGTTGGC	Conservation of the heterologous sgRNA region in PVX progeny
D2069 (R)	GCTACTATGGCACGGGCTGTAC	

*F, forward primer; R, reverse primer.

Table S5. Primers used for Cas9-sgRNA gene editing analysis.

Gene	Primer*	Primer sequence (5'→3')	Purpose
NbFT	D3667 (F)	CTAGAAAACCTATGGCTATAAGGG	Amplification of a 550-bp DNA fragment flanking *NbFT* target region
	D3668 (R)	GTTCTCGAGAGGTATAATATAGGC	Amplification of a 550-bp DNA fragment flanking *NbFT* target region
	D3669 (S)	CACAAGCACGCATAGAAC	Sequencing of the 550-bp PCR product for the analysis of *NbFT* editing
NbPDS3	D3665 (F+S)	GTGGGACAATCTTCTTACACTG	Amplification of a 650-bp DNA fragment flanking *NbPDS3* target region Sequencing of the 650-bp PCR product for the analysis of *NbPDS3* editing
	D3666 (R)	TGGCGAAGAAGTAAGAACC	Amplification of a 650-bp DNA fragment flanking *NbPDS3* target region
NbXT2B	D3670 (F)	TGCACGGTTGTCCGAGTTTG	Amplification of a 750-bp DNA fragment flanking *NbXT2B* target region
	D3671 (R+S)	TGCACGGTTGTCCGAGTTTG	Amplification of a 750-bp DNA fragment flanking *NbXT2B* target region Sequencing of the 750-bp PCR product for the analysis of *NbXT2B* editing

* F, forward primer; R, reverse primer; S, sequencing primer.

CHAPTER II

CRISPR-Cas12a genome editing at the whole-plant level using two compatible RNA virus vectors

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Author contributions:

All authors participated in work conception and design. MU performed the experiments. All authors carried out data analysis and interpretation of the results. MU and JAD wrote the manuscript with input from MVV and DO. All authors provided critical feedback and helped shape the final manuscript.

ABSTRACT

The use of viral vectors that can replicate and move systemically through the host plant to deliver bacterial CRISPR components enables genome editing at the whole-plant level and avoids the requirement for labor-intensive stable transformation. However, this approach usually relies on previously transformed plants that stably express a CRISPR-Cas nuclease. Here we describe successful DNA-free genome editing of *Nicotiana benthamiana* using two compatible RNA virus vectors, derived from *Tobacco etch virus* (TEV; genus *Potyvirus*) and *Potato virus X* (PVX; genus *Potexvirus*), which replicate in the same cells. The TEV and PVX vectors respectively express a Cas12a nuclease and the corresponding guide RNA. This novel two-virus vector system improves the toolbox for transformation-free virus-induced genome editing in plants and will advance efforts to breed more nutritious, resistant, and productive crops.

Keywords: CRISPR-Cas, virus-induced genome editing, DNA-free editing, solanaceous plants

INTRODUCTION

Systems derived from bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins (Cong *et al.*, 2013) have revolutionized biotechnology. In plants, CRISPR-Cas holds great promise for unprecedented genome engineering of both model species and crops (Nekrasov *et al.*, 2013; Zhang *et al.*, 2016; Li *et al.*, 2017; Zhu *et al.*, 2020; Huang and Puchta, 2021). Most common CRISPR-Cas arrangements include a Cas endonuclease, such as *Streptococcus pyogenes* SpCas9, and a single guide RNA (sgRNA), which specifically directs the nuclease to a sequence of interest in the genome. As in other taxonomic groups (Platt *et al.*, 2014; Senís *et al.*, 2014; Lau and Suh, 2017; Xu *et al.*, 2019), virus-derived vectors have been reported as a powerful alternative to express the CRISPR-Cas components at the whole-plant level avoiding the labor-intensive and time-consuming tissue culture approaches required for stable transformation. These strategies are commonly termed as virus-induced genome editing (VIGE) and have focused on the delivery of one or more sgRNAs using RNA or DNA virus vectors in transgenic plants that stably express the Cas nuclease (Ali *et al.*, 2015; Yin *et al.*, 2015; Cody *et al.*, 2017; Ali *et al.*, 2018; Hu *et al.*, 2019; Jiang *et al.*, 2019; Ellison *et al.*, 2020; Lei *et al.*, 2021; Mei *et al.*, 2019).

Expression of a Cas nuclease using a plant virus-derived vector able to move systemically through a plant was long considered unachievable due to cargo constraints. However, the innovative work by Ma *et al.* (2020) demonstrated efficient genome editing by delivering both the sgRNA and SpCas9 at the whole-plant level using a vector derived from *Sonchus yellow net virus* (SYNV; family *Rhabdoviridae*). Despite this unprecedented achievement, additional virus-based systems for the co-expression of Cas nucleases and sgRNAs at the whole-plant level are still required to improve the current toolbox for crop engineering. Notably, each viral vector has its own unique properties, particularly a specific host range.

To expand the virus-based tools for transformation-free genome editing in plants, we co-expressed the Cas nuclease and the guide RNA using two compatible viral vectors that replicate in the same cells and coordinately move systemically through the whole plant. We chose a potyvirus vector to express the Cas nuclease. Potyviruses (genus *Potyvirus*) are the largest group of plus-strand RNA viruses, with more than 200 currently known species that collectively can infect a large range of host plants (Wylie *et al.*, 2017). We also focused on a Cas12a (formerly Cpf1) nuclease, which is a component of a class 2 type V CRISPR system, isolated from *Lachnospiraceae bacterium* ND2006 (LbCas12a) (Zetsche *et al.*, 2015). Genome editing using LbCas12a has been demonstrated in plants (Endo *et al.*, 2016; Tang *et al.*, 2017; Wang *et al.*, 2017; Xu *et al.*, 2017), and the coding sequence corresponding to this nuclease is smaller than that of SpCas9. We also co-expressed the guide RNA using a recently described *Potato virus X* (PVX, genus *Potexvirus*, family *Virgaviridae*) vector, which efficiently induces hereditable gene editing in *Nicotiana benthamiana* plants that stably express SpCas9 (Uranga *et al.*, 2021). Our results demonstrated efficient DNA-free *N. benthamiana* genome editing using the two compatible RNA virus vectors.

RESULTS

A dual virus-based vector system to co-express Cas nucleases and guide RNAs in plants

Initially, we built a TEV recombinant clone in which the cDNA of a human codon-optimized LbCas12a replaced that of the viral NIb protein (TEVΔNIb::LbCas12a). Our previous work showed that this vector could express large exogenous sequences, such as a whole bacterial metabolic pathway to biosynthesize lycopene or the saffron carotenoid cleavage dioxygenase (Majer *et al.*, 2017; Martí *et al.*, 2020). However, since the virus lacks the viral RNA-dependent RNA polymerase NIb, it replicates only in plants that express this protein (Majer *et al.*, 2017). In our recombinant clone, the sequence coding

for LbCas12a replaced most of the NIb cistron and was flanked by the native nuclear inclusion *a* protease (NIaPro) cleavage sites that mediate the release of the nuclease from the viral polyprotein (**Figure 1A** and **Figure S1**).

Next, we built a recombinant version of PVX in which the LbCas12a CRISPR RNA (crRNA) was expressed under the control of the viral CP promoter, and the 29 initial codons of PVX CP were deleted to improve the stability of the recombinant clone (Dickmeis *et al.*, 2014). The viral CP was expressed from a heterologous promoter derived from that of the CP of *Bamboo mosaic virus* (BaMV; genus *Potexvirus*). Based on previous work by Bernabé-Orts *et al.* (2019) assessing the efficiency of the Cas12a-mediated gene editing of several *N. benthamiana* loci, *NbFT* was selected as the target gene (PVX::crFT; **Figure 1B** and **Figure S2**). The 65-nt crRNA was cloned downstream of the PVX CP promoter and consisted of a 23-nt protospacer sequence specific to the target gene flanked on both 5' and 3' ends by a conserved 21-nt scaffold, also known as a direct repeat.

N. benthamiana plants constitutively expressing TEV NIb under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter and terminator(Martí *et al.*, 2020) were co-inoculated with a 1:1 mix of two cultures of *Agrobacterium tumefaciens* C58C1 transformed with plasmids harboring TEVΔNIb::LbCas12a and PVX::crFT constructs. Controls included inoculation of TEVΔNIb::LbCas12a alone and inoculation of TEVΔNIb::crtB, which allows visual tracking of virus systemic movement due to the yellow pigmentation of infected tissue induced by *Pantoea ananatis* phytoene synthase (crtB) (Majer *et al.*, 2017). At 7 dpi, typical symptoms of TEV infection emerged in the upper non-inoculated leaves of all plants. Notably, at 14 dpi irregular chlorotic spots appeared in the leaves of plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT, but not in those inoculated with TEVΔNIb::LbCas12a alone (**Figure 2**). PVX infection is characterized by the appearance of vein banding, ring spots and leaf atrophy

(Loebenstein and Gaba, 2012). Therefore, the observed phenotypic alteration could have been due to LbCas12a-mediated editing of *NbFT* or an effect of the coinfection. Samples from the first systemically infected upper leaf were collected at 7 and 14 dpi, and TEVΔNIb progeny was studied by RT-PCR analysis (**Figure 1C**). An 804-bp specific region of the TEV genome corresponding to the CP cistron was amplified in all virus-inoculated plants, confirming the presence of the virus (**Figure 1C**, top panel). However, for viral vectors carrying big cargos, genomes larger than wild type are likely to recombine to smaller sizes, thus triggering the loss of heterologous genes (Gilbertson *et al.*, 2003). An additional RT-PCR analysis was performed, and a 600-bp cDNA corresponding to a fragment of the LbCas12a coding sequence was exclusively amplified from plants inoculated with TEVΔNIb::LbCas12a alone or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT, regardless of sampling time (**Figure 1C**, bottom panel). A complementary RT-PCR analysis using primers hybridizing on the flanking borders of the viral vector confirmed the presence of full-length LbCas12a coding sequence in the viral progeny at 14 dpi, although also indicated that viral progeny partially lost the cargo at this time point (**Figure S3**). Taken together, these results suggest that the expression of LbCas12a nuclease expands from the onset of viral infection until an undetermined time point in which the viral vector completely lost the heterologous gene.

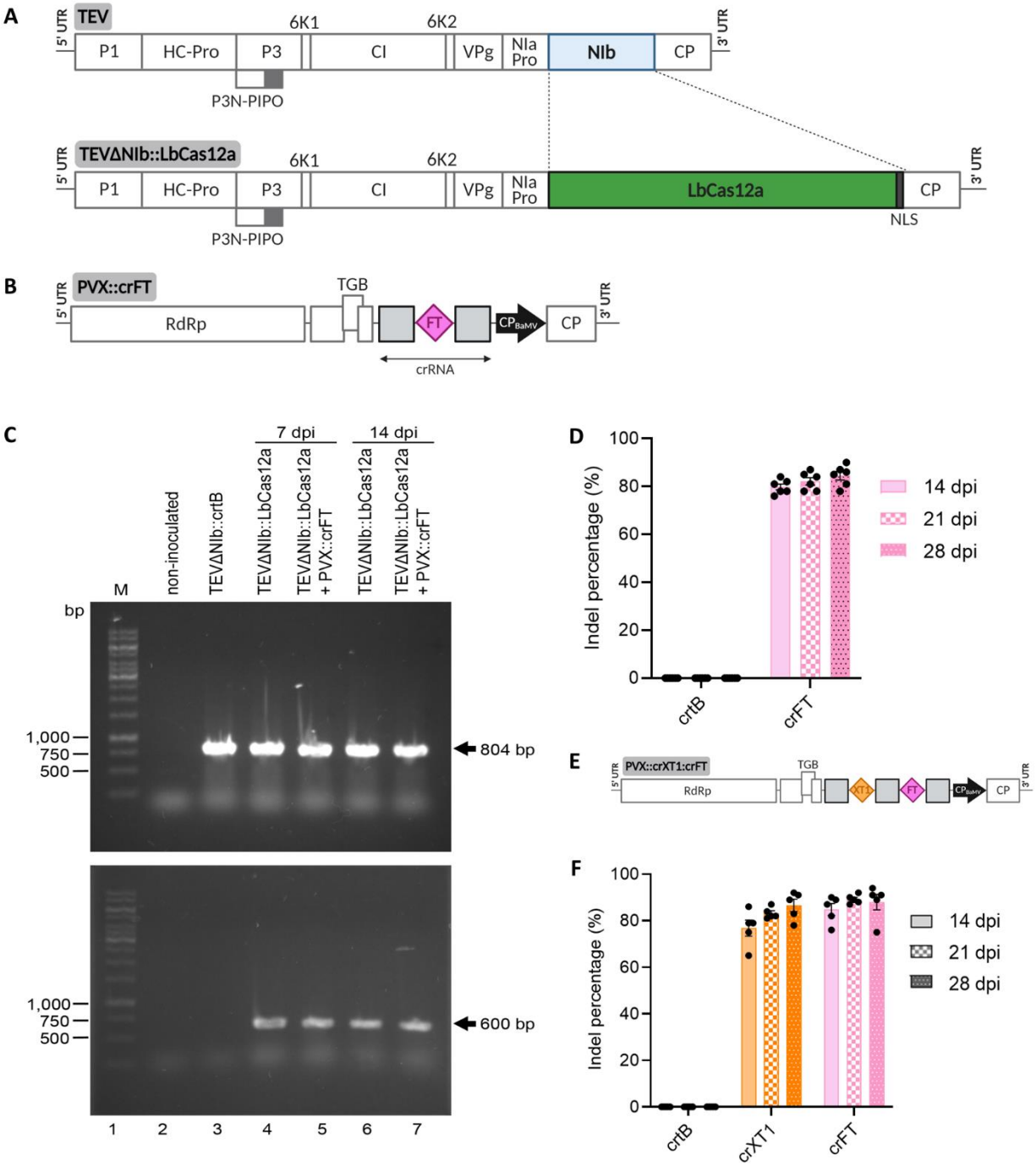

Figure 1. DNA-free gene editing in *N. benthamiana* based on virally delivered LbCas12a and crRNA. (A) Schematic representation of recombinant virus TEVΔNIb::LbCas12a. Cas12a ORF from *Lachnospiraceae bacterium* ND2006 (LbCas12a), containing a carboxy-terminal nucleoplasmin nuclear localization signal (NLS, grey box), replaces the TEV NIb cistron (light blue box) and is flanked by proteolytic processing sites of viral NIaPro. TEV cistrons P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIaPro, NIb, and CP are represented by boxes. 5' and 3' untranslated regions (UTRs) are represented by lines. (B) Schematic representation of recombinant clone PVX::crFT. RNA-dependent

RNA polymerase (RdRp), triple gene block (TGB), and coat protein (CP) are represented by open boxes. Heterologous *Bamboo mosaic virus* CP promoter (CP_{BaMV}) is represented by a black arrow. 5' and 3' UTRs are represented by black lines. crRNA consists of a gene-specific 23-nt protospacer (pink diamond) and conserved 21-nt direct repeats (grey boxes). CP_{BaMV}, protospacer and scaffold are not shown at scale. (C) RT-PCR analysis of TEVΔNIb progeny at 7 and 14 dpi in 35S::NIb *N. benthamiana* plants inoculated with TEVΔNIb::LbCas12a alone or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT. Amplification products were separated by electrophoresis in an agarose gel stained with ethidium bromide. Lane 1, 1-kb ladder DNA marker with the length of some components (in bp) indicated on the left; lane 2, non-inoculated plant; lanes 3 to 7, plants inoculated with TEVΔNIb::crtB (lane 3) or TEVΔNIb::LbCas12a (lanes 4 and 6), and co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (lanes 5 and 7). The amplification products corresponding to cDNA regions of TEV CP (804 bp) (top) or LbCas12a (600 bp) (bottom) are indicated by arrows. (D) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT at the indicated dpi (n=6). Box plot represents indels (%) and standard deviation. TEVΔNIb::crtB was used as a negative control. (E) Schematic representation of recombinant clone PVX::crXT1:crFT. Protospacers for NbXT1 and NbFT are represented by orange and pink diamonds, respectively. Other details are indicated above. (F) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants (n=6) co-inoculated with TEVΔNIb::LbCas12a and PVX::crXT1:crFT at the indicated dpi. Columns and error bars represent average indels (%) and standard error of the mean, respectively.

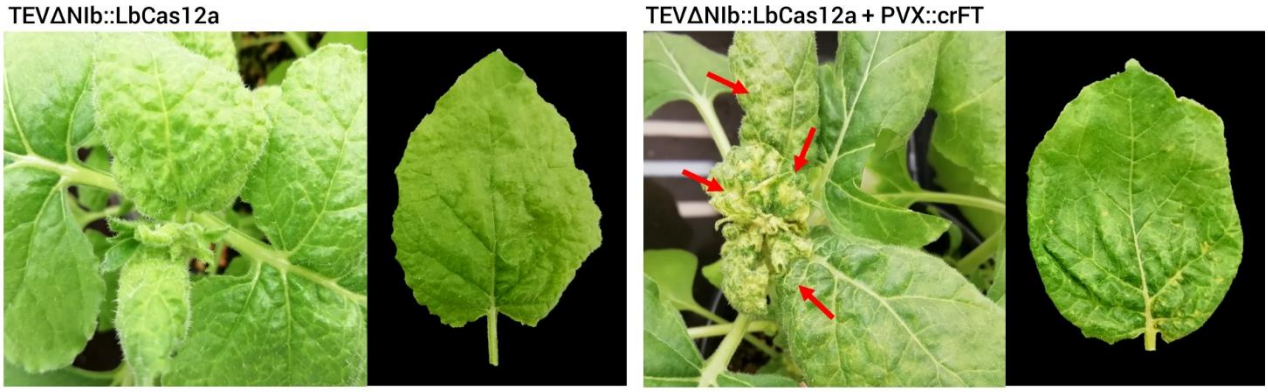

Figure 2. 35S::NIb N. benthamiana plants and representative leaves from these plants at 14 dpi inoculated with TEVΔNIb::LbCas12a (left) or co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (right). Chlorotic spots in leaves from co-inoculated plants are indicated with red arrows.

Next, DNA was purified from leaf samples collected at 7 and 14 dpi, and a 550-bp fragment of the *NbFT* gene covering the LbCas12a target site was amplified by PCR. Sanger sequencing of the PCR products and ICE analysis revealed robust gene editing at 14 dpi in plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT, reaching an indel percentage of up to 75% (**Figure 1D** and **Figure S4**). No significant differences were observed at 21 and 28 dpi. The indel distribution was consistent with the deletion-enriched mutagenesis profile characteristic of Cas12a activity, being mainly 5- to 10-bp deletions (Bernabé-Orts *et al.*, 2019). These results indicate that the simultaneous delivery of CRISPR-Cas12a components through two compatible viral vectors (i.e., TEVΔNIb and PVX) allows highly efficient targeted mutagenesis in *N. benthamiana*.

Multiplex genome editing using the dual virus-based vector system in plants

A key advantage of CRISPR-Cas genome editing is the capacity to target several loci at once by the simultaneous expression of several guide RNAs (i.e., multiplexing). Based on our observations in Cas9-expressing plants (Uranga *et al.*, 2021), we wondered whether the PVX vector could allow the delivery of multiple, functional crRNAs for Cas12a-mediated editing. To investigate this, we selected *Xylosyl transferase 1* (*NbXT1*) as the second target gene. As Cas12a can self-process crRNAs due to its RNase III activity, *NbXT1* and *NbFT* crRNAs were arranged in tandem under the control of the same CP promoter, thus creating the PVX::crXT1:crFT construct (**Figure 1E** and **Figure S2**). *N. benthamiana* plants constitutively expressing TEV NIb were co-inoculated with a 1:1 mix of two *A. tumefaciens* cultures carrying TEVΔNIb::LbCas12a and PVX::crXT1:crFT. *A. tumefaciens* transformed with TEV::crtB was used as a control in this assay. The first systemically infected leaf was sampled at 14, 21, and 28 dpi, following extraction of genomic DNA and PCR amplification of the target sites. ICE analysis revealed efficient gene editing on both *NbXT1* and *NbFT*, with average indel percentages ranging from 76% to 88%, which was maintained regardless of the sampling time (**Figure 1F**). In

addition, the absence of statistically relevant differences in gene editing among *NbXT1* and *NbFT* suggested that LbCas12a can efficiently self-process tandemly arrayed crRNAs. Time-course comparison with the single-crRNA construct, in the case of *NbFT*, also revealed that multiplexing does not affect the editing efficiency, since the mutation rates achieved with both strategies were similar (compare **Figures 1D** and **1F**).

Dual vector CRISPR-Cas genome editing in wild-type plants

The fact that TEVΔNIb infectivity depends on the supplementation of viral NIb from a transgene may be perceived as a limitation of this genome-editing system, as the approach is still bound to a previously transformed plant. An alternative strategy for supplying NIb activity consists of the co-inoculation of TEVΔNIb with a recombinant PVX expressing NIb (Bedoya *et al.*, 2010). We wondered whether a single PVX vector could: (i) provide NIb activity for the replication of TEVΔNIb; and (ii) deliver the crRNA for LbCas12a-mediated gene editing. Thus, the coding sequence of the TEV NIb cistron plus an additional amino-terminal Met was inserted within the expression cassette in PVX. *NbFT*-specific crRNA was added downstream of NIb without any linker sequence, so that both NIb and crFT expression were under the control of the PVX CP promoter (PVX::NIb:crFT; **Figure 3A** and **Figure S2**). Wild-type *N. benthamiana* plants were co-inoculated with a 1:1 mix of two cultures of *A. tumefaciens* carrying TEVΔNIb::LbCas12a and PVX::NIb:crFT. *A. tumefaciens* transformed with TEV::crtB was again used as a control. At 7 dpi, the apical leaves of the co-inoculated *N. benthamiana* plants became symptomatic (i.e., leaf curling). At 14 dpi, necrotic spotting and interveinal mottling were observed in systemic leaves, which became more noticeable over time (**Figure 4**). Samples from the first systemically infected upper leaf were collected at 14 dpi and the 550-bp fragment of the *NbFT* gene covering the LbCas12a target site was amplified by PCR. ICE analysis of the PCR products exhibited 20% indels in plants co-inoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT. These results indicate that a single PVX vector

can supply the viral NIb activity that allows TEVΔNIb to replicate and systemically spread in wild-type *N. benthamiana*, as well as to perform crRNA delivery for LbCas12a-mediated genome editing.

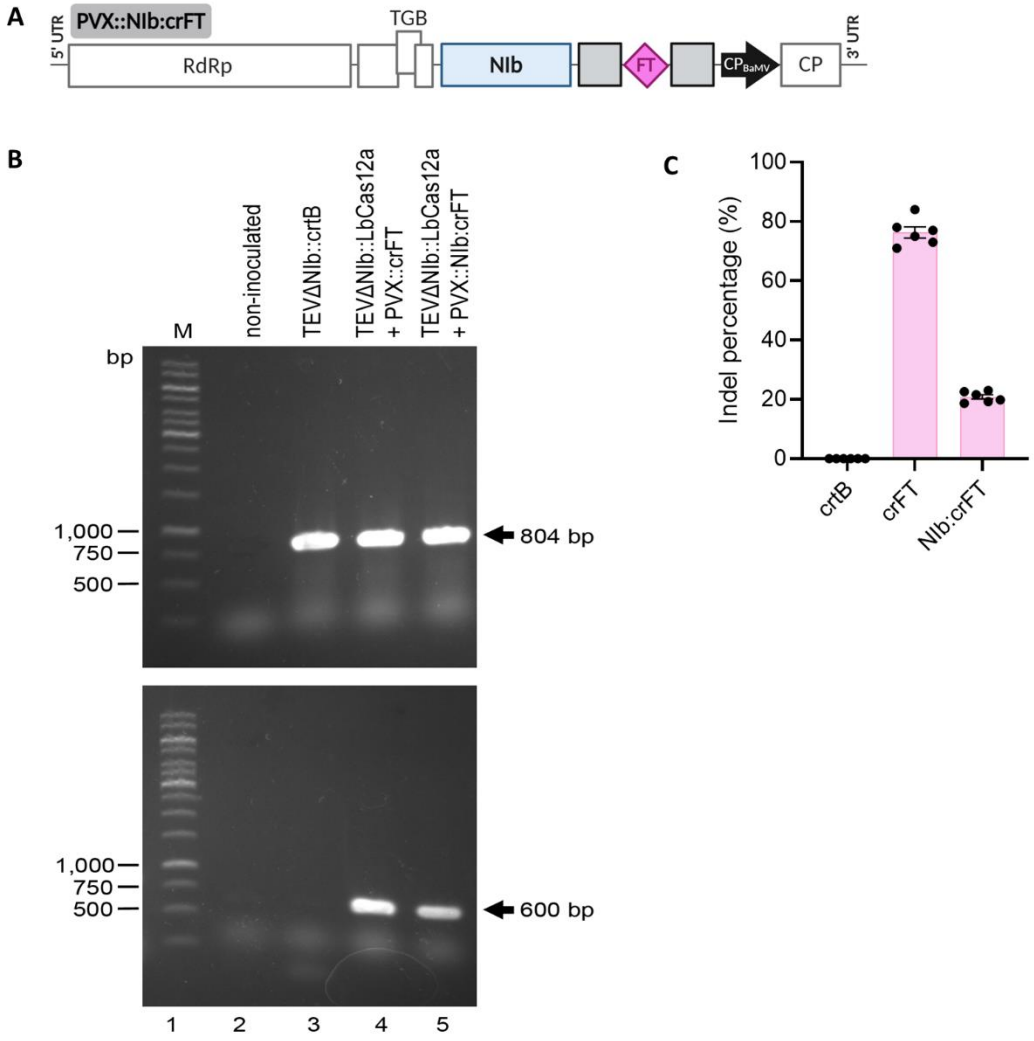

Figure 3. Engineering of a single PVX vector for complementation of defective TEVΔNIb and expression of LbCas12a crRNA. (A) Schematic representation of recombinant virus PVX::NIb:crFT. TEV NIb cistron is represented by a light blue box. Other details are as described in the legend to Fig. 1. (B) RT-PCR analysis of TEVΔNIb progeny at 14 dpi in 35S::NIb *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT or wild-type *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT. Amplification products were separated by electrophoresis in an agarose gel that was stained with ethidium bromide. Lane 1, DNA marker ladder with the length of some components (in bp) indicated on the left; lane 2,

non-inoculated plant; lane 3 to 5, plants inoculated with TEVΔNIb::crtB (lane 3) and co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (lane 4) or with TEVΔNIb::LbCas12a and PVX::NIb:crFT (lane 5). The amplification products corresponding to cDNA regions of TEV CP (804 bp) (top) or LbCas12a (600 bp) (bottom) are indicated by arrows. (C) ICE analysis of the first systemically infected upper leaf of *N. benthamiana* plants co-inoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT at 14 dpi (n=6). TEVΔNIb::crtB was used as a negative control. Columns and error bars represent average indels (%) and standard error of the mean, respectively.

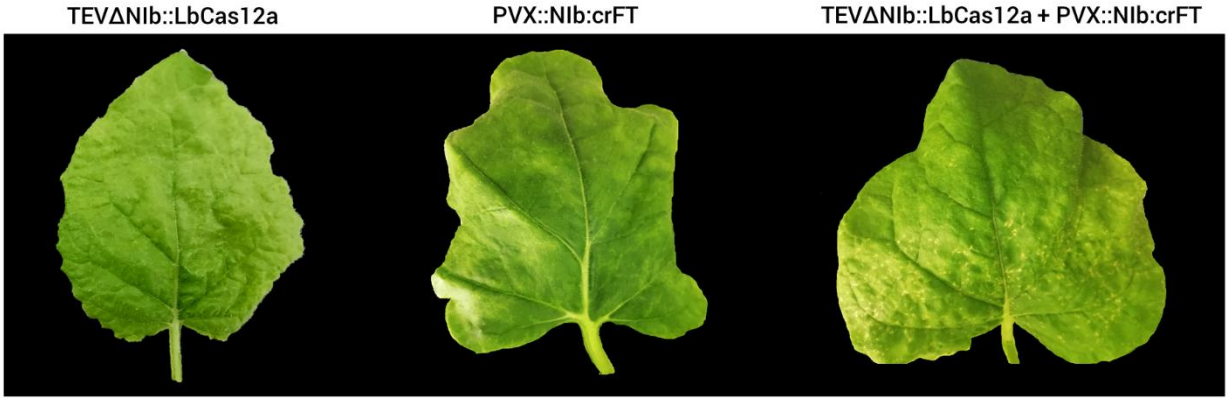

Figure 4. Representative leaves from wild-type N. benthamiana plants at 14 dpi inoculated with TEVΔNIb::LbCas12a alone (left), PVX::NIb:crFT alone (middle), or co-inoculated with TEVΔNIb::LbCas12a and PVX::NIb:crFT (right).

DISCUSSION

In this study we describe the engineering of a dual RNA-virus system for the delivery of CRISPR-Cas12a components for genome editing in plants. The system consists of two compatible plus-strand RNA viruses, specifically the potyvirus TEV and the potexvirus PVX, to express the Cas nuclease and the guide RNA, respectively. Notably, the TEV vector (TEVΔNIb) contains the deletion of the RNA-dependent RNA polymerase NIb to accommodate the large coding sequence of the Cas nuclease. Our previous efforts to express a functional Cas nuclease using a full-length potyvirus vector in plants had been unsuccessful. However, recent works have reported successful Cas9 expression and DNA-free genome editing using single vectors in *N. benthamiana* (Gao *et*

al., 2019; Ariga *et al.*, 2020; Zhang *et al.*, 2020; Ma *et al.*, 2020). These contrasting results emphasize the diverse properties of vectors derived from viruses belonging to different genera and families, and the necessity for a large spectrum of molecular tools, operating in a wide range of host species, to tackle challenging VIGE goals in crop plants. Notably, SYNV is a minus-strand RNA virus whose the inoculation entails some complexity (Peng *et al.*, 2021) and, as with each plant virus, it exhibits a particular host range (Jackson and Christie, 1977).

In this work, we aimed to express a Cas nuclease using a potyvirus vector for DNA-free plant-genome editing. The genus *Potyvirus* is the largest among the plant RNA viruses, and comprises more than 200 species that infect a wide range of host plants from many different botanical families (Wylie *et al.*, 2017). Therefore, it offers a wealth of genetic resources for VIGE. Although we were unable to successfully express the Cas nuclease using a full-length potyvirus vector, we demonstrated that TEVΔNIb allows the transient expression of LbCas12a, and that PVX can perform both single or multiple crRNA delivery as well as providing the NIb activity. Our two-virus delivery system resulted in efficient targeted editing both in NIb-expressing and wild-type *N. benthamiana* plants, reaching indel percentages of up to 80% and 20%, respectively (**Figures 1** and **3**). This dual vector system not only incorporates the enormous genetic resources of potyviruses for VIGE, but also demonstrates that compatible RNA virus vectors can be used to simultaneously deliver several CRISPR-Cas components. This could be useful for more sophisticated arrangements in plant-genome editing and gene-expression regulation studies. However, caution must be taken when implementing these designs because positive-strand RNA viruses, such as potyviruses and potexviruses, are prone to homologous recombination (Lai, 1992). Homology must be avoided in flanking sequences of transgenes and heterologous genes, such as NIb in this work, to avoid undesired acquisition by viral vectors (**Figure S5**). What is also of importance is that our dual RNA virus system is based on two positive-strand viral species that belong to

different families and are able to co-infect the same cells. When designing systems with multiple viral vectors, superinfection exclusion must be taken into consideration because phylogenetically related viruses are generally excluded to co-infect the same cells (Zhang *et al.*, 2018).

Recently, we developed a PVX vector to efficiently express multiplex SpCas9 sgRNAs at the whole-plant level (Uranga *et al.*, 2021). Current results with LbCas12a confirm that PVX can be easily engineered for the simultaneous delivery of guide RNAs regardless of the nature of the Cas nuclease, which highlights its usefulness in a variety of multiplexing approaches. Efficient genome editing in our dual-virus system implies that LbCas12a is able to find and process the crRNA either in the whole PVX genome, in viral subgenomic transcripts or in both. Particularly in the first case, LbCas12a-mediated cleavage will produce replication-incompetent viruses. The observation that LbCas12a expression has no effect on PVX vector infectivity suggests that either the whole viral genome is not the preferred target or cleavage is not that quantitative to abolish infectivity. We obtained similar results when flanked Cas9 guide RNAs with cleavable tRNA sequences in the PVX vector (Uranga *et al.*, 2021).

In contrast to previous reports of DNA-free VIGE that used SpCas9 (Ariga *et al.*, 2020; Ma *et al.*, 2020), we selected Cas12a. This was due to the following unique features of the nuclease (Zaidi *et al.*, 2017): (i) the cleavage of target DNA is directed by a single crRNA shorter than that of SpCas9 sgRNA; (ii) the protospacer adjacent motif (PAM) is T-rich (5'-TTTV-3'); (iii) DNA cleavage results in cohesive ends with 4- or 5-nt overhangs, which might facilitate homology-directed repair (HDR); (iv) it exhibits RNase III activity useful to facilitate multiplex gene editing; and (v) it is smaller than SpCas9 (3.8 kb vs 4.2 kb), which is important in terms of viral delivery. Moreover, the unique characteristics of the CRISPR-Cas12a system, unlike that of Cas9, such as the recognition of a T-rich PAM and the induction of staggered ends that facilitate homologous

recombination, limit the range of target sequences and thus reduce off-target activity (Zaidi *et al.*, 2017). Cas12a orthologues from *Francisella novicida* U112 (FnCas12a), *Acidaminococcus* sp. BV3L6 (AsCas12a) and LbCas12a were first experimentally validated in mammalian cells (Kim *et al.*, 2016; Kim *et al.*, 2017; Zetsche *et al.*, 2017). In plants, targeted mutagenesis was achieved in rice and tobacco using any of the orthologs (Endo *et al.*, 2016; Tang *et al.*, 2017; Wang *et al.*, 2017; Xu *et al.*, 2017). A DNA-free approach based on the delivery of AsCas12a or LbCas12a loaded with crRNA was also validated in wild tobacco and soy-bean protoplasts (Kim *et al.*, 2017). Here we focused on LbCas12a, since previous works reported that this nuclease possesses higher efficiency tha FnCas12a or AsCas12a (Tang *et al.*, 2017; Bernabé-Orts *et al.*, 2019). We showed here that LbCas12a is also effective for plant-genome editing when virally delivered.

In conclusion, our dual RNA-virus-based system broadens the current toolbox for DNA-free VIGE and will contribute to applications in plant functional genomics and crop improvement.

MATERIALS AND METHODS

Viral vectors

Guide RNAs to target *N. benthamiana* Flowering locus T (*NbFT*; SolGenomics Niben101Scf01519g10008.1) and Xylosyl transferase 1 (*NbXT1*; Niben101Scf04205g03008.1) were selected using the CRISPR-P online tool as described in Bernabé-Orts *et al.* (2019). Nucleotide (nt) sequences of recombinant viral clones are shown in **Figures S1** and **S2**. These clones were built using the primers shown in **Tables S2** and **S3.**

Plant inoculation

N. benthamiana wild-type and transformed plants expressing *Tobacco etch virus* (TEV, genus *Potyvirus*) nuclear inclusion *b* (NIb) protein (Martí *et al.*, 2020) were grown at 25°C under a 16 h/8 h day/night photoperiod. Plants that were 4- to 6-weeks-old were agroinoculated as previously described (Bedoya *et al.*, 2010; Uranga *et al.*, 2021). Tissue samples (approximately 100 mg) from the first symptomatic upper non-inoculated leaf were collected at different days post inoculation (dpi), as indicated, for virus progeny and plant genome-editing analyses.

Analysis of viral progeny

RNA was purified from leaf samples using silica gel columns (Uranga *et al.*, 2021). cDNA was synthesized using RevertAid reverse transcriptase (Thermo Scientific) and primer D179 (**Table S4**). Polymerase chain reaction (PCR) with *Thermus thermophilus* DNA polymerase (Biotools) was used to amplify the TEV coat protein (CP) cistron (primers D178 and D211; **Table S4**) or a fragment of the LbCas12a open reading frame (ORF; primers D3604 and D3605; **Table S4**). The presence of full-length LbCas12a coding sequence in the viral progeny was assayed by reverse transcription (RT)-PCR using primers D2567 (RT) and D570-D571 (PCR; **Table S4**). PCR products were separated by electrophoresis in 1% agarose gels followed by staining with ethidium bromide.

Analysis of Cas12a-mediated genome editing

DNA from leaf samples was purified using silica gel columns (Uranga *et al.*, 2021). *N. benthamiana* genome fragments were amplified by PCR using high-fidelity Phusion DNA polymerase (Thermo Scientific; **Table S5**). PCR products were separated by agarose gel

electrophoresis, purified from the gel, and subjected to Sanger sequencing (**Table S5**). The presence of sequence modifications was analyzed using the ICE algorithm (https://www.synthego.com/products/bioinformatics/crispr-analysis).

SUPPORTING INFORMATION

All data relating to Supplementary Figures and Tables can be found at the end of this chapter.

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SUPPORTING INFORMATION

Figure S1. Full sequence of the wild-type *Tobacco etch virus* (TEV-wt), the defective viral vector TEVΔNIb and their derived recombinant viruses, TEVΔNIb::crtB and TEVΔNIb::LbCas12a. TEV-wt sequence corresponds to Genbank accession number DQ986288 including two silent mutations (G273A and A1119G, in red). Limits between TEV cistrons are marked on blue background. cDNAs corresponding to *P. ananatis* **phytoene synthase** (crtB) and **Cas12a** from *Lachnospiraceae bacterium* ND2006 (LbCas12a) are on yellow and dark green backgrounds, respectively. Nucleoplasmin nuclear localization signal (NLS) and human influenza hemagglutinin (3xHA) tag are underlined doubly and dotted, respectively. In the inserted cDNAs, sequences corresponding to native TEV NIaPro **cleavage sites** are in black.

>TEV (DQ986288, G273A and A1119G)

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>TEVΔNIb

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>TEVΔNIb::LbCas12a (insert between positions 6981-6982 of TEVΔNIb)

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Figure S2. Full sequence of wild-type *Potato virus X* (PVX; GenBank accession number MT799816) and its derived recombinant viruses PVX::crFT, PVX::crXT1:crFT and PVX::NIb:crFT. In recombinant PVX clones, heterologous sequences are transcribed from coat protein (CP) promoter and a deleted version of PVX CP, lacking the 29 initial codons, is transcribed from an heterologous promoter derived from Bamboo mosaic virus (BaMV) CP (Dickmeis et al., 2014). **PVX CP promoter** with a ATG-AGG mutation to abolish start codon is underlined. **BaMV CP promoter** is in blue. crRNA sequences corresponding to *NbFT* and *NbXT1* are on fuchsia and red backgrounds, respectively, and **protospacer** region is underlined (dotted). cDNA corresponding to **TEV nuclear inclusion *b* (NIb)** cistron is on dark blue background.

>PVX-wt (MT799816)

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ACAAAGGCAAGAAGAAAGAAGCTCTCAGCGAGCTCACACTAGATGAGCAGGAGGCAATGCTCAAAGCAAGTTGCCTGCGA
CTGTATACGGGAAAGCTGGGAATTTGGAATGGCTCATTGAAAGCAGAGTTGCGTCCAATTGAGAAGGTTGAAAACAACAA
AACGCGAACTTTCACAGCAGCACCAATAGACACTCTTCTTGCTGGTAAAGTTTGCGTGGATGATTTCAACAATCAATTTT
ATGATCTCAACATAAAGGCACCATGGACAGTTGGTATGACTAAGTTTTATCAGGGGTGGAATGAATTGATGGAGGCTTTA
CCAAGTGGGTGGGTGTATTGTGACGCTGATGGTTCGCAATTCGACAGTTCCTTGACTCCATTCCTCATTAATGCTGTATT
GAAAGTGCGACTTGCCTTCATGGAGGAATGGGATATTGGTGAGCAAATGCTGCGAAATTTGTACACTGAGATAGTGTATA
CACCAATCCTCACACCGGATGGTACTATCATTAAGAAGCATAAAGGCAACAATAGCGGGCAACCTTCAACAGTGGTGGAC
AACACACTCATGGTCATTATTGCAATGTTATACACATGTGAGAAGTGTGGAATCAACAAGGAAGAGATTGTGTATTACGT
CAATGGCGATGACCTATTGATTGCCATTCACCCAGATAAAGCTGAGAGGTTGAGTGGATTCAAAGAATCTTTCGGAGAGT
TGGGCCTGAAATATGAATTTGACTGCACCACCAGGGACAAGACACAGTTGTGGTTCATGTCACACAGGGCTTTGGAGAGG
GATGGCATGTATATACCAAAGCTAGAAGAAGAAAGGATTGTTTCTATTTTGGAATGGGACAGATCCAAAGAGCCGTCACA
TAGGCTTGAAGCCATCTGTGCATCAATGATCGAAGCATGGGGTTATGACAAGCTGGTTGAAGAAATCCGCAATTTCTATG
CATGGGTTTTGGAACAAGCGCCGTATTCACAGCTTGCAGAAGAAGGAAAGGCGCCATATCTGGCTGAGACTGCGCTTAAG
TTTTTGTACACATCTCAGCACGGAACAAACTCTGAGATAGAAGAGTATTTAAAAGTGTTGTATGATTACGATATTCCAAC
GACTGAGAATCTTTATTTTCAGTAATAATTTCTACTAAGTGTAGATCAAGATCTATTGGCCTAAGAGTTTAATTTCTACT
AAGTGTAGATTAGGGTTTGTTAAGTTTCCCTTTTTACTCGAAAGATG

Figure S3. RT-PCR analysis of TEVΔNIb progeny at 14 dpi in 35S::NIb (lanes 1 to 3) and wild-type (lane 4) N. benthamiana plants inoculated with several virus combinations as indicated. Amplification products were separated by electrophoresis in an agarose gel stained with ethidium bromide. Lane 1, non-inoculated plant; lanes 2 to 4, plants inoculated with TEVΔNIb::crtB (lane 2) and co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT (lane 3) or with TEVΔNIb::LbCas12a and PVX::NIb:crFT (lane 4); lane 5, DNA marker ladder with the length of some components (in bp) indicated on the right. The amplification product corresponding to the full cDNA region of LbCas12a (3,902 bp) is indicated by an arrow.

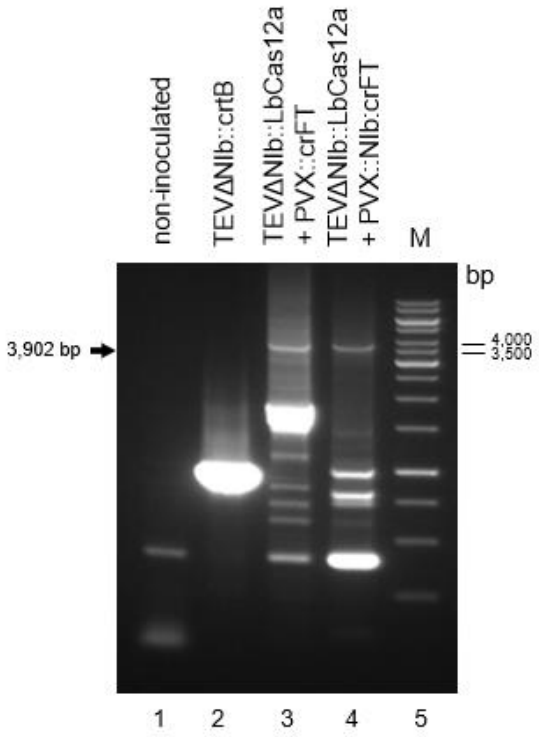

Figure S4. ICE analysis results of target site mutations in 35S::NIb N. benthamiana plants co-inoculated with TEVΔNIb::LbCas12a and PVX::crFT. Examples of sequencing chromatograms (top) and mutagenesis profiles (bottom) are shown.

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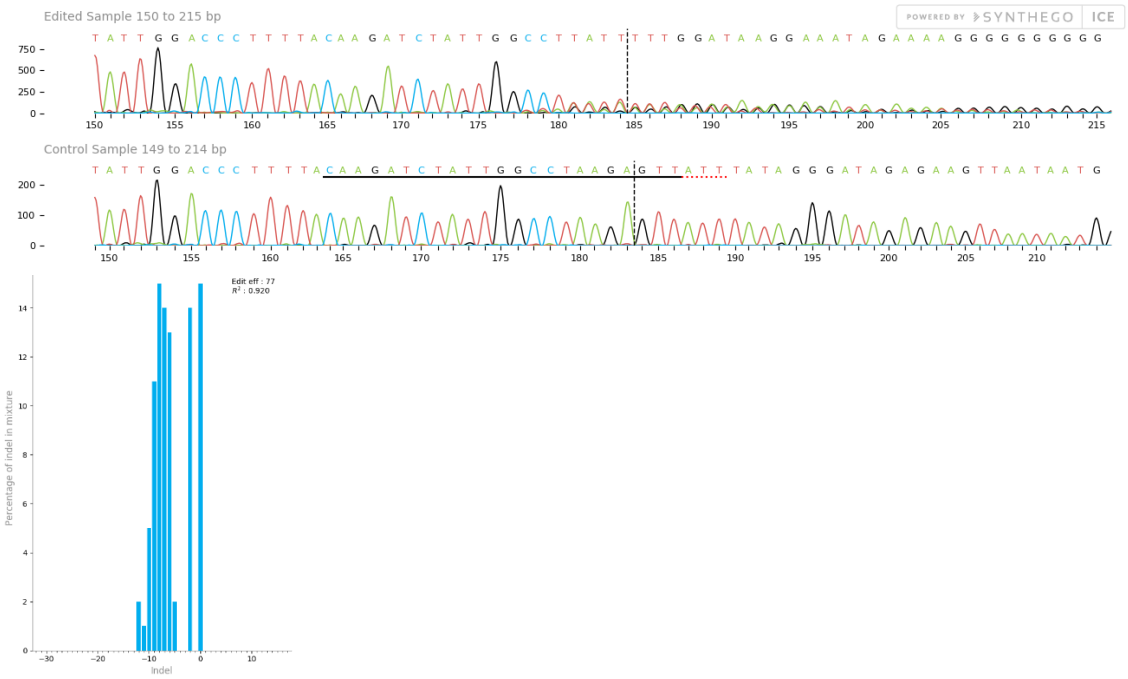

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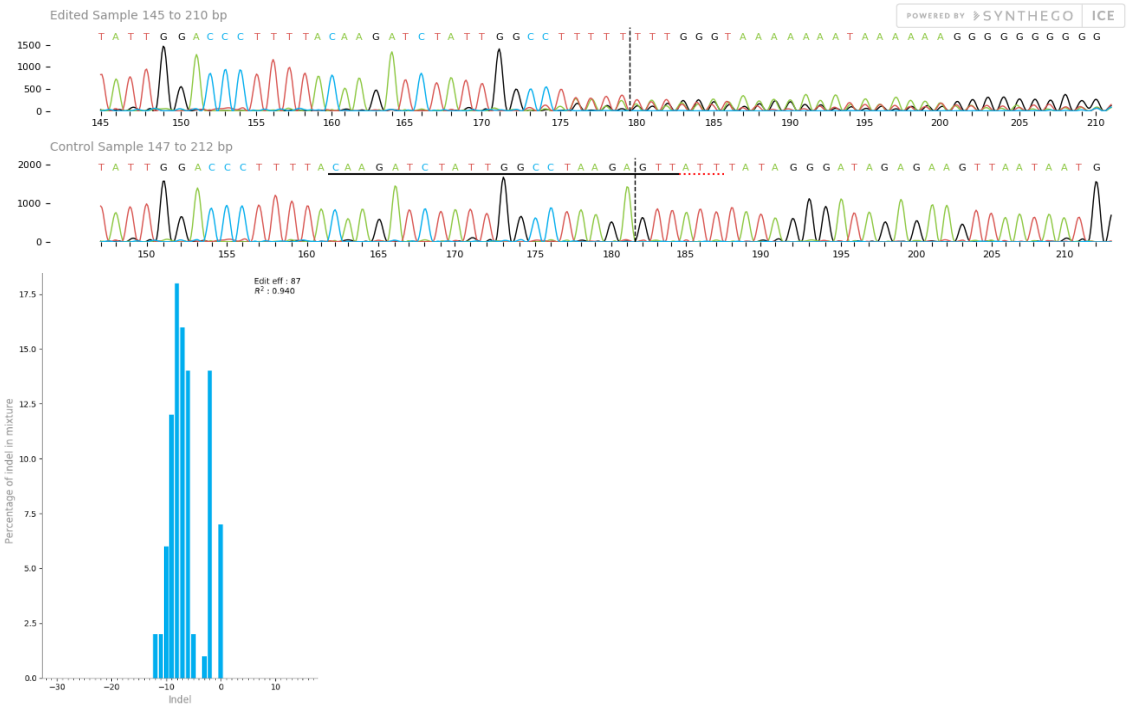


Figure S5. Analysis of potential NIb acquisition by TEVΔNIb. Extracts from three independent 35S::NIb *N. benthamiana* plants infected with TEVΔNIb::LbCas12a (7 dpi) were used to inoculate (A) wild-type and (B) 35S::NIb N. benthamiana plants. TEV infection was diagnosed by RT-PCR at 7 dpi. Amplification products were separated by electrophoresis in an agarose gel stained with ethidium bromide. Lane 1, 1-kbp DNA marker ladder with the length of some components (in bp) indicated on the left; lane 2, non-inoculated control; lanes 3 to 5, plants inoculated with extracts of 35S::NIb *N. benthamiana* plants infected with TEVΔNIb::LbCas12a.

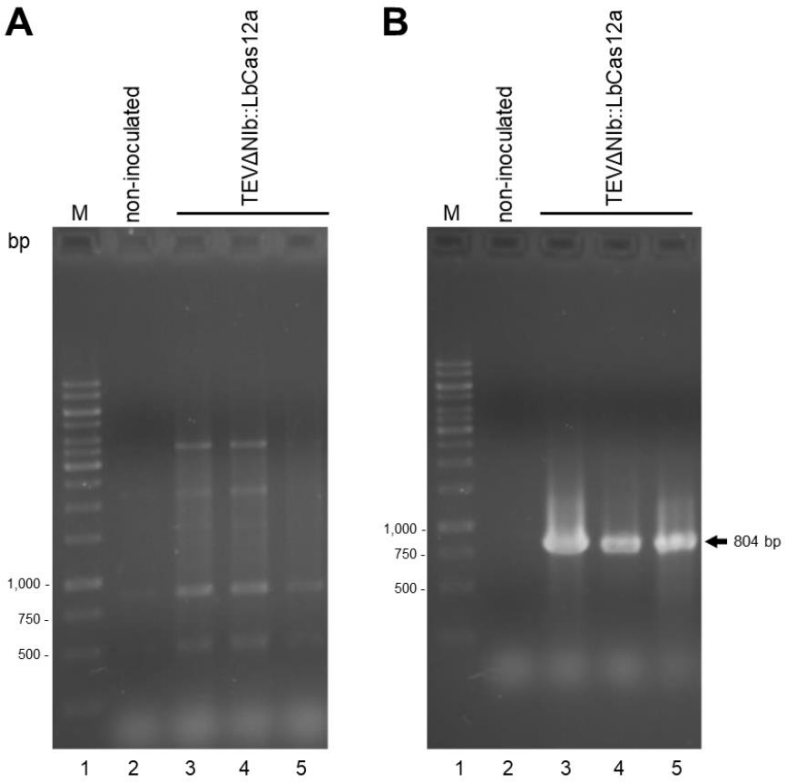


Table S1. *N. benthamiana* genes targeted by the CRISPR-Cas12a system. Note that PAM sequence recognised by LbCas12a nuclease is highlighted in grey.

Target gene	SolGenomics Accession No	Target site
NbFT	Niben101Scf01519g10008.1	**TTTA**CAAGATCTATTGGCCTAAGAGTT
NbXT1	Niben101Scf04205g03008.1	**TTTA**TATGTAGGTGTATTTGGAATTCT

Table S2. Primers used for the construction of recombinant viruses from TEVΔNIb or PVX.

Construct element	Primer*	Primer sequence (5'→3')	Purpose
LbCas12a	D2521 (F)	attggtgtactcgcaagggg agaagATGAGCAAGCTGGAG AAG	Amplification of *LbCas12a* for TEVΔNIb::LbCas12a
	D2508 (R)	tctgaaaataaagattctca gtcgtGGCATAGTCGGGGAC ATC	Amplification of *LbCas12a* for TEVΔNIb::LbCas12a
crFT	D3591 (F)	gaggtcagcaccagctagca TAATTTCTACTAAGTGTAGA TCAAGATCTATTGGCCTAAG	Amplification of crFT for PVX::crFT
	D3592 (R)	gggaaacttaacaaacccta ATCTACACTTAGTAGAAATT AAACTCTTAGGCCAATAGAT	Amplification of crFT for PVX::crFT
	D3712 (F)	ttttcagtaaTAATTTCTAC TAAGTGTAGATCAAGATCTA TTGGCCTAAG	Amplification of crFT for PVX::NIb:crFT
	D3713 (R)	gggaaacttaacaaacccta ATCTACACTTAGTAGAAATT AAACTCTTAGGCCAATAGAT	Amplification of crFT for PVX::NIb:crFT
pBPVX:: crFT	D4036 (F)	TAATTTCTACTAAGTGTAGA TCAAGATCTATTGG	Amplification of pBPVX::crFT for PVX::crXT1:crTFL1:crFT
	D3536 (R)	TGCTAGCTGGTGCTGACC	Amplification of pBPVX::crFT for PVX::crXT1:crTFL1:crFT
crXT1	D4037 (F)	gaggtcagcaccagctagca TAATTTCTACTAAGTGTAGA TTATGTAGGTGTATTTGG	Amplification of crXT1 for PVX::crXT1:crFT
	D4038 (R)	ATCTACACTTAGTAGAAATT AAGAATTCCAAATACACC	Amplification of crTFL1 3.1/14.1 for PVX::crXT1:crFT
NIb	D3710 (F)	gaggtcagcaccagctagca TGGGGGAGAAGAGGAAATG	Amplification of TEV NIb cistron for PVX::NIb:crFT
	D3711 (R)	gtagaaattaTTACTGAAAA TAAAGATTCTCAGTCG	Amplification of TEV NIb cistron for PVX::NIb:crFT

*F, forward primer; R, reverse primer

Table S3. Primer combinations used for the construction of recombinant viruses from TEVΔNIb or PVX.

Recombinant virus	Primers
TEVΔNIb::LbCas12a	D2521 + D2508 (template: pcDNA3.1::hLbCpf1)
PVX::crFT	D3591+ D3592 (no template)
PVX::crXT1:crFT	D4037 + D4038 D4036 + D3536 (template: pBPVX::crFT)
PVX::NIb:crFT	D3710 + D3711 (template: pGTEVa) D3712 + D3713

Table S4. Primers used for TEVΔNIb diagnosis by RT-PCR.

Primer*	Primer sequence (5'→3')	Purpose
D179 (R)	CTCGCACTACATAGGAGAATTAGAC	Reverse transcription of TEVΔNIb mRNA into cDNA
D178 (F)	AGTGGCACTGTGGGTGCTGGTGTTG	Analysis of TEVΔNIb infection
D211 (R)	GGCGCGGCGTCGACCTGGCGGACCCCTAATAG	
D3604 (F)	ATAAGGAGACAGACTATCGGGC	Expression of *LbCas12a* nuclease in TEVΔN progeny
D3605 (R)	TGATCTGTCCGTGATTGTTCTC	
D2567 (R)	TGTAATGTTTCTCTGTAGACCATAC	Reverse transcription of TEVΔNIb RNA into cDNA
D570 (F)	CATGAGTGAATTGGTGTACTCGCAA	Expression of full-length LbCas12a coding sequence in viral progeny
D571 (R)	CAACACCAGCACCCACAGTGCCACT	

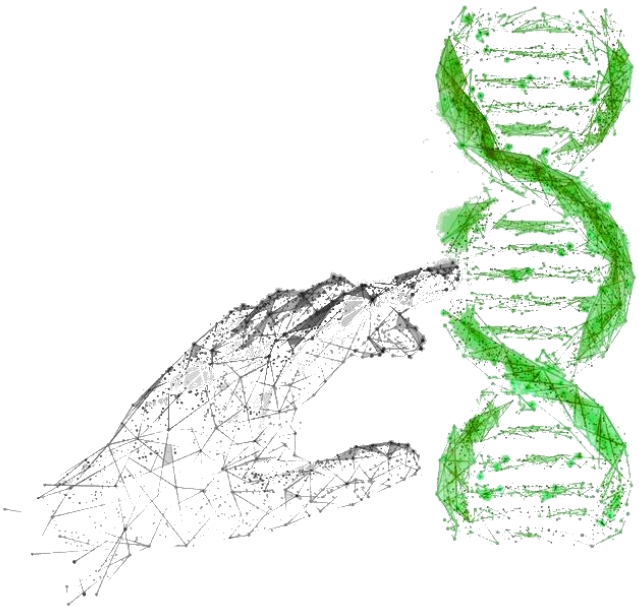
*F, forward primer; R, reverse primer, S, sequencing primer

Table S5. Primers used for Cas12a-crRNA gene editing analysis.

Gene	Primer*	Primer sequence (5'→3')	Purpose
NbFT	D3667 (F)	CTAGAAAACCTATGGCTATAAGGG	Amplification of a 550-bp DNA fragment flanking *NbFT* target region
	D3668 (R)	GTTCTCGAGAGGTATAATATAGGC	Amplification of a 550-bp DNA fragment flanking *NbFT* target region
	D3669	CACAAGCACGCATAGAAC	Sequencing of the 550-bp PCR product for the analysis of *NbFT* editing
NbXT1	JO16 JUN05 (F)	AACCACTTTTCCTCGTCGGAAA	Amplification of a 1286-bp DNA fragment flanking *NbXT1* target region
	JO16 JUN06 (R)	TAACTATTCAACTAAAGCTTCAAACAG	Amplification of a 1286-bp DNA fragment flanking *NbXT1* target region
	JO16 NOV05	TGTTTAATGAAGATTGTCTGG	Sequencing of the 1286-bp PCR product for the analysis of *NbXT1* editing

*F, forward primer; R, reverse primer

GENERAL DISCUSSION



Manipulation of the genetic pool in plants is essential to decipher gene function and tailor agronomic traits for developing novel crop varieties. The recent emergence of CRISPR-Cas technology has revolutionized genome editing due to its unique features such as design simplicity, low cost, versatility, and reproducibility. Nonetheless, the successful application of this system in plants is not free of challenges, being the main one the proper delivery of Cas nuclease and the sgRNA. Conventional delivery approaches like agroinoculation and particle bombardment are frequently associated with unpredicted genomic changes, labor-intensive breeding cycles for foreign DNA segregation, and regulatory restrictions involving transgenesis (Voytas and Gao, 2014; Jupe *et al.*, 2019; Liu *et al.*, 2019) (**Introduction, Figure 4**). Recent studies have demonstrated that plant viruses can be used for the transient delivery of CRISPR-Cas reaction components into plant cells, producing a high editing rate without the incorporation of foreign genetic material in the host (Oh *et al.*, 2021; Varanda *et al.*, 2021) (**Introduction, Table 1**). However, the fact that each viral vector can only infect specific host species poses a great limitation to a broad application of VIGE in plant genome editing. The work developed here aims to contribute to the enrichment of the current VIGE toolbox by engineering plant RNA viruses not proven to date as delivery vectors of CRISPR-Cas reaction components. In the following lines, a discussion is presented about the contributions of the present work to expanding the use of viral vectors for genome editing, as well as the influence of virus biological properties on CRISPR-Cas functioning. Finally, a critical vision is provided on certain ethical and social considerations that VIGE will have to deal with for future applications.

Do not underestimate the power of the little ones: sgRNA engineering is key for efficient genome editing

As a first attempt to expand the VIGE toolbox, in **Chapter I** we engineered a PVX-based sgRNA delivery vector for targeted editing in Cas9-expressing *N. benthamiana*. A

previous study suggested that the host endogenous tRNA-processing machinery could efficiently cleave sgRNA arrays delivered via *A. tumefaciens*, thus avoiding nucleotide overhangs proximal to the protospacer or the scaffold that would lower Cas9 activity *in planta* (Xie *et al.*, 2015). Nevertheless, the presence of long sgRNA overhangs does not negatively affect editing efficiency in our PVX vector, as we observe indel percentages up to 80% regardless of the addition of tRNA sequences (**Chapter I, Figure 2**). What is more, we demonstrate that unspaced tandem sgRNA arrays can be simultaneously delivered from a single PVX genome resulting in a highly efficient multiplex editing (**Chapter I, Figure 3**). As the permissiveness to sgRNA overhangs has only been documented in a few VIGE reports (Cody *et al.*, 2017; Ali *et al.*, 2018; Ellison *et al.*, 2020), we hypothesize that the virus, as a result of imperfect replication or partial RNA degradation, produces a relatively large population of subgenomic sgRNA fragments some of which, by chance, contain the correct 5´ ends for Cas9 activity. This influence of viral vectors in native sgRNA processing will need further research to unravel additional mechanisms that may have a role in this event. Alternatively, extra nucleotide extension at the 5' and 3' may not be that important as previously suggested or, at least, the negative effect is compensated by the large accumulation of imperfectly processed RNAs that result from viral replication.

Even though viral-based sgRNA delivery systems have proven to be efficient for genome editing, the incapacity of most viruses to enter the shoot apical meristem of the host limits modifications exclusively to somatic cells (Qu *et al.*, 2005; Schwach *et al.*, 2005). This means that the inheritance of targeted modifications depends on tissue culture, which underlays several inconveniences: (i) it is a laborious and time-consuming process; (ii) tissue regeneration only works with limited species and genotypes; (iii) some species of agronomic interest are recalcitrant to regeneration; and (iv) it may provoke unintended genomic or epigenomic changes (Altpeter *et al.*, 2016). Hence, it is essential that the editing machinery reaches the germline so that mutations present in parental

plants will be inherited by the offspring. One alternative to improve the access of the sgRNA to the germline relies on using plant endogenous mobile RNA sequences such as *Flowering Locus T* (*FT*) mRNA. In the plant, *FT* is transcribed in leaf vascular tissue and then moves to apical meristems to induce flowering (Notaguchi *et al.*, 2015). By fusing an *FT* mRNA fragment to the 3' end of the sgRNA, Ellison *et al.* (2020) were able to induce germline editing that resulted in heritable biallelic mutations with no evidence of virus transmission to progeny (**Introduction, Figure 5b**). By mimicking this strategy in our PVX vector, we obtained progeny with heterozygous and biallelic mutations in two independent target genes (**Chapter I, Figure 6**). These results were the first evidence confirming that the FT approach can be expanded to other viral vectors, thus emphasizing its robustness to achieve a rapid heritable gene editing bypassing tissue culture (Lei *et al.*, 2021; Li *et al.*, 2021). However, heritable editing at multiple loci seems tricky due to virus-related peculiarities. For the TRV-based vector developed by Ellison *et al.* (2020), unspaced tandem arrays of mobile sgRNA showed a similar editing efficiency to that of single sgRNAs, and resulted in the recovery of progeny with mutations at multiple target sites. Although multiplex heritable editing has been reported by co-inoculating a pool of viruses carrying mobile sgRNAs targeting different loci (Li *et al.*, 2021), this strategy is largely hindered by superinfection exclusion (Zhang *et al.*, 2018). This last finding highlights that the biological properties of a viral vector need to be considered when deciding the editing strategy to be followed (i.e. single vs. multiplex).

A genome tailor needs sharp scissors:
fine-tuning the expression of the Cas nuclease

For a long time, virus-mediated expression of the Cas nuclease was considered challenging, because large foreign genes negatively affect the stability of viral vectors (Avesani *et al.*, 2007). The recent work by Ma *et al.* (2020) overcame this limitation by engineering the rhabdovirus SYNV to deliver the full CRISPR-Cas cassette systemically

and perform gene editing at the whole-plant level. However, rhabdoviruses have a narrow host range that hinders their wide-scale application in genome engineering. In addition, inoculation entails some complication due to minus polarity genome, because viral genome is complementary to the coding RNA strand. Instead, potyviruses can infect plants from diverse botanical families including agronomically relevant species. Former studies from our group demonstrated that TEV can be used for the expression of heterologous sequences with varying sizes (Bedoya *et al.*, 2010; Majer *et al.*, 2017; Martí *et al.*, 2020). In **Chapter II,** we used a modified TEV in which the RNA-dependent RNA polymerase was deleted (i.e. TEVΔNIb) to accommodate the large coding sequence of Cas12a nuclease. By combining this TEV with the PVX-sgRNA delivery vector previously explained, we developed a novel two-virus system for targeted gene editing in plants (**Chapter II, Figures 1** and **2**). To our knowledge, this is the first report that demonstrates the utility of two compatible viral vectors to achieve efficient genome editing in plants. In contrast, other studies focused on embedding all CRISPR-Cas reaction components within a single viral vector (Gao *et al.*, 2019; Ma *et al.*, 2020; Ariga *et al.*, 2020). The use of Cas12a and its derived unique properties compared to that of Cas9 also opens new opportunities for plant genome engineering. What is more, our work highlights the flexibility of PVX for the delivery of single or multiple guide RNAs regardless the nature of the Cas nuclease.

Potyviruses are known to establish synergistic interactions with a broad range of unrelated viruses, resulting in the aggravation of disease symptoms and the accumulation of the non-potyviral partner (Pruss *et al.*, 1997; Shi *et al.*, 1997). In potexvirus/potyviral synergisms, this effect is mediated by the expression of the HC-Pro RNA silencing suppressor encoded close to the 5' terminal end of the potyviral genome. Viral RNA silencing suppressors stimulate virus-mediated expression of heterologous genes, as this may also be useful in VIGE. An increase in gene editing was observed when the potyvirus TuMV was co-inoculated with a FoMV vector carrying CRISPR-Cas reaction components

(Mei *et al.*, 2019). A similar synergistic interaction might be happening in our TEV-PVX system that stimulates PVX replication and, consequently, promotes sgRNA accumulation. We believe that this could partially explain the high editing values obtained through the two-virus system (**Chapter II, Figures 1d** and **1f**).

Motivated by the promising results of our two-virus delivery system, our next step was to perform DNA-free editing to minimize legal and ethical issues regarding transgenic plants. PVX was then reengineered for the simultaneous expression of potyviral NIb as well as the guide RNA, resulting in genome editing of wild-type plants (**Chapter II, Figures 3** and **4**). However, we observed a reduction of indel percentages possibly due to a weaker activity of NIb when supplied by PVX compared to constitutive *in planta* expression, which in turn limits the infectivity of TEVΔNIb and Cas12a levels (**Chapter II, Figure 3c**). A second option for DNA-free editing would be to co-express viral suppressors that inhibit host defense mechanisms, which has been shown to enhance the infectivity of viral vectors carrying CRISPR-Cas reaction components. Indeed, several reports documented that the p19 suppressor from TBSV significantly increased Cas9 levels in young tissues, thus promoting genome editing (Mao *et al.*, 2018; Mei *et al.*, 2019). Application of this strategy with a full-length TEV carrying Cas12a coding sequence did not work in our case, thus reinforcing the fact that each plant virus establishes specific interactions with the host that lead to different effects.

Transforming the "bad guy" into a good one:
a potential application of viral vectors in crop protection

CRISPR-Cas technology holds a very promising future for protecting crops against biotic stress by creating "custom-model" plants. Plant viruses are responsible for nearly half of all plant diseases, leading to massive losses in agricultural production every year (Zaidi *et al.*, 2016). Initial studies on the use of genome editing for disease resistance

focused on disrupting essential viral replication genes to inhibit virus spread (**Figure 1, left panel**) (Baltes *et al.*, 2014; Baltes *et al.*, 2015; Ji *et al.*, 2015; Ali *et al.*, 2016). Nevertheless, such an approach is limited exclusively to diseases caused by DNA viruses due to the inability of Cas9 to cut other nucleic acids. Aiming to achieve resistance against RNA viruses, researchers started to target host genes including (**Figure 1, right panel**): (i) receptors located on the plant cell surface that recognize pathogen effectors and mediate its entry into the host cell; or (ii) negative regulators of immunity and host proteins involved in plant developmental pathways (i.e. susceptibility factors) that, when controlled by the pathogen, lead to the suppression of defense mechanisms. Host receptors tend to have a narrow recognition spectrum, and pathogens can get easily adapted to new 'resistant' varieties by effector diversification (Dodds and Rathjen, 2010). Besides, targeted editing of susceptibility factors has the potential to generate a long-lasting resistance in the field due to its recessive nature (Hashimoto *et al.*, 2016; Garcia-Ruiz, 2018). Viral vectors like the two systems developed throughout this thesis can be particularly useful to obtain foreign DNA-free edited plants by transient expression of the CRISPR-Cas machinery. However, it is important to consult the literature when choosing the susceptibility factor to be edited, as it may compromise essential developmental or physiological processes within the plant.

Nevertheless, the use of viruses in biotechnological applications comprises several biosafety implications. Gene drive based on CRISPR-Cas technology can efficiently spread the desired genetic elements through populations via sexual reproduction. This technique could be used to eliminate invasive species including pests and weeds or to introduce new traits into existing populations. However, the dispersion of modified viruses by insect vectors could cause massive gene insertion events, or it could even be used for developing insect-delivered bioweapons. Social responsibility must prevail when using genetically altered organisms that might have an advantage over native individuals. To minimize such concerns, essential to use viruses with identified biological vectors or

known virus-insect molecular interactions. The viruses used in our work can be considered biosafe vectors for various reasons. PVX is exclusively transmitted by mechanical contact between plants, so physical barriers should be enough to avoid its propagation from experimental fields to nature (Adams *et al.*, 2004). On the other hand, TEVΔNIb infectivity depends on the supplementation of the potyviral NIb either by a transgenic plant or an additional recombinant virus, which prevents the infection of wild-type individuals. In any case, these biotechnological tools must be used under proper confinement and in authorized installations.

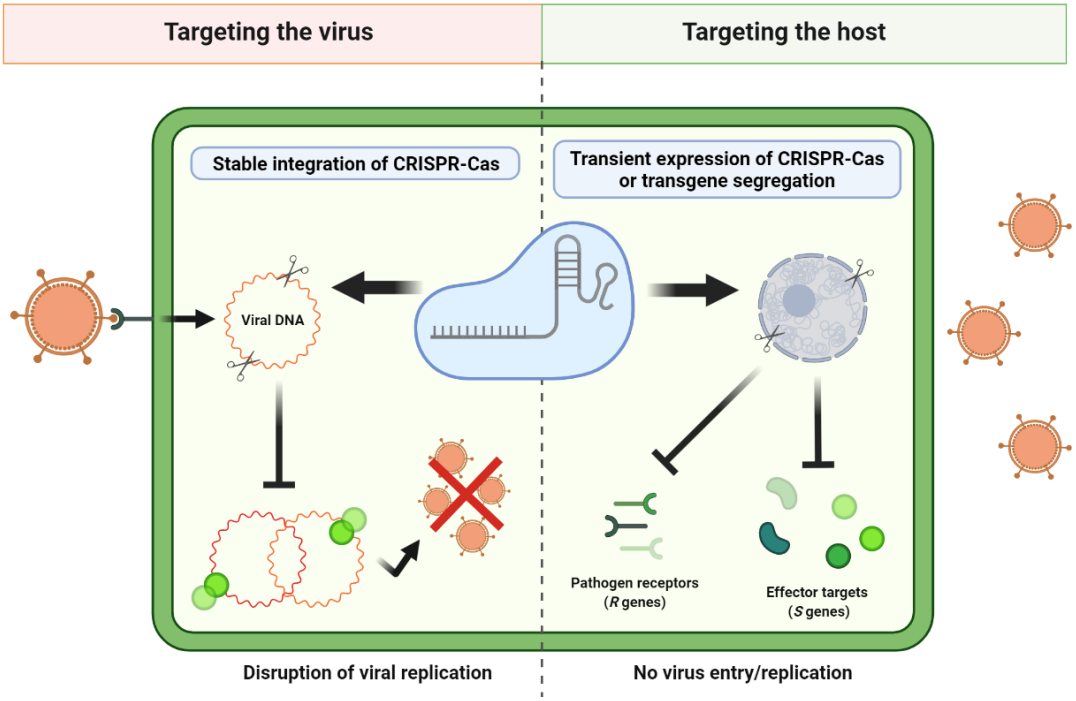


Figure 1. Development of CRISPR-Cas mediated virus resistance in plants. Protection against DNA viruses can be obtained by disruption of essential viral replication genes, which in turn inhibits virus spread (left). The CRISPR-Cas machinery needs to be stably integrated into the plant genome to edit the pathogen's genome whenever it enters the host. Protection against either DNA or RNA viruses can be obtained by editing either receptors (*R* genes) located on the plant cell surface that recognize the pathogen and trigger defense, or host proteins (*S* genes) that are recruited by the pathogen to complete the infection cycle (right). When host genes are targeted, the CRISPR-Cas transgene can be transiently expressed or segregated to obtain non-transgenic edited plants. Adapted from Khatodia *et al.* (2017).

Not everything is a bed of roses:

genome editing technologies still need improvements

As previously mentioned, the main drawback of VIGE is the limited cargo capacity of viral vectors. Recently characterized hypercompact effectors such as CasΦ (Pausch *et al.*, 2020) and CasMINI (Xu *et al.*, 2021) hold great potential for their delivery via viral vectors without affecting their infectivity. Both nucleases have been reported to generate genome editing levels comparable to that of Cas9 or Cas12a in mammalian cells. Aiming to explore the applicability of CasΦ and CasMINI in plants, we followed the two-virus system strategy in **Chapter 2**, but unfortunately, we were unable to observe gene editing at all. These new Cas effectors probably have a strong dependency on the sgRNA targeting sites, as it has been observed for other nucleases like Cas12a (Zetsche *et al.*, 2015). Hence, future work such as genome-wide screening analysis is needed to elucidate the principles for an efficient sgRNA design on hypercompact editing systems.

Despite the many algorithms developed for sgRNA design, there is still unexplained variability in observed efficiencies. The latest evidence suggests that chromatin state, that is, the way DNA is modified and packaged in the cell nucleus, can significantly influence Cas9 activity (**Figure 2)**. Understanding such a role in genome engineering is especially relevant when chromatin states differ between experimental models and potential applications. One of the best-studied factors influencing heterochromatin-euchromatin differences is DNA methylation. Even though it does not seem to have a direct effect on Cas9 activity (Hsu *et al.*, 2013; Fujita *et al.*, 2016), the presence of 5-methylcytosine can stimulate the recruitment of methyl-binding proteins and chromatin remodelers. Indeed, several studies have documented that nucleosomes show a robust ability to block functional Cas9 binding, albeit access can be gained upon natural remodeling and unwrapping of nucleosomal DNA (Horlbeck *et al.*, 2016; Isaac *et al.*, 2016). A more extensive investigation based on allele-specific chromatin states suggested that

heterochromatin can significantly hinder Cas9 activity, especially under suboptimal conditions such as low nuclease expression or partial sgRNA mismatches (Kallimasioti-Pazi *et al.*, 2018). Besides, transcription has been shown to promote gene editing, since cut ends are more exposed for error-prone repair when Cas9 is dislodged by the RNA polymerase (Clarke *et al.*, 2018).

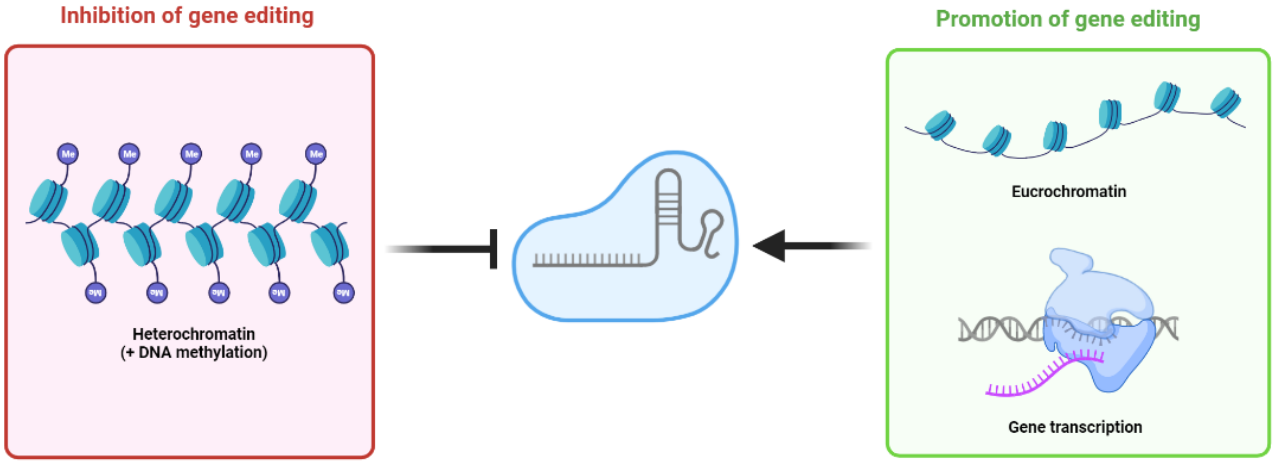


Figure 2. Factors affecting chromatin state and their influence on CRISPR-Cas functioning. Heterochromatin is consisted of densely packed nucleosomes and blocks Cas9 binding to the target loci. DNA methylation increases heterochromatin formation by stimulating the recruitment of nucleosomes and metal-bonding proteins. However, chromatin segments can be unpacked upon in vivo nucleosome remodeling, which promotes Cas9 access to target cleavage. Gene editing is also promoted during transcription since Cas9 dislodging by the RNA polymerase exposes cut ends for error-prone repair. Purple circles represent methyl groups at histone tails and are not in scale. Adapted from Verkuijl and Rots (2019).

Beyond gene knock-out, the greatest potential of CRISPR-Cas technology lies in HDR-mediated modifications of the target gene. In plant cells, HDR frequency is low because it requires simultaneous induction of DSBs along with the delivery of large amounts of repair templates. This mechanism can be promoted by manipulating the expression of critical proteins involved in DNA repair pathways, as it has been reported in mammals (Mateos-Gomez *et al.*, 2015; McVey *et al.*, 2016). Additionally, geminivirus vectors have been shown to deliver high quantities of repair templates that result in

efficient gene-targeted DNA integration (Baltes *et al.*, 2014; Butler *et al.*, 2016; Gil-Humanes *et al.*, 2017; Dahan-Meir *et al.*, 2018). However, the capacity for systemic movement is lost when large heterologous sequences are inserted into the viral genome, so further research is needed to develop DNA virus vectors able to perform HDR-mediated gene knock-in at whole plant level.

Off-target events lead to modifications of unintended loci on account of sgRNA mismatches and continuous expression of Cas nuclease. Even though this phenomenon is considerably less frequent in plants compared to other organisms (Feng *et al.*, 2014; Zhang *et al.*, 2015; Tang *et al.*, 2018; Liu *et al.*, 2021), it can cause phenotypic or metabolic effects wrongly associated with modifications of the target gene. An advantage of VIGE over conventional CRISPR-Cas strategies is that the viral vector can be deployed when editing negatively affects plant fitness, as editing occurs before the onset of severe physiological effects. Moreover, the restriction of CRISPR-Cas machinery only to plant tissues invaded by the virus may contribute to decreasing off-target activities (Ji *et al.*, 2018). Anyway, off-targeting in plant biotechnology seems to be more of an academic concern than a real issue. Outcrossing to other varieties is a routine during product development and could be used to eliminate off-target effects before commercialization. Additionally, gene editing can be temporarily controlled by using phage-derived inhibitors of CRISPR-Cas immunity known as anti-CRISPR proteins (Acr) (Borges *et al.*, 2017; Trasanidou *et al.*, 2019; Marino *et al.*, 2020). To inactivate CRISPR-Cas systems, Acr proteins can either (i) directly interact with a Cas nuclease to prevent DNA binding, crRNA loading or DNA cleavage; or (ii) interfere with effector-complex formation. Recently, Calvache *et al.* (2021) successfully demonstrated the potential of Acrs for post-transcriptional regulation of CRISPR-Cas systems in plants. Moreover, it was proven that viral-mediated delivery of Acrs completely abolished the high gene editing levels obtained via the PVX::sgRNA system.

Looking into the crystal ball:
what is next about virus-mediated gene editing

When plant viruses were first used as heterologous gene expression vectors more than thirty years ago, the scientific community could not imagine the diversity of applications they would have. Throughout this thesis, we have focused on the potential of viruses for CRISPR-Cas-mediated gene editing. Their capacity to replicate and systemically spread through the whole plant leads to fast and highly efficient genome editing, constituting an ideal screening tool to assess the effectiveness of sgRNAs and analyze the phenotypic effects of editing. If technical hurdles are soon overcome, such as achieving an effective nuclease delivery or increasing the frequency of HDR-mediated gene knock-in, the application of VIGE will broaden across crop species. At the disposal of plant breeding, farmers will have the power to create crops *a la carte* that meet consumer's expectations. Our understanding of plant viruses as biotechnological tools is about to change, so let's not limit our imagination and just keep working.

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CONCLUSIONS

1. *Potato virus X* (PVX) can be engineered to deliver unspaced guide RNA arrays in the model plant species *N. benthamiana*, achieving highly efficient genome editing (nearly 80% indels). Indel formation lowers in the multiplexing strategy compared to single sgRNA delivery.

2. Virus-free edited progeny with a high rate of heritable biallelic mutations can be obtained either from plants regenerated from infected tissue, or infected plant seeds. This last approach offers a simple and rapid strategy to obtain edited plants bypassing tissue culture.

3. A two-virus vector system composed by the potyvirus *Tobacco etch virus* (TEV) and the potexvirus PVX can simultaneously express all CRISPR-Cas reaction components (i.e. Cas12a nuclease and guide RNA, respectively). These two viruses replicate in the same cells and allow transformation-free genome editing in *N. benthamiana*.

4. PVX is suitable for the delivery of unspaced guide RNA arrays regardless of the nature of the nuclease (i.e. Cas9 versus Cas12a), which highlights its usefulness in a variety of editing approaches.

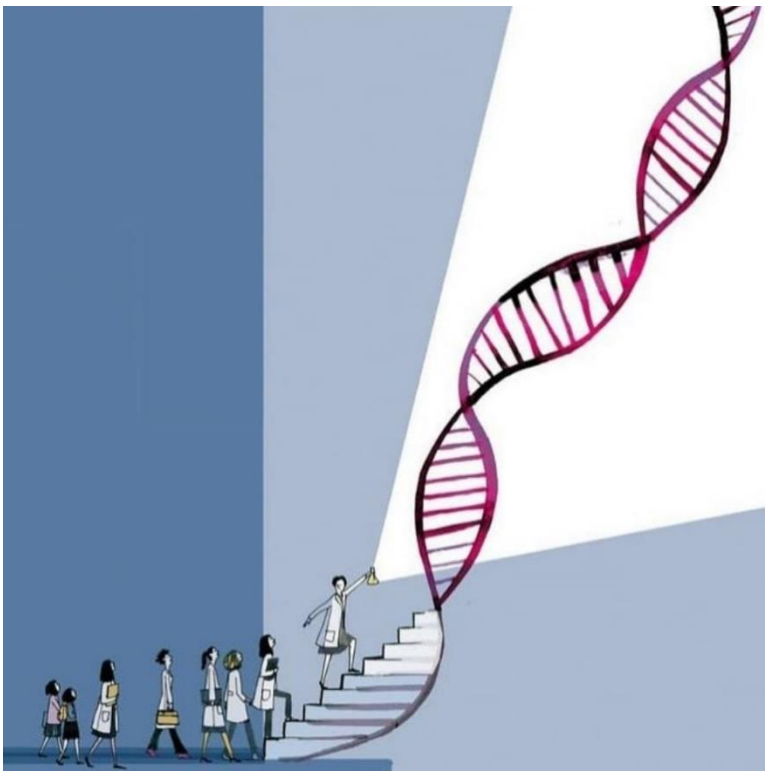
5. A single PVX vector can supply potyviral NIb activity for the replication of a defective TEV, as well as deliver the guide RNA for genome editing in wild-type *N. benthamiana*. However, indel production is reduced possibly due to a weaker activity of NIb compared to its constitutive *in planta* expression.

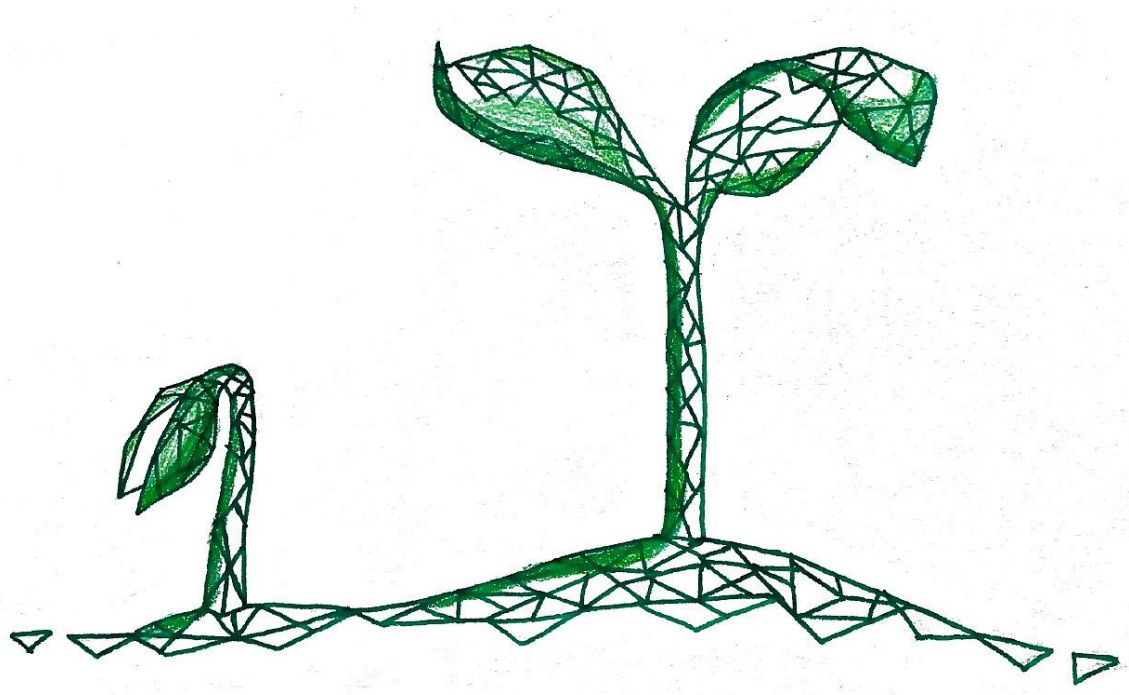
6. The wide host range that both TEV and PVX show, particularly in the family Solanaceae, highlights their potential for future applications in VIGE-mediated functional genomics studies and crop improvement.

"Una investigación básica de calidad es fundamental para un posterior desarrollo, porque de ella saldrán resultados no previsibles a priori"

"La mujer ocupará en el mundo científico el puesto que le corresponda de acuerdo con su capacidad, sin necesidad de cuotas"

— Margarita Salas





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