

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Advancing the application of viral vectors for CRISPR/Cas genome editing and virus-induced gene silencing in plants

PhD Thesis

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Valencia, November 2025







PhD in Biotechnology

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CERTIFICA:

Que D. Arcadio García Pérez, Graduado en Biotecnología por la Universitat Politècnica de València, ha realizado bajo su dirección el trabajo con título "Advancing the application of viral vectors for CRISPR/Cas genome editing and virus-induced gene silencing in plants" que presenta para optar al grado de Doctor en Biotecnología por la Universitat Politècnica de València.

Y para que así conste a los efectos oportunos, firma el presente certificado en Valencia a 27 de noviembre de 2025.

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Summary

The considerable increase in the world population, coupled with the effects of climate change, highlights the urgent need to increase agricultural production and productivity worldwide. To this end, humans have at their disposal a wide range of biotechnological tools that allow them to modify crops almost at will. Among these tools, CRISPR/Cas technology stands out. This system is derived from a natural defence mechanism present in bacteria and archaea that has been adapted for genome editing in different organisms, including plants. This valuable tool can be combined with the use of viral vectors to express the necessary components for gene editing in plants. The overall objective of this doctoral thesis has been to delve deeper into the use of viral vectors and their application in the field of plant genome editing (using the CRISPR/Cas system), as well as in virus-induced gene silencing (VIGS), a tool widely used in studies of functional genomics.

To achieve this goal, a vector derived from potato virus X (PVX) was first developed to express guide RNAs (gRNAs) in Micro-Tom tomato plants that constitutively expressed the SpCas9 nuclease. Using this system, editing efficiencies of up to 46% were achieved in systemic tissue. Solid mutants were regenerated from infected tissue, and once the progeny of these plants had been analysed, the heritability of the mutations generated was confirmed. These results were also transferred to traditional tomato cultivars.

With the aim of expanding the range of viral vectors used to express editing components in plants, a vector derived from tobacco etch virus (TEV) was developed to express gRNAs in *Nicotiana benthamiana* plants that constitutively expressed the LbCas12a nuclease. In this case, edited plants were obtained in the next generation after self-pollinating the inoculated plants, without the need of tissue culture regeneration. The validity of this viral vector for expressing gRNAs and inducing somatic mutations was also verified in crops of agronomical interest, such as tomato and tobacco. In this way, gRNAs were successfully expressed in plants for the first time using a potyviral vector.

VIGE technology has recently been extended to a large number of crops of agronomical interest, although it has not yet been possible to use it in a crop of economic importance, such as melon. Therefore, in this Thesis, we developed a platform that combines stable transformation mediated by *Agrobacterium tumefaciens* with the expression of gRNAs using viral vectors in different melon varieties. Although no editing events were achieved, certain advances have been made in this platform, and transgenic plants with a relatively high transformation efficiency (0.95%) have been obtained.

With the aim of continuing to exploit and improve the well-known VIGS methodology, a silencing strategy was developed using a vector derived from tobacco rattle virus (TRV). The size of the insert homologous to the gene to be silenced was minimised, thereby simplifying the process of cloning and obtaining the viral vector. It was demonstrated that it is possible to produce gene silencing that leads to phenotypic alterations in *N. benthamiana* plants using inserts of only 24 nt. In addition, it was verified that the system developed is transferable to species of agronomical interest such as tomato and scarlet eggplant (*Solanum aethiopicum*).

Overall, the advances made in this doctoral thesis expand the application of viral vectors in VIGE and VIGS techniques, contributing to functional genomics studies not only in model plants, but also in plants of agronomical interest.

Resumen

El considerable aumento de la población mundial, unido a los efectos causados por el cambio climático, ponen de manifiesto la urgente necesidad de incrementar la producción y la productividad agrícola a nivel mundial. Para ello, el ser humano tiene a su alcance una amplia gama de herramientas biotecnológicas, que permiten modificar los cultivos casi a nuestro antojo. Dentro de esta gama de herramientas, desataca la tecnología CRISPR/Cas, un sistema derivado de un mecanismo de defensa natural presente en bacterias y arqueas que ha sido adaptado para la edición genómica de diferentes organismos, entre los que se encuentran las plantas. Esta valiosa herramienta, puede combinarse con el uso de vectores virales para expresar en planta, los componentes necesarios para la edición. El objetivo general de esta tesis doctoral ha sido profundizar en el uso de vectores virales y su aplicación en el campo de la edición genómica vegetal (mediante el sistema CRISPR/Cas), así como en el campo del silenciamiento génico ("virus-induced gene silencing", VIGS), herramienta ampliamente utilizada en estudios de genómica funcional.

Para lograr este objetivo, primeramente, se desarrolló un vector derivado del virus X de la patata (PVX), para expresar guías de RNA (gRNA) en plantas de tomate del cultivar Micro-Tom que expresaban de manera constitutiva la nucleasa SpCas9. Con este sistema, se consiguió obtener porcentajes de edición de hasta un 46% en tejido sistémico. Se regeneraron mutantes sólidos a partir de tejido infectado, y una vez analizada la progenie de estas plantas, se confirmó que las mutaciones generadas podían heredarse. Estos resultados se pudieron transferir a cultivares tradicionales de tomate.

Con el objetivo de ampliar la gama de vectores virales utilizados para expresar los componentes de edición en planta, se desarrolló un vector derivado del virus del grabado del tabaco (TEV) para expresar gRNAs en plantas de *Nicotiana benthamiana* que expresaban de manera constitutiva la nucleasa LbCas12a. En este caso, se consiguieron plantas editadas en la siguiente generación tras autofecundar las plantas inoculadas, sin necesidad de recurrir al cultivo de tejidos. La validez de este vector viral para expresar gRNAs e inducir mutaciones somáticas, también fue contrastada en cultivos de interés agronómico como el tomate y el tabaco. De esta manera, se consiguieron

expresar por primera vez en planta los gRNAs mediante un vector derivado de un potyvirus.

La tecnología VIGE se ha ampliado recientemente a una gran cantidad de cultivos de interés agronómico, pese a que aún no ha podido ser empleada en un cultivo con importancia económica, como lo es el melón. Por ello, en este trabajo desarrollamos una plataforma que combina la transformación estable mediada por *Agrobacterium tumefaciens*, con la expresión de gRNAs por medio de vectores virales, en diferentes variedades de melón. Pese a no conseguir ningún evento de edición, se han hecho ciertos avances en esta plataforma, y se han conseguido obtener plantas transgénicas con una eficiencia de transformación relativamente alta (0.95%).

Con el propósito de seguir explotando y mejorando la conocida metodología de VIGS, se desarrolló una estrategia de silenciamiento utilizando un vector derivado del virus del cascabel del tabaco (TRV). Se consiguió minimizar el tamaño del inserto homólogo al gen a silenciar, simplificando de esta manera el proceso de clonación y obtención del vector viral. Se demostró que es posible producir silenciamiento génico que da lugar a alteraciones fenotípicas en plantas de *N. benthamiana* utilizando insertos de tan solo 24 nt. Además se verificó que el sistema desarrollado es transferible a especies de interés agronómico como el tomate y la berenjena escarlata (*Solanum aethiopicum*).

En conjunto, los avances realizados en esta tesis doctoral expanden la aplicación de los vectores virales en técnicas de edición genética (VIGE) y de silenciamiento génico (VIGS), para contribuir en estudios de genética funcional no solo en plantas modelo, sino también en plantas de interés agronómico.

Resum

El considerable augment de la població mundial, unit als efectes causats pel canvi climàtic, posen de manifest la urgent necessitat d'incrementar la producció i la productivitat agrícola a nivell mundial. Per a això, l'ésser humà té al seu abast una àmplia gamma de ferramentes biotecnològiques, que ens permeten modificar els cultius quasi al nostre antull. Dins d'esta gamma de ferramentes, desataca la tecnologia CRISPR/Cas, un sistema derivat d'un mecanisme de defensa natural present en bacteris i arqueges que ha sigut adaptat per a l'edició genòmica de diferents organismes, entre els quals es troben les plantes. Esta valuosa ferramenta, pot combinar-se amb l'ús de vectors virals per a expressar en planta, els components necessaris per a l'edició. L'objectiu general d'esta tesi doctoral ha sigut aprofundir en l'ús de vectors virals i la seua aplicació en el camp de l'edició genòmica vegetal (mitjançant el sistema CRISPR/Cas), així com en el camp del silenciament gènic ("virus-induced gene silencing", VIGS), ferramenta àmpliament utilitzada en estudis de genòmica funcional.

Per a aconseguir este objectiu, primerament, es va desenrotllar un vector derivat del virus X de la creïlla (PVX), per a expressar guies d'RNA (gRNA) en plantes de tomaca del cultivar Micro-Tom que expressaven de manera constitutiva la nucleasa SpCas9. Amb este sistema, es va aconseguir obtindre percentatges d'edició de fins a un 46% en teixit sistèmic. Es van regenerar mutants sòlids a partir de teixit infectat, i una vegada analitzada la progènie d'estes plantes, es va confirmar la heredabilidad de les mutacions generades. Estos resultats es van poder transferir a cultivars tradicionals de tomaca.

Amb l'objectiu d'ampliar la gamma de vectors virals utilitzats per a expressar els components d'edició en planta, es va desenrotllar un vector derivat del virus del gravat del tabac (TEV) per a expressar gRNAs en plantes de *Nicotiana benthamiana* que expressaven de manera consitutiva la nucleasa LbCas12a. En este cas, es van aconseguir plantes editades en la següent generació després d'autofecundar les plantes inoculades, sense necessitat de recórrer al cultiu de teixits. La validesa d'este vector viral per a expressar gRNAs i induir mutacions somàtiques, també va ser contrastada en cultius d'interés agronòmic com la

tomaca i el tabac. D'esta manera, es van aconseguir expressar per primera vegada en planta els gRNAs mitjançant un vector derivat d'un potyvirus.

La tecnologia VIGE s'ha ampliat recentment a una gran quantitat de cultius d'interés agronòmic, malgrat que encara no ha pogut ser emprada en un cultiu amb importància econòmica, com ho és el meló. Per això, en este treball hem desenrotllat una plataforma que combinara la transformació estable mediada per *Agrobacterium tumefaciens*, amb l'expressió de gRNAs per mitjà de vectors virals, en diferents varietats de meló. Malgrat no aconseguir cap esdeveniment d'edició, s'han fet uns certs avanços en esta plataforma, i s'han aconseguit obtindre plantes transgèniques amb una eficiència de transformació relativament alta (0.95%).

Amb el propòsit de continuar explotant i millorant la coneguda metodologia de VIGS, es va desenrotllar una estratègia de silenciament utilitzant un vector derivat del virus del cascavell del tabac (TRV). Es va aconseguir minimitzar la grandària de la inserció homòloga al gen a silenciar, simplificant d'esta manera el procés de clonació i obtenció del vector viral. Es va demostrar que és possible produir silenciament gènic que dona lloc a alteracions fenotípiques en plantes de *N. benthamiana* utilitzant insercions de tan sols 24 nt. A més es va verificar que el sistema desenrotllat és transferible a espècies d'interés agronòmic com la tomaca i l'albergínia escarlata (*Solanum aethiopicum*).

En conjunt, els avanços realitzats en esta Tesi doctoral amplien l'aplicació dels vectors virals en tècniques d'edició genètica (VIGE) i de silenciament gènic (VIGS), per a contribuir en estudis de genètica funcional no sols en plantes model, sinó també en plantes d'interés agronòmic.

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Abbreviations

Viruses

ACMV: African cassava mosaic virus
ALSV: apple latent spherical virus
BeYDV: bean yellow dwarf virus
BNYVV: beet necrotic yellow vein virus
BSMV: barley stripe mosaic virus
BYSMV: barley yellow striate mosaic virus
FoMV: foxtail mosaic virus
PEBV: pea early browning virus
PVX: potato virus X
SYNV: sonchus yellow net virus
TEV: tobacco etch virus
TMV: tobacco mosaic virus
ToMV: tomato mosaic virus
TRV: tobacco rattle virus
TSWV: tomato spotted wilt virus

Others

aa: amino acid
ABE: adenine base editor
AGO: Argonaute
CBE: cytosine base editor
CGBE: cytosine to guanine base editor
CRISPR: clustered regularly interspaced short palindromic repeats
CRISPRa: CRISPR activation
CRISPRi: CRISPR interference
crRNA: CRISPR RNA
dCas: dead Cas
DCL: Dicer-Like
DNA: deoxyribonucleic acid
dpi: days post inoculation
ds: double-stranded
DSB: double-stranded break
gRNA: guide RNA
GVR: geminiviral replicon
HDR: homology-directed repair
nCas9: Cas9 nickase
NHEJ: non-homologous end joining
nt: nucelotides
pegRNA: prime editing guide RNA
PTGS: posttranscriptional gene silencing
RISC: RNA-induced silencing complex
RNA: ribonucleic acid
RNAi: RNA interference
RNP: ribonucleoprotein
sgRNA: single guide RNA
siRNA: small interfering RNA
ss: single-stranded
TGS: transcriptional gene silencing
Ti: tumor-induced
tracrRNA: trans-activating crRNA
VIGE: virus-induced genome editing
VIGS: virus-induced gene silencing
vsRNA: virus-derived small RNA

General introduction

CRISPR/Cas technology used for gene editing purposes

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas; CRISPR/Cas) nucleases is a novel system that has revolutionized the world of biotechnology, due to its applicability in the field of genetic engineering. The basis of its functioning comes from the natural system present in most bacteria and archaea, which acts as a defence mechanism to phages that invade the prokaryotic cell (Barrangou et al., 2007; Le Rhun et al., 2019). Firstly named as short regularly spaced repeats (SRSR) (Mojica et al., 2000), CRISPR systems consist of invader DNA fragments ranging from 24 to 40 nucleotides (nt) (Mojica et al., 2005), acquired over time and sorted in repetitive arrays, whose transcription and processing gives rise to CRISPR RNAs (crRNAs). These molecules behave as guides that target the invader DNA, leading to its destruction by means of Cas nucleases (Pacesa et al., 2024).

The adaptation of this natural system for genomic engineering purposes derives from the capability of one of its components (Cas nuclease), to generate double-stranded DNA breaks (DSB) at targeted positions. Of the wide range of Cas proteins used today in biotechnology, each with its characteristics and properties, the Cas9 nuclease, originating from *Streptococcus pyogenes*, stands out for its remarkable activity and specificity. This was the first nuclease engineered to be used in genome editing, and remains the most widely used these days (Knott and Doudna, 2018; Pacesa et al., 2024). CRISPR/Cas9 constructs used for gene editing purposes are composed of different regions. The main part is a single guide RNA (sgRNA) which joins to the endonuclease protein Cas9 and targets a specific sequence of DNA. The sgRNA is composed of two RNA segments. Firstly, the CRISPR RNA (crRNA), which presents a 17-20 nucleotide region located in its 5´ end that binds to the target region by base complementarity (Borrelli et al., 2018), and is the responsible of specific target recognition (Jinek et al., 2012). Secondly, trans-activating crRNA (tracrRNA), which is base-paired to crRNA (**Figure 1**). This tracrRNA is required for the housekeeping RNase III to cleave the pre-crRNA, that will finally give rise to the mature and active crRNA (Charpentier et al., 2015). When the targeted DNA region is recognized by the crRNA, the endonuclease Cas9 creates a DSB. This break can be repaired by non-homologous end joining (NHEJ), which is usually

associated with the origination of small insertions and deletions (indels) (Rodgers and McVey, 2015). When a DNA template with homology to the DSB region is provided in the same CRISPR/Cas9 construct, another repair mechanism occurs (homology-directed repair, HDR) integrating the template in the genome (Zaidi et al., 2018). For this interaction to be effective, a protospacer adjacent motif (PAM) is needed. This short DNA sequence acts as a recognition signal for the CRISPR/Cas complex. In the case of Cas9 nuclease, this PAM region is composed of three nucleotides, usually NGG. This PAM sequence is juxtaposed to the complementary strand in the target DNA. The DSB is generated by Cas9 nuclease three nucleotides upstream of the PAM sequence (Jinek et al., 2012).

From the spectrum of nucleases adapted to be used in the field of genome editing, we can also highlight the endonuclease Cas12a, previously known as Cpf1, and smaller than Cas9 (Zetsche et al., 2015). The Cas12a nuclease is capable of processing the pre-crRNA into mature crRNA, because it has not only endonuclease activity (like Cas9), but also endoribonuclease activity (Charpentier et al., 2015; Fonfara et al., 2016). Therefore, to be functional, it does not need the presence of a tracrRNA, but a short repeat region, a direct repeat ranging from 19 to 21-nt that forms the 5´ region of the crRNA conforming a pseudoknot, composed of a stem and a loop region (Bandyopadhyay et al., 2020; Paul and Montoya, 2020) (**Figure 1**). This self-processing capacity makes it an ideal nuclease for expressing several crRNAs at the same time (multiplexing), and thus editing several genes at once. Cas12a cleaves both DNA strands, generating staggered ends, with 4 or 5-nt at PAM distal part. This endonuclease requires a T rich PAM (TTTV, being 'V' a A, C, or G) (Zetsche et al., 2015).

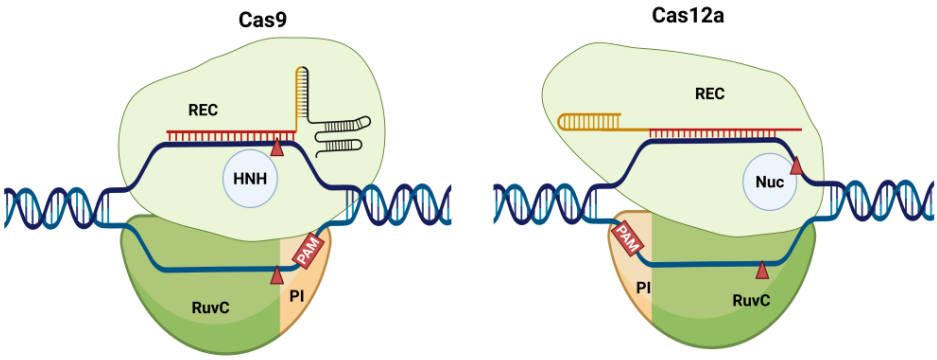


Figure 1. Schematic representation of the main CRISPR/Cas systems used in biotechnology in their native forms. CRISPR/Cas9 system (left) requires for its proper functioning the combination of an effector Cas9 protein and two small RNAs. The crRNA, which is represented in red and orange, and the tracrRNA (in black). The 20-nt guide (spacer) located at the 5´ end of the crRNA (red) mediates the DNA binding to the target site. The combination of the crRNA and the tracrRNA is known as sgRNA. Cas9 generates blunt-ended DSB, 3 bp upstream of the PAM sequence (NGG). The recognition of the target-sgRNA complex is mediated by the recognition lobe (REC), while the recognition of the PAM sequence is mediated by the PAM interacting (PI) domain. The cleavages of the DNA strains are performed by the nuclease domains; HNH in the target strain, and RuvC in the complementary strain. CRISPR/Cas12a system (right) requires for its functioning the combination of an effector Cas12a protein and a crRNA molecule (red and orange sequences). The recognition of the target DNA sequence takes place downstream of the PAM (TTTV), and it is mediated by the 23-25-nt guide (spacer) located at the 3´ end of the crRNA (displayed in red). Cas12a nuclease generates staggered-ended DSB in the distal region of the PAM site. As in the case of Cas9, the REC lobe mediates the recognition of the target-crRNA complex, and the PI domain recognizes the PAM sequence. The nuclease domains of Cas12a are the Nuc domain, which cleaves the target strand at the position 18 or 19 downstream the PAM (depending on the nuclease) and the RuvC domain, which cleaves the complementary strand 23-nt downstream the PAM. Cutting sites are represented by red triangles in both nucleases. Adapted from Schindele et al., 2018. Figures of this introduction were created with Biorender (https://biorender.com/).

Taking the previous into account, the use of CRISPR/Cas genome editing is limited by the presence of a specific PAM in the target region of the gene to be edited. The SpCas9 nuclease, which requires an NGG PAM, is the most widely used nuclease currently in the field of genome editing. Theoretically, it has been calculated that a target site could be obtained every 8 nucleotides. Even so, there are many regions of the genome that present sequences with a high A/T content, which makes it difficult to find a suitable target site and, consequently, to use the SpCas9 nuclease to edit those regions (Pacesa et al., 2024). To overcome this situation, researchers have tried to identify different Cas9 orthologs, with different PAM sites. SaCas9, from *Staphylococcus aureus* is more than 300 amino acids shorter in size, and presents a similar editing efficiency to SpCas9 in mouse cells (Ran et al., 2015). However, this nuclease requires a NNGRR PAM (being R a G or A), which is more specific, and so, more difficult to find than that required by SpCas9. Another ortholog that was proved to have a similar editing efficiency than SpCas9 in human cells is NmCas9, from *Neisseria meningitidis* (Esvelt et al., 2013), which requires a very specific PAM as well (NNNNGATT) (Zhang et al., 2013). In addition, while being 28% shorter than SpCas9 in amino acid terms, CjCas9 from *Campylobacter jejuni* does also have very specific requirements for its PAM sequence to achieve genome editing in humans cells (5´-NNNNACAC-3´) (E. Kim et al., 2017). Thus, there are many orthologs of SpCas9 present in nature that can be engineered to be used with genome editing purposes. Most of them have very specific PAM requirements, which provides a higher targeting specificity, reducing off-target activity; this refers to genome editing events in non desired regions. However, this often leads to suboptimal DNA cleavage and editing efficiencies (Pacesa et al., 2024).

New CRISPR/Cas nucleases are being incorporated into the toolbox every year, with possible application in the genome engineering field. They are worth to be mentioned, the three systems belonging to class II identified by Zhang's group C2c1, C2c2 and C2c3. While C2c1 and C2c3 present endonuclease domains distantly related to Cas12a and belong to type V, C2c2 (Cas13a) has been classified in a type VI CRISPR/Cas cluster (Shmakov et al., 2015). This nuclease targets single-stranded (ss) RNA instead of double-stranded (ds) DNA, as

nucleases from C2c1 and C2c3 groups do (Lewis and Ke, 2017; Nakade et al., 2017).

In addition, C2c2 nuclease is said to present dual RNase activities. While one domain of the protein is in charge of cleaving RNA when coupled to a crRNA sequence, another domain is in charge of CRISPR RNA maturation. Thus, C2c2 is able to give rise to various mature crRNAs from a single CRISPR transcript, a characteristic that is shared with Cas12a (Abudayyeh et al., 2016; East-Seletsky et al., 2016). Other Cas nucleases that can target ssRNA molecules are Cas12a2 (which also targets ssDNA and dsDNA) (Dmytrenko et al., 2023) and Cas12g (which also targets ssDNA) (Yan et al., 2019). The ability of these nucleases to degrade mRNAs provides extremely high value in the field of genome editing mediated by CRISPR/Cas systems, since they allow the control of the expression of genes in a very specific manner. Furthermore, in the case of the C2c2 nuclease, its crRNAs processing capacity makes it a very suitable nuclease to be used to control the expression of different genes at the same time (another case of multiplexing), and so, to be used in programming cell circuits. However, every nuclease has particular limitations, the most important being the restrictive PAM sequences, different ranges of specificities, and different ranges of off-target profiles (Pacesa et al., 2024). As a result of these properties, the nuclease of choice in each particular case must be carefully selected.

CRISPR interference and CRISPR activation

To take advantage of the ability of nucleases to localize a short and specific region of the genome when accompanied by an RNA molecule (guide RNA, gRNA), they have been engineered to give rise to dead nucleases (dCas), which lack nucleolytic activity (Whinn et al., 2019), dCas9 being the most widely studied (Brezgin et al., 2019). They have been used to modulate gene expression in a transient manner, by means of two approaches known as CRISPR interference (CRISPRi), and CRISPR activation (CRISPRa). When dCas9 is fused to a transcriptional repressor domain such as the Krüppel-associated box (KRAB) domain of Kox1, endogenous genes can be silenced in humans cells,

with an outcome efficiency comparable to the obtained using RNA interference (RNAi) approaches, and with minimum off target gene repression (Gilbert et al., 2013) (**Figure 2**). Very recently, a similar system was also proved to function in plants (*Cucumis sativus* and *Arabidopsis thaliana*). In this case, the transcriptional repression was promoted by the N-terminal domain of a TEN protein (histone acetyltransferase) fused to a dCas9 (B. Wang et al., 2024). On the other hand, dead nucleases can be fused to specific enzymes, or even to particular domains, to promote the expression of targeted genes (CRISPRa) (**Figure 2)**. Linking a synthetic VP64 activation domain to the C terminus of a dCas9, it was proved to increase the expression of several targeted genes in human cells (Maeder et al., 2013). This strategy has also been proved to be effective to get detectable upregulation (twofold) of up to ten genes at the same time, which highlights its possible application to manipulate cellular circuits (Konermann et al., 2015). Gene activation can also be achieved by fusing dCas9 to demethylases or acetyltransferases, enzymes that alter chromatin states (Hilton et al., 2015; Kearns et al., 2015).

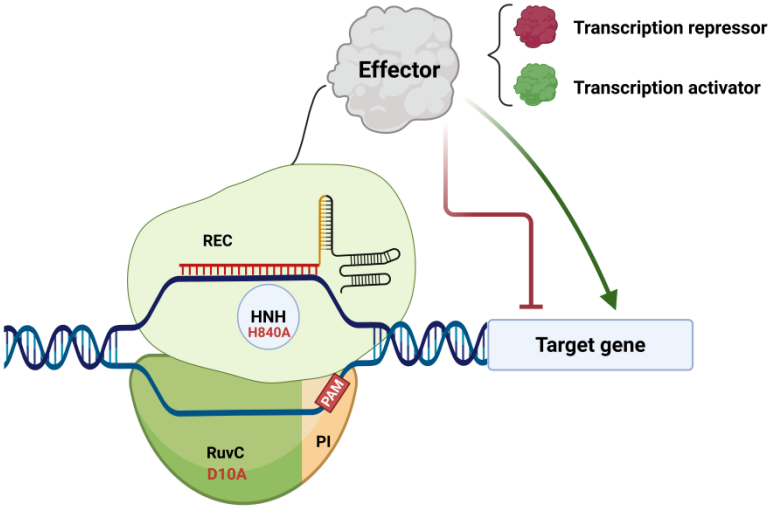


Figure 2. CRISPR interference and CRISPR activation complex based on Cas9 nuclease. H840A and D10A point mutations inactivate the HNH and the RuvC catalytic domains, respectively, generating a dCas9 nuclease. This dCas9 is fused to an effector domain, either a transcription repressor (red) or a transcription activator (green), that leads to target gene downregulation or

upregulation, respectively. The rest of the components of the figure are described in **Figure 1**. Adapted from Gilbert et al., 2013.

Base editing and prime editing

Through years and thanks to the study of the functioning and the structure of nucleases, CRISPR technology has evolved, and with it, the possibilities for genome editing that it can offer to us have multiplied. One of the newest and most sophisticated applications of Cas9 nucleases is base editing. This approach relies on a Cas9 nickase (nCas9) which lacks essential catalytic amino acid residues in certain domains of the nuclease (**Figure 3A**). Thus, nCas9 is still able to recognize the sequence targeted by the sgRNA, but it is only able to crate single-strand breaks (Lee et al., 2023). The other essential component of base editing system is a nucleotide deaminase enzyme. Base editing relies on the ability of the mentioned enzyme to remove an amino group from a specific nucleotide, resulting in a change of base. First base editors described were based on cytidine deaminase enzymes (named as cytosine base editors, CBEs), and they effected a C to T (or G to A) substitution (Komor et al., 2016; Nishida et al., 2016) (**Figure 3B**). In addition, when nCas9 is fused to an adenosine deaminase, thus giving rise to adenine base editors (ABEs), a conversion from A-T base pairs into G-C can be obtained (Gaudelli et al., 2017; Xiao et al., 2024) (**Figure 3B**). Furthermore, both CBEs and ABEs have been efficiently combined in a single system, working both in human and plant cells (Grünewald et al., 2020; Li et al., 2020; Sakata et al., 2020; Xiaohui Zhang et al., 2020). Finally, C to G base editors (CGBEs) have also been proved to induce base transversions in an efficient manner in human cells (Kurt et al., 2021). While the base editors systems composed of Cas9 nuclease are the most broadly used, Cas12a has also been used for C to T base editing (CBEs) in human cells (Kleinstiver et al., 2019). Taking into account all of this, anyone can easily realise the powerful tool that base editing is. This subtle technology allows introducing single nucleotide polymorphisms (SNPs) in desired areas of the genome, thus creating really small changes, that can have huge consequences. Its applicability ranges from plant genome engineering (Li et al*.,* 2020), to mammalian cell engineering with medical purposes (Alves et al., 2023; Musunuru et al., 2021).

While the sophisticated base editing technology allows manipulating the genome in a precise manner, its application is limited to creating SNPs. In this way, we can only obtain the following five substitutions (C to T, G to A, A to G, T to C and C to G). However, in 2019, David Liu's laboratory developed a new variant of the CRISPR/Cas system, called prime editing, which allowed to generate more than 175 different types of editing events in human cells, including targeted deletions and insertions, and all the 12 different types of base substitutions (Anzalone et al., 2019). Prime editing is mediated by a complex composed by a prime editor (formed by the fusion of an RNA-guided DNA-nicking domain, like an nCas9, and a reverse transcriptase domain) and a prime editing guide RNA (pegRNA). This long RNA molecule, is able to direct the complex to the target site, and also encodes the desired modification (Anzalone et al., 2019). The wide range of possibilities offered by the prime editing system means that it has been rapidly developed for its use in the field of medicine (Liu et al., 2020) to treat diseases such as Alzheimer's (Davis et al., 2024). In the field of plant biotechnology, prime editing technology has also been optimized based on codon usage, and the application of promoters adapted to each species, thus achieving effective genome editing in both monocot species, such as rice and wheat (Lin et al., 2020; Xu et al., 2020), and in dicots such as *A. thaliana* and tomato (Vu et al., 2024).

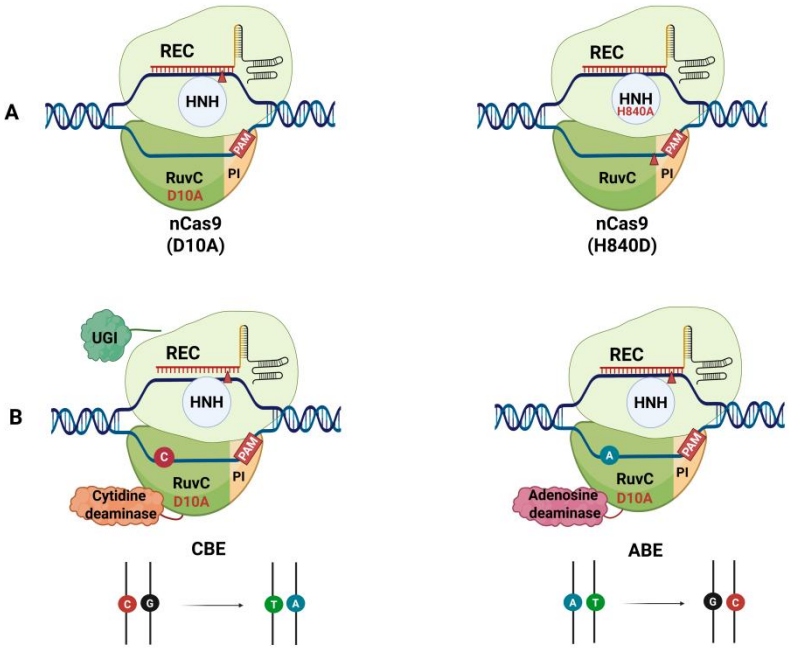


Figure 3. Schematic representation of some base editing approaches. (**A**) D10A point mutation in RuvC domain, converts Cas9 nuclease in a nickase (nCas9) that cleaves the target strand (left); while H840A point mutation in HNH domain generates a nCas9 cleaving the non-target DNA strand (right). (**B**) Cytidine base editors present a cytidine deaminase domain fused to the nCas9, represented by an orange lobe (left). In addition, second generation CBEs also present a uracil glycosylase inhibitor (UGI) fused to the nCas9. This domain blocks the natural base excision repair mechanism, preventing the reversion of the expected editing. CBEs effect a C to T (or G to A) substitution. Adenosine base editors present an adenosine deaminase domain fused to the nCas9 represented by a pink lobe (right). ABEs effect an A to G (or T to C) substitution. The rest of the components of the figure are described in **Figure 1**. Adapted from Lee et al., 2023 and from Li et al., 2020.

CRIPSPR/Cas delivery into plants

The effectiveness of genome editing mediated by the CRISPR/Cas system depends on the ability of the complex formed by the nuclease and the gRNAs to reach the nucleus of the cell to be edited. In the case of plant biotechnology, it could be said that the greatest challenge is to achieve an efficient delivery method for CRISPR reaction components. In particular, plants have some characteristics that make it difficult to effectively express the CRISPR/Cas system, and therefore, to obtain solid mutants (plants that present the same mutation in all the cells). Firstly, the polyploidy of many plant species, as well as other genome re-arrangements results in plants having a larger and often more complex genome than other living organisms. In addition, the presence of a rigid cell wall makes it extremely difficult for ribonucleoproteins (RNPs) to penetrate the cell. Finally, many of the CRISPR/Cas genome editing protocols require *in vitro* regeneration to obtain edited plants, something that can take many months of work, or is even impossible in recalcitrant species (Laforest and Nadakuduti, 2022).

The most common method to deliver CRISPR/Cas components into plant cells (also taking into account the newest versions of the system, based on base editing and prime editing), relies on the use of *Agrobacterium tumefaciens* (Lin et al., 2020) (**Figure 4**). This pathogenic bacterium has been well known in the agricultural field for more than 100 years as the cause of crown-gall disease, the symptoms of which are obvious tumors formed in plants (Nester, 2015). The ability to form tumors is due to the presence of a so-called tumor-induced (Ti) plasmid. Although the genes present in each Ti plasmid depend on the bacterial strain, in general, all of them present a vir region, and a transferred DNA or T-DNA. The vir region encodes around 35 different proteins such as VirA, VirC, VirG and VirF, which regulate the T-DNA transfer process, and 20 of them are involved in tumor formation (Azizi-Dargahlou and Pouresmaeil, 2024; Nester, 2015). The other region of the Ti plasmid, the T-DNA is a virulent DNA that causes the specific crown-gall phenotype, by an increase of cellular hyperproliferation and opine production (Křenek et al., 2015). In *A. tumefaciens* strains that have been engineered for biotechnological use, that is, non pathogenic strains, genes involved in tumors formation have been removed,

without affecting the ability to transfer DNA. This modified plasmid is known as a disarmed Ti plasmid (Kiyokawa et al., 2009).

While the infection proceeds, the T-DNA is delivered into the plant cell, and it is randomly integrated in the genome of the host (Chilton et al., 1977). However, the expression of T-DNA is not only related to the stable integration in the genome, but also to transient expression (Janssen and Gardner, 1990). It is thought that in early stages of infection, *A. tumefaciens* delivers a higher amount of copies of T-DNA molecules than those that are finally integrated in the genome, explaining in this way the decrease in the transient expression usually observed 2-4 days post infection (Křenek *et* al., 2015).

Most of the times, the use of *A. tumefaciens* as a delivery vector for CRISPR/Cas components, relies on the expression of the nuclease and the gRNAs inserted in the T-DNA of the disarmed Ti plasmid. Explants of aseptic plants are cocultured with *Agrobacterium* suspensions, and whole plants are later regenerated by means of tissue culture. There are numerous examples of the effective use of this technology to obtain edited plants, and the results are widely demonstrated. In an informal manner, this type of genetic transformation is known as "classical transformation". In the case of model plants, *Nicotiana benthamiana* and *A. thaliana*, edited plants have been successfully generated using both Cas9 and Cas12a nucleases (Angulo et al., 2023; Bernabé-Orts et al., 2019; Matsuo and Atsumi 2019). However, when transforming *A. thaliana*, floral dip strategy is preferred, bypassing then the tissue culture regeneration process, thus saving a substantial amount of time. However, this method cannot be used in all the species (Desfeux et al., 2000). In addition, crops of agronomical interest can be successfully edited using the described methodology, with both mentioned nucleases. Tomato, rice and grapevine, are simply some examples of them (Bernabé-Orts et al., 2019; Lv et al., 2024; Moffa et al., 2024; Nekrasov et al., 2017; Ren et al., 2023; Távora et al., 2022; Vu et al., 2021).

The other widely used method for plant genetic transformation, which is comparable in terms of efficiency to *A. tumefaciens* transformation is particle bombardment or biolistics (**Figure 4**). Although it has mainly been used in

monocots, to date, a huge number of particle bombardment applications have been successfully used in different plant species (Ozyigit and Kurtoglu, 2020). The most used variation of this approach to obtain CRIPR/Cas editing also consists of the integration of foreign DNA in the plant genome. Transformed plants are later regenerated by tissue culture techniques. The DNA to be transferred is mainly formed by a cassette that expresses the CRIRPR/Cas reaction components, coating particles (dense metal particles are usually preferred, such as gold and tungsten) that are fired into plant cells using a pressure gradient (Fu et al., 2000; Ozyigit and Kurtoglu, 2020). Particle bombardment has also been efficiently used in transient expression assays, being of great importance to study gene expression and function in plants (Mookkan, 2018; Sivamani et al., 2009). While T-DNA can only target nuclear DNA, particle bombardment is considered the first reported method used for mitochondria and chloroplast transformation (Svab et al., 1990; Altpeter et al., 2005). This opens the door to the possible future application of CRISPR/Cas technology to edit plastid genomes, something that has not been achieved so far (Nakazato and Arimura, 2024).

A third method used nowadays to transfer CRISPR/Cas components into the cell is the delivery of preformed RNP complexes, composed of guide RNAs and the Cas protein (**Figure 4**). In this approach, no recombinant DNA is used if gRNAs are chemically synthesized, thus resulting in transgene free edited plants. Furthermore, the use of RNPs avoids random integration of foreign DNA into the genome of the host. In addition, RNPs are degraded by endogenous proteases and nucleases after a short period of time, something that avoids continuous expression of the CRISPR/Cas system (Kim et al., 2014). Taking both these aspects into account, the number of off-target events is strongly reduced (Kouranova et al., 2016; Zaidi et al., 2018). Typically, RNPs are delivered into plant protoplasts by means of polyethylene glycol (PEG)-mediated transfection (Zhang et al., 2021). The use of both Cas9 and Cas12a nucleases to edit protoplasts by means of RNPs complexes has been reported in different plant species (H. Kim et al., 2017; Wu et al., 2020). However, protoplast isolation and later plant regeneration is usually a tedious and long lasting process. Particle bombardment can also be used to deliver RNPs into explants such as embryos and leaf discs. While avoiding protoplast isolation and maintenance, this

method also has to deal with plant regeneration by tissue culture (Liang et al., 2017; Svitashev et al., 2016).

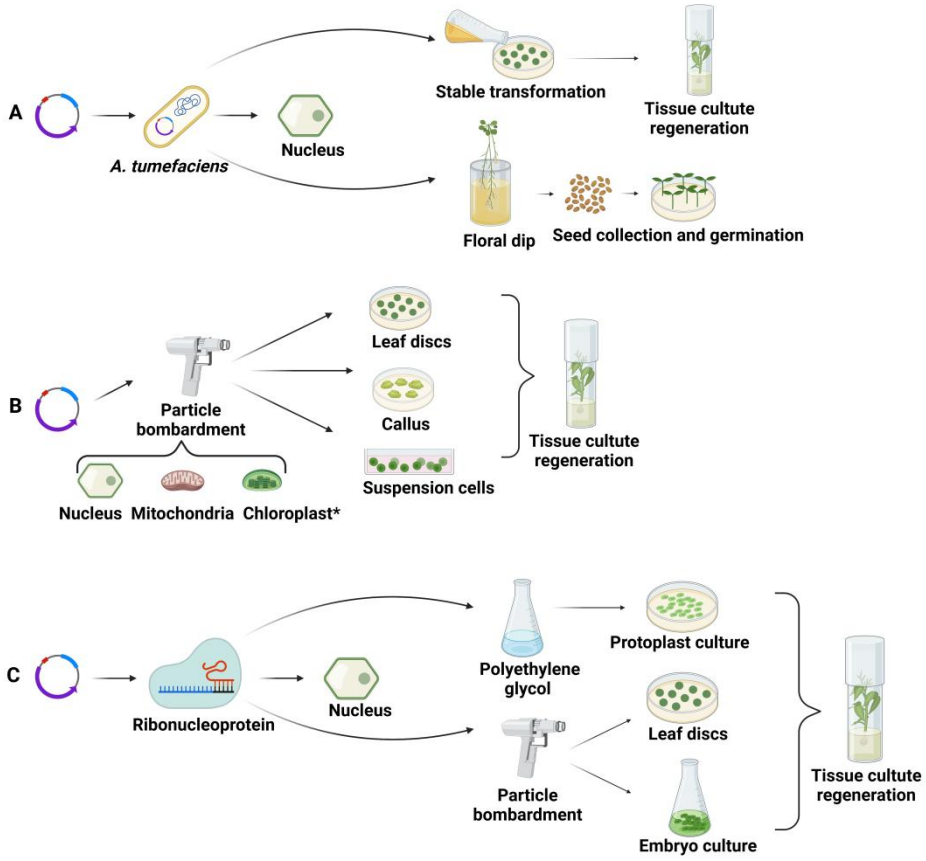


Figure 4. Delivery mechanisms of CRISPR/Cas reaction components to plants. (**A**) The most common mechanism to deliver CRISPR/Cas components to plants is based on the use of *A. tumefacines*, which transfers the T-DNA to the nucleus of the plant cell, where it is integrated. Stable transformation is the desired method to obtain solid mutants, in which cotyledons or hypocotyls can serve as explant source, most of the time. Solid mutants can be obtained after tissue culture regeneration. In the case of *A. thaliana* floral dip transformation can also be used, bypassing then the tissue culture regeneration process. (**B**) The other widely used method for plant genetic transformation, is particle bombardment or biolistics. This method has been implemented to target nuclear genome as well as to perform mitochondria and chloroplast transformation (although no CRISPR/Cas editing of plastid genome has been

achieved so far*). Leaf discs, callus cultures and suspension cells are usually used for particle bombardment transformation. Complete plants are later regenerated by tissue culture. (**C**) Preformed ribonucleoprotein (RNP) complexes, composed of guide RNAs and the Cas protein can also be directly delivered to plant cells, to target the nuclear genome. RNPs can be delivered to plant protoplast by means of polyethylene glycol (PEG)-mediated transfection. Particle bombardments can also be used to deliver RNPs to leaf discs or embryo cultures. Complete plants are later regenerated by tissue culture. Adapted from Uranga and Daròs, 2023.

Virus-induced genome editing (VIGE)

The use of viruses to express heterologous genes in plants has been reported since more than 30 years ago. Firstly used as expression vectors for reverse genetics studies, and later implemented in RNA silencing assays, the versatility of plant viruses and their applicability to plant biotechnology has been widely proved (Cody and Scholthof, 2019). The so called virus-induced genome editing (VIGE) relies on the capacity to manipulate viral genomes to express CRISPR/Cas reaction components that are delivered into plants. These vectors are designed to be delivered by means of *A. tumefaciens*, something that helps to initiate transcription of the viral clone in a homogeneous way. There are several studies that strongly support VIGE strategy as a tool to obtain edited plants (Ellison et al., 2020; Kang et al., 2025; Uranga, Aragonés, et al., 2021; Zhao et al., 2024). The main advantages when choosing VIGE over classical transformation, will be summarised in the following lines. First of all, when expressing CIRSPR/Cas components with viral vectors, their expression is boosted due to viral replication, something that has as a result an increased and fast editing efficiency. Secondly, when RNA viruses are used as vectors, there is no integration of foreign DNA in the host cells. This considerably reduces the amount of off target effects, and also facilitates the path towards the commercialisation of the edited organisms obtained, as they are not considered transgenic. Finally, if we are able to edit the germline of the plant, thus obtaining edited plants in the next generation, we could bypass the tissue culture regeneration step, saving a considerable amount of time (Uranga and Daròs, 2023; Zhang et al., 2022) (**Figure 5**).

On the other hand, one of the main disadvantages of using viral vectors with genome editing purposes is their limited cargo capacity (Wu et al., 2024). The large size of nucleases means that not all plant viruses are capable of expressing them. Because of this, VIGE is usually classified in two different categories. Firstly, editing events achieved when gRNAs are expressed by a viral vector, in a transgenic plant that already expresses a particular nuclease. These cases are much more frequent in literature, and are not subjected to the cargo limitations of the vector, since the reduced size of the gRNA makes it easily expressed. Secondly, although more technically demanding, both gRNA and Cas nucleases can be simultaneously expressed either by a unique or by two viral vectors at the same time. In this case, there is no requirement of previously obtaining a transgenic line (Zhang et al., 2022) (**Figure 5**). The main advances achieved to date in VIGE in plants will be discussed below.

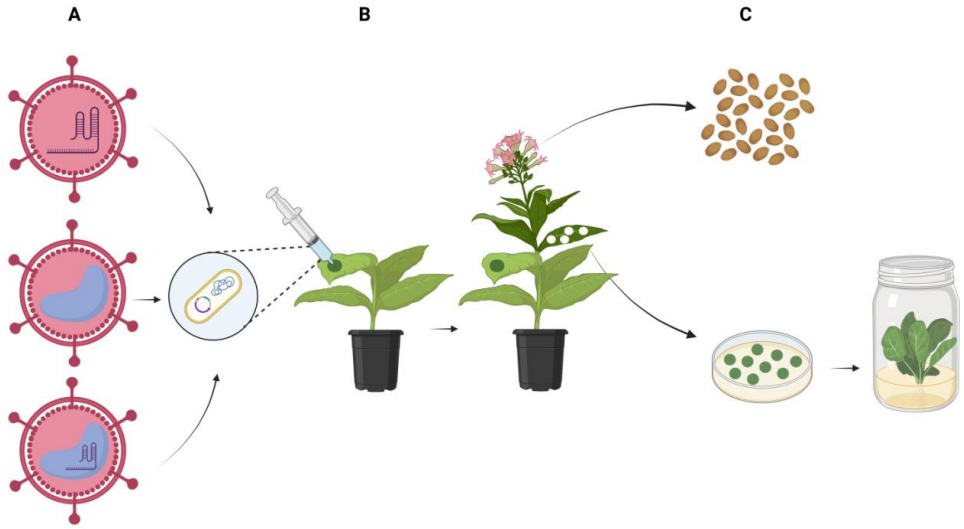


Figure 5. Schematic representation of some VIGE approaches. (**A**) Viral vectors have been engineered to express gRNAs in transgenic plants expressing Cas nucleases (top). Despite their cargo limitations, some viral vectors can also express Cas nucleases (middle) or both Cas nucleases and gRNAs at the same time (bottom). (**B**) Viral vectors are delivered to the plants by means of *A. tumefaciens*-mediated inoculation. (**C**) While infection develops, the virus spreads to systemic uninoculated tissue. If the virus is able to reach and edit the germline, edited progeny can be obtained after selfing. When this milestone is not achieved, solid mutant plants can be obtained after plant

regeneration by means of tissue culture. Adapted from Uranga and Daròs, 2023.

Viruses expressing gRNAs

Most VIGE events in plants are related to viral vectors expressing sgRNAs in transgenic lines that constitutively overexpress a Cas9 nuclease (**Figure 5**). Biological characteristics of each virus, such as its mode of replication, cargo capacities and host range, make each case study unique. Over the years, scientists have been able to efficiently express gRNAs using both RNA and DNA viruses.

Tobacco rattle virus (TRV; genus *Tobravirus*) is a positive single-stranded RNA (+ssRNA) virus, with a bipartite genome, split in two RNA molecules (RNA1 and RNA2) (Hamilton et al., 1987). TRV presents a wide host range, and an easily manipulated genome. These characteristics have made the use of this virus very popular not only in VIGE assays, but also in virus induced gene silencing assays (VIGS) (Y. Liu, Schiff, and Dinesh-Kumar, 2002; Dong et al., 2007). Expressing sgRNAs with a TRV vector, genome editing of the reporter gene *Phytoene desaturase* (*NbPDS*) has been achieved in *N. benthamiana* plants overexpressing Cas9 (Ali et al., 2015). In order to obtain a solid mutant, we can either regenerate complete plants from infected tissue by means of tissue culture, or we can try to edit the germline and obtain mutants in the next generation (**Figure 5**). The ability to edit the germline varies among different viruses, and also among different hosts. Although present, the amount of edited plants in the next generation was scarce in the mentioned study (Ali et al., 2015).

In order to increase this mutation heritability, different approaches can be followed. As a proof of concept, Firedrich Kragler´s group proved that the fusion of tRNA-like sequence motifs to Cas9 and gRNA transcripts can enhance RNA mobility from transgenic rootstocks to grafted wild-type shoots in wild-type *A. thaliana* and *Brassica rapa* (Yang et al., 2023). Ellison et al. (2020) reported an increase of heritable edits higher than threefold when expressing gRNAs targeting *NbPDS* gene with a TRV vector in *N. benthamiana* overexpressing Cas9 plants. These gRNAS transcripts presented the sequence of

the *Arabidopsis* FLOWERING LOCUS T (FT) mRNA fused to its 3´ end (Ellison et al., 2020).

The efficiency of TRV vector to deliver gRNAs has also been proved in tomato, a crop of agronomical interest. Very recently, Kang et al. (2025) used a very similar approach, and fused mobile sequences to gRNAs targeting *SlPDS* in transgenic Cas9 tomato plants (cv. Moneymaker). Although they achieved editing of systemic tissue, no edited progeny was obtained (Kang et al., 2025). Authors hypothesized that this lack of heritable editing could be related to the use of the 35S promoter controlling the expression of the Cas9 nucelase in their transgenic lines, having this promoter species-specific activity in germ cells. Kim and coworkers solved this issue by generating a tomato line that expresses Cas9 under the control of the tomato ubiquitin 10 (*SlUbi10*) gene promoter. gRNAs were fused to the *SlFT* gene fragment and expressed by a TRV vector, that also targeted *SlPDS* gene. Edited progeny was obtained after selfing, even achieving albino seedlings, a characteristic phenotype derived from complete *PDS* gene inactivation (Lee at al., 2025).

Another methodology has been recently developed in Daniel Voytas´ group, to eliminate the dependency on tissue culture work when using viral vectors to deliver gRNAs. In this case, TRV vector expressed gRNAs along with the developmental regulator isopentenyl transferase gene in Cas9 tomato plants. The viral construct was injected into the axillary tissues and the first appearing shoots were removed. The newest shoots that appeared during the second month of the infection presented edited alleles at the targeted genes. These shoots were regenerated into adult plants, and their progeny happened to be edited at both targeted genes (*UGT75C1* and *Hairless*) (Liu et al*.,* 2024).

The use of other +ssRNA viruses as gRNAs deliverers has also been very contrasted. Potato virus X (PVX; genus *Potexvirus*) can infect more than 60 different plant species, among which are crops of agronomic importance from the Solanaceae family (Zhang et al., 2022). A vector derived from PVX was able to simultaneously express (multiplexing) three different sgRNAs targeting *NbPDS3*, *NbFT* and *NbXT2B* in *N. benthamiana* Cas9 expressing plants. Genome editing of these three genes was observed in systemic uninoculated tissue from

14 days post inoculation (dpi) on (Uranga, Aragonés, et al., 2021). Targeting *NbXT2B* in an individual manner, 22% of seedlings from infected plants contained biallelic mutations, thus proving heritability in this model plant. A similar approach has also been used in a tomato line expressing Cas9. However, no mutant progeny was found when expressing sgRNAs with PVX in this species (Kang et al., 2025).

In the same category of +ssRNA viruses, also stands barley stripe mosaic virus (BSMV; genus *Hordeivirus*) for being able to express multiple gRNAs in monocot crops. This virus, which presents a tripartite genome, was used to achieve the first VIGE ever obtained in wheat and maize in 2019. Hu et al. (2019) reported a mutation efficiency of 78% when targeting *GASR7* in wheat Cas9 plants and 48% when targeting *TMS5* in maize Cas9 plants, although no edited progeny was found (Hu et al., 2019). Also using BSMV to express gRNAs, Li et al. (2021) made a significant advance in this field, when years later they managed to obtain mutated progenies of Cas9 wheat plants at frequencies ranging from 12.9% to 100% in three different varieties. Similarly, there has also been reported a strategy to achieve heritable multiplexed editing in genes of agronomic interest in wheat, in this case, expressing gRNAs cloned in the BSMVγ chain, transcribed prior inoculation (Wang et al., 2022). Heritable genome editing has also been reported using BSMV to express gRNAs in barley (Tamilselvan-Nattar-Amutha et al., 2023).

Another useful viral vector used in monocots is foxtail mosaic virus (FoMV; genus *Potexvirus*). The efficacy of this virus to express gRNAs was first tested in *N. benthamiana* Cas9 expressing plants. After that, the same approach was followed to target *CA2* gene in a Cas9 foxtail (*Setaria viridis*) line, obtaining an editing efficiency of 60% in upper uninoculated tissue. In addition, this vector was also proved to generate genome editing in maize by expressing gRNAs targeting *HKT1* gene (Mei et al., 2019). The list of +ssRNA viruses that have been engineered as viral vectors to deliver gRNAs in plants remains continuously increasing. As prominent examples, the following viruses can be mentioned: pea early browning virus (PEBV; genus *Tobravirus*) (Ali et al., 2018); apple latent spherical virus (ALSV; genus *Cheravirus*) (Luo et al., 2021); beet necrotic yellow vein virus (BNYVV; genus *Benyivirus*) (Jiang et al., 2019) and

tobacco mosaic virus (TMV; genus *Tobamovirus*) (Cody et al., 2017). These viruses were tested to efficiently deliver gRNAs in *N. benthamiana* plants expressing Cas9 either constitutively or transiently.

Although not so frequently employed, DNA viruses have also been used in VIGE assays to deliver gRNAs, particularly geminiviruses. This family of plant viruses presents a circular single-stranded DNA genome. Their reduced genome size makes them appealing to be engineered as biotechnological tools. However, it is considered a disadvantage when trying to express heterologous genes because of their cargo limitations (Lozano-Durán, 2016).

Viruses expressing Cas proteins

When trying to express longer heterologous genes, such as the ones coding Cas nucleases, cargo constrains of viral vectors play a very important role. To express large inserts in plants, positive-strand RNA viruses and DNA viruses are not usually considered as the best options, due to their considerable cargo limitations and genetic instabilities (Gao et al., 2019). On the other hand, negative-strand RNA viruses possess certain characteristics that make them more suitable for foreign gene expression. The stability of their genome is guaranteed because of the encapsidation mediated by a linear nucleocapsid, thus avoiding disruptive recombination (Luo et al., 2020). However, the application of delivery vectors derived from negative single-stranded RNA (-ssRNA) viruses has not been so extensively investigated compared to +ssRNA and DNA viruses, at least until recent years (Jackson and Li, 2016).

In the extensive group of -ssRNA viruses, the ones belonging to the *Rhabdoviridae* family stand out for their capacity of expressing large foreign genes. In vertebrate cells, these viruses are able to express genes up to 6 kb in size (Jackson and Li, 2016). Unsegmented rhabdoviruses infecting plants were classified into two different genera, which comprise the *Cytorhabdovirus* and the *Nucleorhabdovirus* genus, according to their cytoplasmic or nuclear replication. Since 2019, the genus *Nucleorhabdovirus* was split into the three genera *Alphanucleorhabdovirus*, *Betanucleorhabdovirus* and *Gammanucleorhabdovirus* (Dietzgen et al., 2020). Barley yellow striate mosaic virus (BYSMV; genus *Cytorhabdovirus*) has been engineered as a vector to

express both sgRNAs and a zCas9 (*Zea mays* codon-optimized Cas9) in *N. benthamiana* plants (Gao et al., 2019). Gao and co-workers reported efficient editing of the transgenic GFP gene in infected tissue of this model plant. Another virus from the same family, which has been used in VIGE assays is the sonchus yellow net virus (SYNV; genus *Betanucleorhabdovirus*). Ma et al. (2020) proved to be able to express the entire CRISPR/Cas9 cassette (sgRNA and spCas9) in the same model plant, using SYNV as a delivery vector. In this case, authors efficiently achieved multiplexed mutagenesis of endogenous genes and chromosome deletions, and proved that the viral vector remained stable after mechanical transmission. However, plant rhabdoviruses have not been reported to invade the meristem or germline (Jackson et al., 2005). This can explain why the authors of the previous study failed to obtain genome editing events in the progeny of infected plants. Solid mutants were obtained by regenerating complete plants by means of tissue culture. The edited progeny of regenerated plants was also virus free, due to the non-seed transmissibility of plant rhabdoviruses (Jackson et al., 2005).

Another -ssRNA virus with very interesting biological characteristics to be used in VIGE experiments is tomato spotted wilt virus (TSWV; genus *Orthotospovirus*). This plant pathogen presents a tripartite genome and is able to infect more 1000 plant species, among dicotyledonous and monocotyledonous crops. In the field of genome engineering, Liu and coworkers made great advances by removing genes involved in insect transmission from their TSWV viral vector, thus increasing its cargo capacity, but without compromising its replication. Using this system, these authors were able to obtain gene editing by delivering CRISPR/Cas9 reagents, CRISPR/Cas12 reagents, adenine base editors and cytosine base editors not only in *N. benthamiana* plants, but also in crop plants such as tobacco, tomato, chili pepper, sweet pepper or peanut (Liu et al., 2023). The lack of germline invasion of TSWV made it impossible for the authors to find heritable gene editing events. However, they developed a general protocol for plant regeneration by tissue culture, and virus elimination with ribavirin, applicable to several plant species and tested in tobacco, tomato and pepper, and so promoting the use of this virus in VIGE assays (Lou et al., 2024; Zhao et al., 2024).

The limited cargo capacity of +ssRNA viruses makes them less suitable to express the CRISPR/Cas nucleases in plants. To overcome this limitation, researchers have developed different approaches, some of them with very positive outcomes. Kaya et al. (2017) explored different Cas9 that could be expressed by +ssRNA viruses. They managed to clone a *Staphylococcus aureus* Cas9 (SaCas9) (which is smaller in size than the widely used SpCas9) in a tomato mosaic virus derived vector (ToMV; genus *Tobamovirus*) using a "split approach". The protein was split in two inactive fragments, which might later reassemble to form an active protein (Kaya et al., 2017). One of the split fragments was delivered to *N. benthamiana* plants by means of the ToMV vector, while the other fragment was transiently expressed by *A. tumefaciens*. Efficient mutagenesis of the targeted gene was achieved in infiltrated tissue. However, these authors failed to express the complete sequence of SaCas9 in their ToMV vector. Using two compatible viruses, Uranga, Vazquez-Vilar, et al. (2021) managed to express an LbCas12a protein (from *Lachnospiraceae bacterium*), and to obtain genome editing in *N. benthamiana* plants. In this study, authors removed the *NIb* gene, encoding the viral RNA-dependent RNA polymerase from a tobacco etch virus vector (TEV; genus *Potyvirus*). In this way, the viral vector was able to increase its cargo capacity, and express large exogenous sequences in an efficient manner (Martí et al., 2020). The procedure followed consisted on expressing the complete LbCas12a with the modified TEV vector, while a PVX vector expressed the *NIb* gene required for TEV replication and the gRNA (Uranga, Vazquez-Vilar, et al., 2021). Using this combinatorial system, efficient editing of the *NbFT* gene was achieved in upper non inoculated tissue. PVX vector has also been used to deliver both Cas9 nuclease and sgRNA at the same time in a single construct, in *N. benthamiana* plants (Ariga et al., 2020). When targeting the endogenous gene *TOBAMOVIRUS MULTIPLICATION 1* (*NbTOM1*), authors were able to achieve a high amount of edited shoots (62%) regenerated from infected tissue. In addition, FoMV has also been used with similar purposes. Two different FoMV vectors expressing each a Cas9 nuclease or a sgRNA targeting *NbPDS* gene could simultaneously infect the same cell, leading to gene editing in the endogenous reporter gene. However, no heritable editing was achieved following this strategy (Xian Zhang et al., 2020).

Finally, DNA viruses have also been modified to be used as vectors to deliver large Cas nucleases. Most of the cases rely on the use of geminiviral replicons (GVR; usually known as deconstructed virus) which present an increased cargo capacity (Gleba et al., 2004). When using a full virus delivery approach, the coat protein can be removed from the viral vector (it is not required for cell-to-cell movement in some bipartite begomoviruses) (Gardiner et al., 1988). When using a deconstructed virus approach, both movement protein and coat protein sequences are usually removed, making it impossible viral cell-to-cell movement. This lack of virus spread can be bypassed using specific delivery methods for the viral vector, like particle bombardment (Baltes et al., 2014). The first geminiviral vector used to express both sgRNAs and the Cas9 nuclease was derived from bean yellow dwarf virus (BeYDV; genus *Mastrevirus*). This GVR could efficiently target the *NtALS* gene in *Nicotiana tabacum* plants (Baltes et al., 2014). In addition, replicons based on the same virus have also been used to obtain CRISPR mutants in other non-model plant species, such as potato (*Solanum tuberosum*) (Butler et al., 2016) and tomato (Čermák et al., 2015; Dahan-Meir et al., 2018). In addition, GVRs derived from wheat dwarf virus (WDV; genus *Mastrevirus*) have been implemented to edit the genome of monocots, such as wheat and rice, by means of CRISPR/Cas technology. When using WDV replicons carrying Cas9 nucleases and sgRNAs to target endogenous genes in wheat, genome editing of frequencies 12-fold greater than when using non-viral delivery methods can be obtained (Gil-Humanes et al., 2017).

The use of miniature Cas nucleases

The application of viral vectors as a delivery tool for CRISPR/Cas reagents has mainly focused on the use of Cas9 and Cas12a nucleases, which usually range from 1000 to 1500 amino acids (aa) in size. Taking into account the cargo limitations of viral vectors, it makes sense to try to address strategies that minimize the size of the inserts. One of the alternatives which is being adapted to plant biotechnology is the use of miniature Cas nucleases, which have already been tested in mammalian cells. One of the most commonly used compact nucleases is Cas12f (Cas14). While its wild version is not active in mammalian cells, Xu et al. (2021) generated an engineered version of 529 aa

named as CasMINI, which efficiently enables gene editing in human cells. They also proved that CasMINI can be used for base editing purposes and presents a similar gene activation efficiency to Cas12a (**Figure 6**). Hino et al. (2023) developed two versions of Cas12f from *Acidibacillus sulfuroxidans* with enhanced activity, named as enAsCas12f. They expressed these variants in mice cells by means of an adeno-associated virus (AAV) vector, and efficiently accomplished generating knock-in and knock-out of targeted genes, as well as transcriptional activation.

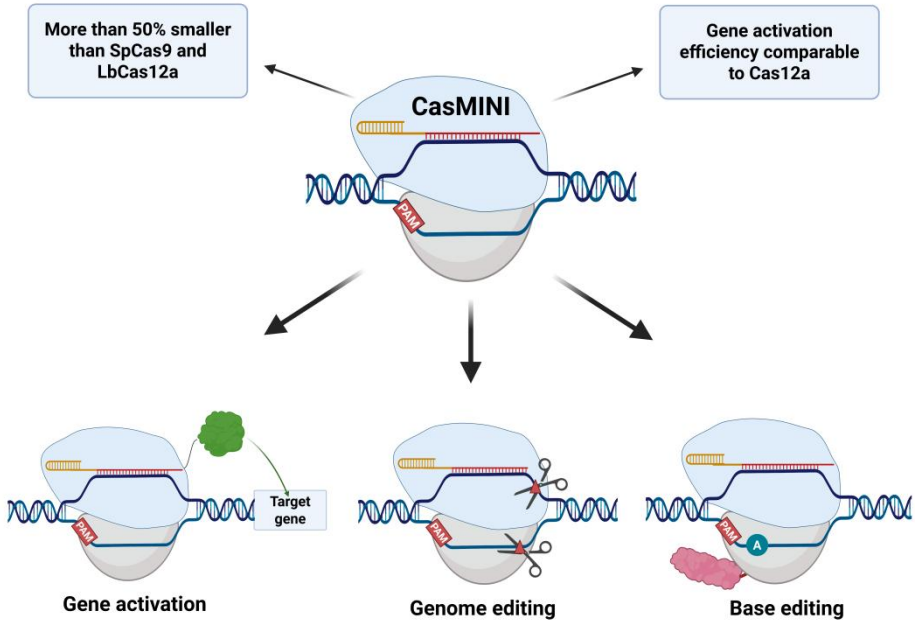


Figure 6. Schematic representation of the different applications of CasMINI tested in mammalian cells. The compact size of CasMINI as well as its high gene activation efficiency makes this nuclease a perfect option for different genomic assays in mammal cells. When fused to a transcription activator (green domain), CasMINI can induce gene expression of the target gene (left). Genome editing by means of DSB (center) and base editing when fused to an adenosine deaminase domain (right) can also be achieved. The efficiency of CasMINI to edit plant genomes has also been demonstrated, with the engineered version CasMINIv3.1. Particular components of the figure are described in **Figure 1** and **Figure 3**. Adapted from Xu et al., 2021.

The application of these compact Cas nucleases has already been demonstrated in plants as well. Sukegawa et al. (2023) developed a strategy based on the use of Cas12f nuclease (497 aa) from *Syntrophomonas palmitatica* (SpCas12f), to obtain genome editing in rice. Targeting two endogenous genes, the authors were able to obtain mutation frequencies of 28.8% and 55.6% in transformed calli. The mutations present in regenerated plants were later transferred to the next generation, thus proving their heritability. Furthermore, no off-target effects were observed in this study. A few years before, Bigelyte et al. (2021) had reported the first genome editing event in crops, in this case, in maize, using SpCas12f. However, the ability of the mentioned nuclease to generate the DSB relied on the use of heat pulses at 45°C, which were not required in Sukegawa et al. (2023) study.

The capacity of these systems to be transferred to plant viral vectors has been recently demonstrated. The first application of miniature CRISPR/Cas systems for a VIGE approach relied on the use of a deconstructed geminiviral DNA replicon, derived from BeYDV (Gong et al., 2024). The authors of this study tested the efficiency of four different engineered miniature nucleases: CRISPR/Cas12j-2 (CasΦ) and three Cas12f orthologs, SpCas12f1, AsCas12f1, and CasMINIv3.1 in *N. benthamiana* plants. Although at lower efficiencies than SpCas9, Gong et al. (2024) proved that CasΦ, SpCas12f1 and CasMINIv3.1 can generate gene editing of an endogenous gene (*NbPDS*) when expressed by a GVR. The use of a PVX vector has also been reported to express miniature CRISPR/Cas systems in plants. Ishibashi et al. (2024) inoculated *N. benthamiana* plants with a PVX vector expressing an AsCas12f variant targeting the *NbPDS* gene. Authors achieved highly efficient genome editing compared to Cas9 not only in inoculated leaves, but also in systemic tissue. However, no mutant progeny was recovered, although solid mutant plants were obtained after regenerating shoots from infected tissue.

Virus-induced gene silencing (VIGS)

Currently, VIGE technology is on the rise due to the tremendous advancement in knowledge and applicability of CRISPR/Cas genomic editing techniques in recent years. However, viral vectors have been used for a long time for other purposes. Such is the case of virus-induced gene silencing (VIGS), an approach that has been used for decades in gene function analysis, applicable to a wide spectrum of species. The mechanism of action of this technique, in which plant endogenous genes are silenced, derives from the adaptation of a plant defence system (RNAi), considered the first line of defence against viral pathogens (Rössner et al., 2022). The core mechanism derives from the processing of a viral double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) (Baulcombe, 2015).

When an RNA virus infects the plant cell and replicates in the cytoplasm, viral particles are assembled and spread as full viruses. During this process, double-stranded virus-derived small RNAs (vsRNAs) are generated, which are recognized by Dicer-Like proteins (DCL) and cleaved into 21-24-nt siRNAs. DCL2, for example, generates 22-nt siRNAs, while DCL4 generates 21-nt siRNAs (Aregger et al., 2012). These siRNAs are loaded into Argonaute (AGO) proteins, which activate antiviral silencing (**Figure 7**). AGO proteins are part of the RNA-induced silencing complex (RISC), which targets complementary viral RNAs, cleaving them and leading to posttranscriptional gene silencing (PTGS) (Carbonell and Carrington, 2015). Another pathway in which viral genomes are silenced is by translational inhibition, which is caused by 22-nt siRNAs, products of DCL2 (**Figure 7**). In the case of DNA viruses that replicate in the nucleus, overlapping transcripts including dsRNA segments are generated after bidirectional open reading frame (ORF) transcription. These dsRNAs are recognized by DCL3, and cleaved into 24-nt siRNAs, which are loaded into AGO4, leading to methylation of viral DNA, and thus, to transcriptional gene silencing (TGS) (Rössner et al., 2022). This silencing mechanism is also combined with the above mentioned PTGS.

The RNA silencing signal is latter spread through the plant, by means of cell to cell transport of siRNAs via plasmodesmata, which can later be loaded onto the

RISC complexes (**Figure 7**). The RISC products are converted to dsRNAs by means of RNA-dependent RNA polymerases (RDR), and are later processed by DCLs, resulting in a new RNA silencing cycle (Muhammad et al., 2019). siRNAs migration through the plant´s distal parts occurs faster than viral infection, priming systemic tissue for viral infection.

The application of this plant defence system to silence endogenous genes is based on the expression of a homologous gene fragment (normally corresponding to part of the coding sequence) by means of a viral vector (VIGS). Generally, when the viral vector is delivered to the cell (most of the time by *A. tumefaciens* transformation), the T-DNA is integrated into the host genome. After transcription in the nucleus and translation in the cytoplasm, dsRNAs are generated, triggering the cascade of events described above and leading to the downregulation of the targeted gene (Rössner et al., 2022) (**Figure 7**).

The first employment of this technique was done in 1995 by Kumagai et al. (1995). In this study, the authors expressed a fragment of the *NbPDS* coding sequence, by means of a tomato mosaic virus derived vector, and showed the downregulation of the targeted gene, accompanied by the expected white leaf phenotype in *N. benthamiana* plants. Since then, VIGS technology has been refined and improved. It also has certain characteristics that make it perfectly compatible with the coexistence of genomic editing technologies. VIGS strategies are often preferred in species where transformation protocols are inefficient, or in species with very long reproductive cycles, such as trees. Additionally, the transient nature of the effects caused by VIGS becomes an advantage when studying genes whose knockout may be lethal.

Viral vectors used for VIGS are most commonly derived from single-stranded RNA viruses, or DNA viruses, with some of the most representative being TRV (Y. Liu, Schiff, and Dinesh-Kumar, 2002; Y. Liu, Schiff, Marathe, et al., 2002) and the African cassava mosaic virus (ACMV) (Beyene et al., 2017), respectively. Other viruses used in VIGS assays are PEBV (Grønlund et al., 2010), PVX (Lacomme and Chapman, 2008) and BSMV (Manmathan et al., 2013).

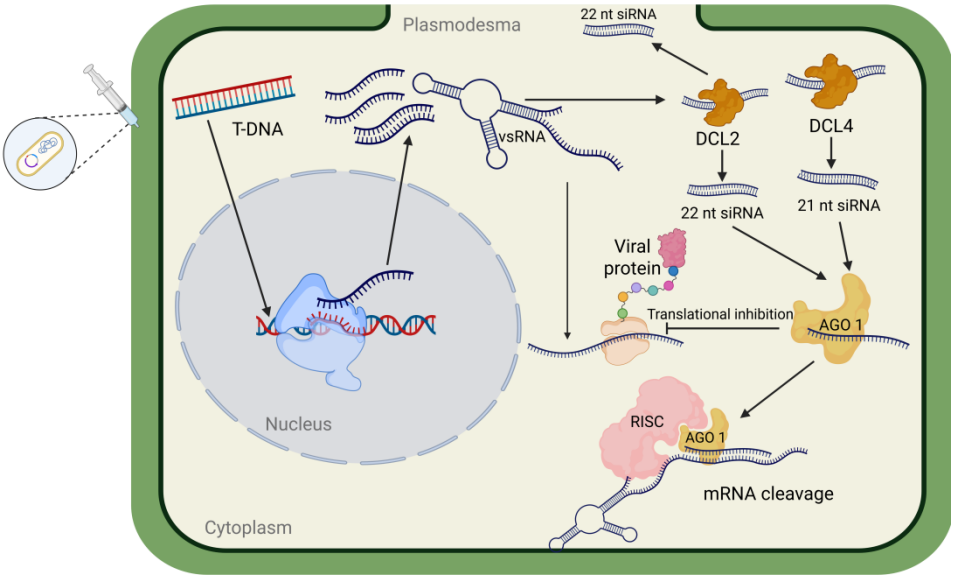


Figure 7. Simplified representation of the VIGS mechanism when using an ssRNA virus as a vector. The viral vector is delivered to the cell by means of *A. tumefaciens* agroinoculation. The T-DNA migrates to the nucleus, and it is transcribed to RNA by RNA polymerase II, forming vsRNA molecules. Once in the cytoplasm, vsRNAs are translated into viral proteins, which are assembled as particles, and spread to other cells. During this process of transcription (in the nucleus of the first infected cell, and also in the cytoplasm of distal cells infected by the virus), dsRNA molecules are generated. These RNAs are recognized by DCL proteins, with one of the most common ones being DCL2, which generates 22-nt siRNAs and DCL4, which generates 21-nt siRNAs. Produced siRNAs are recognized by AGO proteins (such as AGO1), which is part of the RISC complex. This complex has the ability to cleave homologous viral RNA, as well as the endogenous target mRNA expressed in the VIGS vector, leading so to downregulation of the targeted gene. In the natural defence mechanism, 22-nt siRNAs generated by DCL2 trigger translational inhibition of viral genomes. These siRNAs also have the ability to migrate to distal cells via plasmodesmata, spreading the silencing signal, and leading to a new RNA silencing cycle. Adapted from Rössner et al., 2022.

Transferring knowledge from model plants to cultivated crops

The available knowledge about model plants used for basic research (mainly *A. thaliana* and *N. benthamiana*) is extremely extensive and luckily, detailed online resources are easily accessible. One of the main purposes of plant biotechnology, however, is transferring this knowledge, in combination with the developed techniques, to crops of agronomical interest. This approach is not always an easy task, mainly because of the basic biology of cultivated crops (long reproductive cycles, recalcitrance to *in vitro* regeneration or complex ploidy levels as an example). When talking about crops of agronomical importance, two large families stand out from the rest: Solanaceae and Cucurbitaceae.

Family Solanaceae

The Solanaceae family contains more than 3000 species, many of them being of great agricultural relevance (tomato, potato, pepper and eggplant for example) (Knapp and Peralta, 2016). Inside this family, *Solanum* is the most economically important genus, tomato being one of the most economically important vegetable crops around the world. Cultivated tomato (*Solanum lycopersicum L*.) was imported to Europe in the 16th century from the Andean region and its closest relatives are *Solanum pimpinellifolium* and *Solanum cheesmaniae* (Bergougnoux, 2014). Their ancestors were originated in the South American center of origin formed by Peru, Ecuador and Bolivia (Casas et al., 2019). Biological characteristics of tomato make it a source of valuable nutrients like lycopene, β-carotene and vitamin C. This vegetable is not only sold as fresh fruit in the market, but also as soup, juice, sauce or powder. These characteristics have had great importance in the fact that tomato production has doubled in the last decades. The world production in 2023 was more than 192 million metric tons, cultivated in 5.41 million hectares. Of the total production, 62.7% is concentrated in Asia, with China being the country that produces the largest amount of tomatoes in the world, followed by India and Turkey (FAOSTAT, 2025). Spain is considered the ninth world producer, and the second in Europe, only surpassed by Italy (**Figure 8**).

Cultivated tomato presents a high level of homozygosity, due to its autogamous biology (Fentik, 2017). The directionality of tomato crosses can be controlled, because of the relative ease of manual pollination and hybridization of individuals. Furthermore, this crop can also be propagated in an asexual way, by means of grafting (Melnyk, 2016), by which explants from different parts of the plant can give rise to a complete organism (Bergougnoux, 2014). The use of this method is usually recommended when trying to avoid the propagation of certain pathogens. Tomato is a diploid species with 12 pairs of chromosomes (2n=24) and it has a relatively small genome (around 900 Mb) lacking gene duplication (Bergougnoux, 2014; Fentik, 2017). Certain estimations suggest that cultivated tomato presents less than 5% of the genetic variation that is present in its wild relatives (Fentik, 2017). The study of the tomato genome has been a topic of great interest for researchers around the world for many decades. The deep study of the genotypic and phenotypic diversity of the traits in between cultivated and wild tomatoes performed to date, make this crop an ideal plant for quantitative trait loci (QTL) analysis. Because of the linkage maps obtained, molecular markers, generated mutants, and introgression lines developed, tomato can serve as a model organism for the Solanaceae family. In addition, tomato is the only model plant with compound leaves of which most of its genome is known (Kimura and Sinha, 2008).

Breeding objectives of tomato have changed and evolved during years, adapting to market and consumers' needs. Around 1970, breeding strategies were based on increasing total yield in the field, changing to improving shelf life around 1980. Years later, the main objective was improving fruit taste, and in the recent years the most desired characteristic is nutritional value (Bergougnoux, 2014). A huge focus trait in tomato breeding is resistance to pest and pathogens. Many of the resistances are inherited and there are several examples of resistance genes (R genes) introgressed in modern cultivars from wild tomato relatives (Chitwood-Brown et al., 2021). The most used method to obtain resistant plants has been hybridization or crossing, followed by a pedigree selection procedure. To recover the desired genotype after introgression, backcross selection is usually required (Sharma et al., 2019). Obtaining a new cultivar derived from the cross of two elite lines could take from 5 to 7 years, while introducing a new trait from a wild relative, followed

by backcross genotype recovering could take around 20 years (Bergougnoux, 2014). Although it is an autogamous crop, hybrid breeding has also been implemented in tomato (Fentik, 2017).

When using *S. lycopersicum* as female, crosses with most of the species in the tomato clade are successful, although embryo rescue is needed in some occasions (Bedinger et al., 2011). Mutation breeding is also another technique used in tomato to achieve resistance mainly to abiotic stress (Bergougnoux, 2014; Sharma et al., 2019). In recent decades, these classic methods have been combined with modern molecular techniques, which have benefit from the publication of the fully sequenced tomato genome in 2012 (Sato et al., 2012). This event and the ease of tomato to be transformed with *A. tumefaciens* favors the possibility of obtaining new cultivars with desired traits using transgenic technology. It should also be said that Flavr Savr tomato was the first commercially grown crop obtained by genetic engineering that was sold for human consumption (Fentik, 2017).

The use of VIGS technology in functional genetic studies has been widely validated in this species, demonstrating the ease of using viral vectors for tomato breeding purposes. This is largely due to the broad spectrum of viruses capable of infecting this crop. All of this has led to several studies demonstrating the use of VIGE technology in tomato, the ultimate goal being the production of transgenic-free edited plants.

Family Cucurbitaceae

Another family of plants that stands out for its great economic importance is Cucurbitaceae. More than 800 species grown globally belong to this family, being an important part of the diet in both developed and developing countries (Rolnik and Olas, 2020). Cucurbit´s edible plant belong to five main genera: *Cucurbita*, *Cucumis*, Citrullus, *Legenaria*, and *Sechium* (Ardabili et al., 2011). Usually considered as major crops, the most important cultivated cucurbits are cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), summer squash and zucchini (*Cucurbita pepo*), pumpkins and squash (*Cucurbita maxima*).

The cultivation of cucurbits for human consumption started more than 3000 years ago in Asia, while different species present different domestication regions (Rolnik and Olas, 2020). Summer squash and zucchini are native from North America, while pumpkins and squash were domesticated in Peru. The center of domestication of watermelon was Africa, and melon was domesticated twice, once in Asia and once in Africa (Chomicki et al., 2020).

Cucurbit production has traditionally been one of the strong points of Mediterranean countries agriculture. In 2023, Spain ranked second in Europe in both melon cultivated area (more than 16.5 thousand hectares) and total production (516 thousand metric tons), surpassing major producers such as France and Greece, and only surpassed by Italy in the Old Continent. Worldwide, 75.4% of total production is concentrated in Asia, being China the largest global producer, leaving far behind its closest competitor, India, with a production 9.5 times higher (FAOSTAT, 2025) (**Figure 8**).

Melon is a dicotyledonous diploid species (2n=24) with a genome size of 454 Mb. Compared to its close relative cucumber, melon presents a considerable larger genome probably due to transposon amplification (Garcia-Mas et al., 2012). Commercial melon varieties usually present unisexual flowers, occurring both male and females flowers in the same individual, although hermaphrodite flowers can also occur. Through ages, melon breeding objectives have evolved based on social needs. Human selection for larger melon fruits has been notable for decades. Fruit size is mainly controlled by three processes in the ovary: cell differentiation, cell division and cell expansion. However, fruit size regulation is a complex process in cucurbits (Chomicki et al., 2020). Most melon fruits present a 1:1 ratio of length/breadth, existing flexuosus types, which exhibit a ratio close to 4:1 (Burger et al., 2009). In terms of organoleptic characteristics, selection for sweetness, loss of bitterness and carotenoid accumulation has occurred during domestication (Chomicki et al., 2019). In addition, other primary breeding objectives in this crop are high female flower to male flower ratio, small seed cavity, attractive flesh colour and pathogen resistance (Kesh and Kaushik, 2021).

Classical breeding techniques have had huge relevance in melon breeding across years. To direct crosses among individuals, breeders are favored from male sterile plants, thus lacking the necessity to emasculate the female parental. Several recessive genes related to male sterility have been known for decades, although their transfer to new lines through crossing methods is really slow (Kesh and Kaushik, 2021). However, many economical important traits in *C. melo* show polygenic inheritance. The appearance and application of molecular markers to cucurbit breeding has allowed the generation of a huge variety of mapping populations, such as recombinant inbred lines (RILs), near-isogenic lines (NILs) or double haploids (DHs), adding in a very consistent way QTL mapping and so, melon breeding (Monforte et al., 2004; Moreno et al., 2008; Sáez et al., 2017).

Melon genome sequencing and annotation in 2012 brought with it the possibility of applying new genome editing techniques, such as CRISPR/Cas technology (Garcia-Mas et al., 2012). This species is considered recalcitrant for stable genetic transformation, which explains the low efficiency of CRISPR/Cas trials, as well as the smaller amount of literature available, in relation to other species of agronomical interest such as tomato. The first report of gene editing in *C. melo* was in 2019, obtaining an editing efficiency of 1.77% (Hooghvorst et al., 2019). Since then, there have been diverse approaches to increase transformation efficiency and edit genes of agronomical interest, with very positive outcomes (Nonaka et al., 2023; Shirazi Parsa et al., 2023; Z. Wang et al., 2024). These promising results encourage continued support for this type of research in a crop of great importance in our country, such as melon.

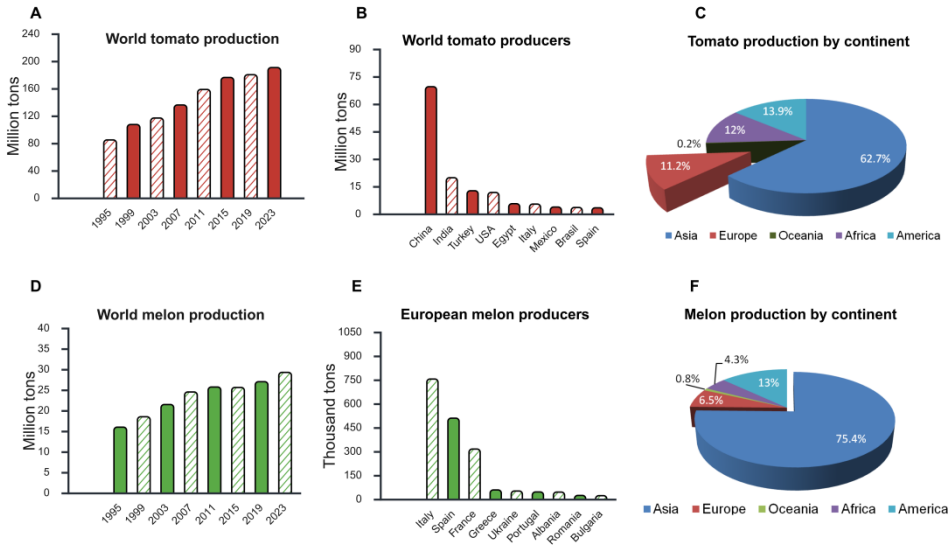


Figure 8. Schematised data of tomato and melon worldwide cultivation. Top part of the figure shows data related to global tomato cultivation. (**A**) World tomato production in metric tons from 1995 to 2023. During these years, tomato production has doubled, surpassing the 160 million metric tons in 2023. (**B**) Main world producers. Spain is considered the ninth world producer, and the second in Europe. (**C**) Global tomato production distributed by continents. Most of the tomato production worldwide is concentrated in Asia, with more than 62% of the total, being China the biggest producer in the world. Bottom part of the figure shows data related to global melon cultivation. (**D**) World melon production in metric tons from 1995 to 2023 which has kept increased among years, and has almost doubled in the mentioned 28 years period. (**E**) Main European producers. As in the case of tomato, Spain is considered the second European producer, surpassed by Italy. (**F**) Global melon production distributed by continents. As in the case of tomato, Asia is considered the biggest producer, covering more than 75% of world production. Data taken from FAOSTAT, 2025.

Objectives

In a world where exponential population growth is gradually diminishing our available natural resources, it is imperative to turn to science and technology to find ways to optimize the tools at our disposal and maximize crop yield. Since the beginning of the application of biotechnological tools to plant cultivation, various techniques have evolved that have allowed humans to develop crops of agronomical interest with characteristics and features that improve their performance and productivity. One of the most widely used techniques in this field is the use of viral vectors as delivery systems. The general objective of this doctoral thesis was to delve into the use of viral vectors and their application in the field of plant genome editing (CRISPR/Cas technology) as well as in the well-known area of gene silencing (virus-induced gene silencing, VIGS). To this end, the following specific objectives were established:

1. To validate, the use of a PVX-derived vector for sgRNA expression and the generation of CRISPR edits in Cas9-tomatoes.

2. To validate, the use of potyviral vectors as crRNA deliverers in Cas12-expresing model plants (*Nicotiana benthamiana*), as well as in crops of agronomical interest (tomato and lettuce).

3. To develop a CRISPR/Cas editing platform based on the combinatorial use of *Agrobacterium tumefaciens* stable plant transformation and the expression of gRNAs by means of a potyviral vector in melon, a species which presents serious difficulties when it comes to plant transformation and regeneration.

4. To develop a universal VIGS system that minimises the size of the insert homologous to the target gene, simplifying the cloning procedure, and increasing the applicability of the mentioned technique.

Chapter I

RNA virus-mediated gene editing for tomato trait breeding

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All authors participated in the work conception and design. M.U., V.A., Ar.G., S.M., S.G. and S.P. performed the experiments. All authors participated in the result and data analyses. M.U., F.P. and J.-A.D. wrote the manuscript with input from the rest of the authors. All authors discussed and revised the manuscript.

Abstract

Virus-induced genome editing (VIGE) leverages viral vectors to deliver CRISPR/Cas components into plants for robust and flexible trait engineering. We describe here a VIGE approach applying an RNA viral vector based on potato virus X (PVX) for genome editing of tomato, a major horticultural crop. Viral delivery of single guide RNA into Cas9-expressing lines resulted in efficient somatic editing with indel frequencies up to 58%. By proof-of-concept VIGE of *PHYTOENE DESATURASE* (*PDS*) and plant regeneration from edited somatic tissue, we recovered loss-of-function *pds* mutant progeny displaying an albino phenotype. VIGE of *STAYGREEN 1* (*SGR1*), a gene involved in fruit colour variation, generated *sgr1* mutant lines with recoloured red-brown fruits and high chlorophyll levels. The obtained editing events were heritable, overall confirming the successful breeding of fruit colour. Altogether, our VIGE approach offers great potential for accelerated functional genomics of tomato variation, as well as for precision breeding of novel tomato traits.

Keywords: CRISPR/Cas, virus-induced gene editing, tomato, trait breeding.

Introduction

Tomato (*Solanum lycopersicum* L.) is a major horticultural crop grown worldwide (https://www.fao.org/faostat/) and a model plant for studying the genetics of fleshy fruit and domestication traits. To address the limited genetic diversity of modern tomatoes and facilitate the development of new varieties with enhanced traits, novel breeding approaches are needed (Li et al., 2023; Pons et al., 2023).

Owing to their specificity and versatility, clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein (Cas) systems are widely used for efficient genome editing in numerous plant species, including tomato (Tiwari et al., 2023; Zsögön et al., 2018; Li et al., 2018; Alonge et al., 2020). Current genome editing methods mainly use the *Streptococcus pyogenes* Cas9 nuclease and single guide RNA (sgRNA) molecules that are delivered into plant cells through stable *Agrobacterium*-mediated transformation of constructs for the simultaneous Cas9 and sgRNA expression. Although powerful, these methods may require careful sgRNA design and combinatorial T-DNA construct assembly, selection of stable transformation events to recover transgenic plants, as well as complex breeding schemes to eventually obtain the desired mutant lines (Yang et al., 2023).

Virus-induced genome editing (VIGE) leverages viral vectors to transiently deliver CRISPR/Cas components into plants (Ali et al., 2015). Compared to alternative methods, VIGE (i) allows efficient and fast genome editing for rapid prototyping of sgRNA designs, (ii) minimizes the plant genome integration of exogenous DNA if based on RNA viral vectors, (iii) allows recovery of virus-free edited progeny under optimized conditions (Uranga and Daròs, 2023). VIGE has been successfully applied to model plants (Ellison et al., 2020; Ma et al., 2020; Uranga et al., 2021; Aragonés et al., 2022; Nagalakshmi et al., 2022), but not-yet reported for horticultural trait breeding of tomato. Given the VIGE great potential for advanced breeding strategies of this important crop, we developed and describe here a VIGE approach based on an RNA viral vector for tomato genome engineering. We show that the obtained modifications at

target genomic loci are heritable, and our VIGE approach can be used for horticultural trait breeding of tomato.

Results

Proof-of-concept VIGE of tomato *PHYTOENE DESATURASE* (*PDS*)

We previously reported efficient multiplex, heritable editing in *Nicotiana benthamiana* by infection of a transgenic line constitutively expressing Cas9 with a potato virus X (PVX; genus *Potexvirus*) vector for sgRNA expression in plant cells (Uranga et al., 2021; Uranga et al., 2023). PVX infects a wide range of Solanaceae species. To translate our reported VIGE approach to tomato, we first generated a transgenic Cas9-expressing line of the cultivar Micro-Tom (MT-Cas9) by *Agrobacterium*-mediated stable transformation. T_0 plants were selected on kanamycin-supplemented medium and by visual tracking of the expression of a reporter gene (DsRed). Transgene integration was confirmed by PCR amplification of a T-DNA fragment from plant genomic DNA. We next engineered pLX-PVX, an enhanced PVX vector based on a mini binary vector of the pLX series (Pasin et al., 2017) for autonomous replication in *Escherichia coli* and *Agrobacterium*. pLX-PVX allows *Agrobacterium*-mediated inoculation (agroinoculation) of PVX and virus-mediated sgRNA expression in plants (**Figure 1A**).

We reasoned that PVX-mediated sgRNA expression in MT-Cas9 would result in editing events of the tomato genome. For proof-of-concept VIGE assays, we assembled pLX-PVX::sgPDS for expression of a sgRNA targeting *PHYTOENE DESATURASE* gene (*PDS*) (**Figure S1A**; **Table S1** of supporting information), whose loss-of-function mutants have a reported photobleaching phenotype (Pan et al., 2016). Cotyledons of MT-Cas9 seedlings were agroinoculated with pLX- PVX::sgPDS, and samples were collected from upper uninoculated leaves after 6 weeks. PVX infectivity in inoculated plants was confirmed by RT–PCR detection of a viral fragment (**Figure 1B**). Although no photobleaching could be observed (**Figure S1B**), PCR amplification of a plant genomic fragment spanning the sgRNA target site followed by Sanger trace deconvolution revealed the presence of multiple *PDS* alleles with insertion–deletion (indel) mutations and

cumulative indel frequencies of 34.9– 58.0% (**Figure 1C**). These results confirm that RNA virus-mediated sgRNA delivery can produce somatic mutations in a tomato Cas9-expressing line.

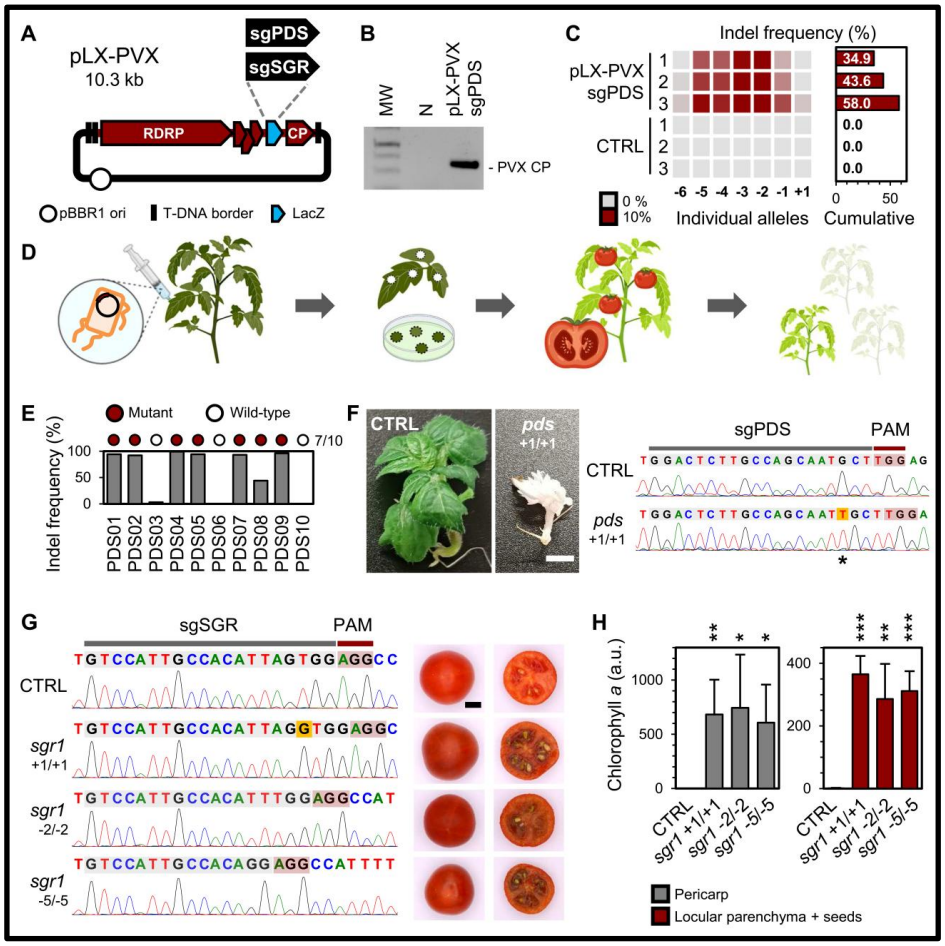


Figure 1. RNA virus-CRISPR/Cas9 editing in tomato. (**A**) Scheme of pLX-PVX, a mini T-DNA vector for agroinoculation of recombinant potato virus X (PVX) constructs for sgRNA delivery. The derivatives pLX-PVX::sgPDS and pLX-PVX::sgSGR include single guide RNAs (sgRNAs) targeting the tomato *PHYTOENE DESATURASE* (*PDS*) and *STAYGREEN 1* (*SGR1*), respectively. (**B**) *Agrobacterium*-mediated inoculation of pLX-PVX in tomato. RT–PCR detection of a PVX genomic fragment (CP) in a Cas9-expressing tomato cv. Micro-Tom (MT-Cas9) plant agroinoculated with pLX-PVX::sgPDS; samples were collected from upper uninoculated leaves; MW, DNA size standards; N, negative control. (**C**) Virus-

induced genome editing (VIGE) of *PDS* in tomato somatic cells. MT-Cas9 plants were agroinoculated with pLX-PVX::sgPDS; after 6 weeks, VIGE was assessed in upper uninoculated leaf samples by PCR of a *PDS* genomic fragment and Sanger trace deconvolution. Indel frequency percentages are shown; CTRL, uninoculated MT-Cas9 plants. (**D**) Experimental workflow for tomato trait breeding using the PVX-CRISPR/Cas9 system. sgRNAs are delivered into MT-Cas9 by pLX-PVX agroinoculation, whole plants are regenerated *in vitro* from upper uninoculated leaf tissue, and progeny is screened for mutant line identification. (**E**) VIGE of *PDS* in regenerated tomato plants. Indel frequency percentages and genotypic classification of 10 regenerated plants are shown. (**F**) Rescue of homozygous *PDS* mutant progeny. Phenotype and Sanger trace of the progeny line PDS02.3 with a homozygous loss-of-function *PDS* allele (*pds* +1/+1); CTRL, control condition. (**G**) Rescue of homozygous *SGR1* mutant progeny. Sanger traces and mature fruit phenotypes of progeny lines with homozygous loss-of-function *SGR1* alleles; CTRL, unedited MT-Cas9. (**H**) Chlorophyll quantification in fruits of *SGR1* mutants. Normalized fluorometric amounts of chlorophyll *a* in mature fruit samples are plotted (mean ± SD, n = 4); significance levels versus the control unedited MT-Cas9 (CTRL) as per Student's *t*-test are shown; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Given the high editing efficiency detected in somatic cells, we conceived an experimental work flow for heritable trait breeding using the PVX-CRISPR/Cas9 system in tomato (**Figure 1D**). At 21 days post inoculation (dpi), we collected upper uninoculated leaves of infected plants, detected *PDS* indel frequencies of 19.7–46.9% ($\bar{x}$ = 28.5; **Figure S1C**), and regenerated whole plants by tissue culture (**Figure 1D**). Genotyping of randomly picked green plants indicated that 70% (7/10) of them had *PDS* mutant alleles (**Figure 1E**). Among these, the line PDS02 had a 1-bp insertion allele (+1) and a 3-bp deletion allele (–3) in *PDS* (**Table S2**). To confirm the mutation heritability, fruits were collected from the self-pollinated PDS02 line and progeny was analysed. The recovered lines PDS02.1 and PDS02.2 were bi-allelic (+1/–3) or homozygous (–3) mutants showing the green, wild type phenotype (**Figure S1D**; **Table S2**). Remarkably, the progeny line PDS02.3 showed a photobleaching phenotype and the homozygous +1 allele (*pds* +1/+1; **Figure 1F**; **Table S2**), which caused the *PDS* complete inactivation. These findings demonstrate that our VIGE approach yields, through tissue culture regeneration, heritable editing events and can be used to recover loss-of-function mutant progeny.

VIGE of tomato *STAYGREEN 1* (*SGR1*) yields fruits with the *green-flesh* phenotype

Fruit colour is an important trait for fresh market tomatoes, and it is influenced by multiple genes that control pigment accumulation in the pericarp and peel (Yang et al., 2023; Zhu et al., 2018; Gianoglio et al., 2022). Heirloom accessions show a wide range of fruit colour variation; among others, Purple Calabash, Cherokee Chocolate, Black Krim, and Black Prince are mutants of *STAYGREEN 1* (*SGR1*) (Barry et al., 2009). *SGR1* inactivation inhibits degradation of chlorophyll *a*; simultaneous chlorophyll and lycopene accumulation during ripening produces a *green-flesh* phenotype characterized by red-brown fruits (Yang et al., 2023; Gianoglio et al., 2022).

Supported by the *PDS* editing results, we wondered if our VIGE approach could be used for *SGR1* editing and breeding of fruit color. We thus assembled pLX-PVX::sgSGR to target *SGR1* (**Figures 1A**, **S2A**; **Table S1**), and agroinoculated it into MT-Cas9 seedlings. At 21 dpi, Sanger trace deconvolution analysis revealed an average *SGR1* indel frequency of 21.4% in upper uninoculated leaves (**Figure S2B**), which were then used for *in vitro* regeneration of whole plants. 60% (9/15) of them had *SGR1* mutant alleles (**Figure S2C**). Subsequent fruit collection and progeny analysis allowed the identification of homozygous lines, with the same mutant allele of *SGR1* each (*sgr1* +1/+1, *sgr1* –2/–2, and *sgr1* –5/–5), and whose fruits displayed the *green-flesh* phenotype (**Figure 1G**); no PVX could be detected in these lines (**Figure S3**). Fluorometric and absorbance-based quantification revealed a significant increase of chlorophyll *a* amount in the pericarp and locular parenchyma plus seed samples of *sgr1* mutant lines compared to the control, unedited MT-Cas9 (**Figures 1H**, **S4A**). Results of the two complementary methods were highly consistent (r = 0.967, p < 0.0001; **Figure S4B**), overall confirming the *SGR1* inactivation. Altogether, our VIGE results prove the successful recovery of virus-free, *SGR1* loss-of-function mutants and thus horticultural trait breeding of tomato.

PVX-based VIGE of traditional tomatoes

We asked if our PVX-based VIGE approach could be applied to cultivated tomatoes. Traditional varieties show high stability of agro-morphological traits (Pons et al., 2023) and include Muchamiel and Pera, two cultivars popular in

Spain. PVX infectivity was confirmed in Muchamiel and a derived line with introgressed *Tm-2*^{2}, *Sw-5*, and *Ty-1* loci conferring resistance to tobamoviruses, orthotospoviruses, and geminiviruses, respectively (**Figure S5**). We then generated transgenic Cas9-expressing lines of Pera (Pera-Cas9; **Figure S6A**) that were agroinoculated with pLX-PVX vectors for sgRNA expression. Analyses of upper uninoculated leaves confirmed the PVX infectivity (**Figure S6B-C**), and revealed *PDS* and *SGR1* indel frequencies of 26–39% ($\bar{x}$ = 32.8%) and 30–57% ($\bar{x}$ = 44.9%), respectively (**Figure S6D**), overall, supporting the portability of our VIGE approach to cultivated tomato varieties.

Discussion

We reported here (i) the generation of a transgenic Cas9-expressing line of tomato cv. Micro-Tom (MT-Cas9); (ii) use of pLX-PVX, a T-DNA plasmid with an enhanced RNA viral vector, for sgRNA delivery into MT-Cas9 expressing plants; (iii) heritable, proof-of-concept VIGE of *PHYTOENE DESATURASE* and recovery of albino progeny; (iv) recovery of *green-flesh* coloured fruits by VIGE of *STAYGREEN 1*; and (v) generation of Cas9-expressing lines of a traditional cultivar and the successful editing of two genomic loci. Altogether, the results indicate that the presented VIGE approach can be readily applied for breeding of tomato traits with horticultural interest.

Simultaneous stable transformation of Cas nucleases and sgRNAs requires months until the DNA construct functionality is confirmed. Once edited lines are obtained, the continuous expression of sgRNA(s) and the selection marker used can limit subsequent transformation events and breeding for multiple traits. However, our Cas9-expressing lines with uncoupled sgRNA delivery would enable rapid prototyping of sgRNA designs within weeks, as well as unconstrained trait stacking through multiple VIGE rounds.

Zhenghe Li and co-workers recently reported an orthotospovirus vector system for viral delivery of Cas nucleases and their corresponding CRISPR guide RNAs, which enabled VIGE in a variety of non-transgenic crops (Liu et al., 2023). Our potexvirus-based VIGE approach presents a valuable breeding resource for

modern varieties with resistances that constrain the applicability of systems based on orthotospoviruses, tobamoviruses, and geminiviruses.

In sum, our work reinforces VIGE as a flexible and robust technology for accelerating functional genomics of tomato variation, as well as for precision breeding of novel traits to tackle global food supply (Kwon et al., 2020; Huang et al., 2021; Zhu et al., 2023) and health problems (Li et al., 2022).

Materials and methods

Plasmid construction

The binary vector GB2234 for plant expression of a codon-optimized *S. pyogenes* Cas9 (hCas9) sequence under the control of the cauliflower mosaic virus (CaMV) 35S promoter and *Agrobacterium tumefaciens* nopaline synthase terminator (P_{35S}:hCas9:T_{nos}) was reported (Uranga et al., 2021; Vazquez-Vilar et al., 2021). GB2234 includes a kanamycin resistance gene (*npt*II) and a red fluorescent protein gene (DsRed) for plant selection.

pLX-PVX was used for recombinant viral vector assembly. Potato virus X (PVX) genomic cDNA were amplified from pPVX (Uranga et al., 2021), a pgR107-derivative based on pGreen0000 which lacks autonomous replication in *Agrobacterium* (Lu et al., 2023). The obtained viral sequence was inserted into pLX-B2 (GenBank: KY825137; Addgene: 160636), a mini T-DNA binary vector of the pLX series (Pasin et al., 2017) for autonomous replication in *E. coli* and *Agrobacterium*. The T-DNA of pLX-PVX includes a full-length PVX cDNA flanked by the CaMV 35S promoter and terminator, single guide RNA (sgRNA) expression is driven by the PVX coat protein (CP) promoter, whereas a PVX CP truncated version (Dickmeis et al., 2014) is expressed from a heterologous promoter derived from bamboo mosaic virus. pLX- PVX includes the *LacZ* alpha peptide reporter for white-blue screen of recombinant vectors.

The sgRNA sequences of sgPDS and sgSGR, used to, respectively, target the tomato *PHYTOENE DESATURASE* (*PDS*; Solyc03g123760. 2.1) and *STAYGREEN 1* (*SGR1*; Solyc08g080090.2), are shown in **Table S1**. Recombinant pLX-PVX

derivatives for sgRNA expression were assembled using standard molecular biology techniques (Uranga et al., 2023). Briefly, fragments, including sgPDS and sgSGR fused to a truncated *Arabidopsis thaliana FLOWERING LOCUS T* sequence (Ellison et al., 2020), were obtained by PCR reactions including high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), pPVX::sgXT2B-tFT as the DNA template (Uranga et al., 2021), and the primer pairs D4747/D3898 and D4748/D3898, respectively (**Table S3**). Gel-purified fragments were inserted into MluI-linearized pLX-PVX by Gibson assembly reactions with the NEBuilder HiFi DNA assembly master mix (New England Biolabs). Recombinant plasmids were purified from white *E. coli* colonies selected on agar medium plates supplemented with kanamycin and 5-bromo-4-chloro-3- indolyl *β*-d-galactopyranoside (X-Gal). The obtained viral vectors pLX-PVX::sgPDS and pLX-PVX::sgSGR were verified by Sanger sequencing.

Plant materials

The tomato cultivar 'Micro-Tom' and the Spanish traditional accessions 'De la Pera 21' (Pera) and 'Muchamiel 18' (M18) were reported, as well as UMH 1200, an M18-derived line with the introgressed *Tm-2*^{2}, *Sw-5*, and *Ty-1* loci (Rubio et al., 2016).

Tomato stable transformation was done using GB2234 as described (Gianoglio et al., 2022). Transformants of Micro-Tom (MT-Cas9) and Pera (Pera-Cas9) were identified by kanamycin selection and monitoring of the DsRed reporter expression under an epifluorescence stereoscope (Leica DMS1000). Genomic DNA was purified from plant samples using silica gel columns (Zymo Research), and stable transgene integration was confirmed by PCR amplification of a 792-bp T-DNA fragment using the primer pair D3663/D3664 (**Table S3**). Plants were grown in greenhouse chambers at ∼24°C under a 16-hour day/8-hour night photoperiod.

Viral vector agroinoculation and analysis of virus infectivity

A. tumefaciens C58C1 cells including a disarmed pTi were electroporated with viral vector plasmids; colonies were selected on plates supplemented with rifampicin and kanamycin. Suspensions of the transformed bacteria were

prepared as described (Uranga et al., 2023) and used to inoculate fully expanded cotyledons from 10-day-old seedlings.

Total RNA was purified from upper uninoculated leaf samples using silica gel columns as described (Uranga et al., 2023). cDNA was synthesized using RevertAid reverse transcriptase (Thermo Fisher Scientific) and the primer D2409 (**Table S3**); the PVX CP sequence was amplified in PCR reactions including the obtained cDNA, Tth DNA polymerase (Biotools) and the primer pair D2410/D3436 (**Table S3**). RT-PCR products were analysed by electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, pH 7.2), and visualized by ethidium bromide staining.

***In vitro* regeneration of tomato plants**

At 21 dpi, systemic leaves showing symptoms of viral infection were detached, surface-sterilised by submersion in sterilisation solution (10% bleach, 0.02% Nonidet P-40) for 10 min, and then rinsed three times in sterile water. One-cm leaf slices were obtained and plated on to callus induction medium including 1× Murashige & Skoog salts (MS) with vitamins, 0.5 g/l 2-(N-Morpholino)ethanesulfonic acid hydrate (MES), 20 g/l glucose, 10 g/l phytoagar, 0.1 µg/ml 3-indolyl acetic acid (IAA), 0.75 µg/ml trans-zeatin, pH 5.7. Leaf slices were transferred to fresh plates every 2–3 weeks until shoots emerged. The shoots were then transferred to elongation medium (1× MS with vitamins, 0.5 g/l MES, 20 g/l glucose, 10 g/l phytoagar, 0.1 µg/ml trans-zeatin, pH 5.7) for 1–2 weeks until they reach a length of 1–2 cm. Subsequently, shoots were cut and transferred to rooting medium (0.5× MS with vitamins, 0.5 g/l MES, 20 g/l glucose, 10 g/l phytoagar, 0.1 µg/ml IAA, pH 5.7). Rooted plantlets (>5 cm) were transferred to soil and covered with plastic cups to keep moisture in. After acclimation to soil conditions, plastic cups were removed for adequate plant growth. Fruits were harvested from each plant for progeny analysis.

Genome editing analysis

Genomic DNA was purified from leaf samples using silica gel columns (Uranga et al., 2023). PCR using high-fidelity Phusion DNA polymerase, and the primer pairs D4762/D4763 and D4766/D4767 (**Table S3**) were respectively used to amplify tomato genomic fragments spanning the sgPDS and sgSGR target sites.

PCR products were separated by agarose gel electrophoresis, purified, and subjected to Sanger sequencing. Genome editing efficiency was quantified by Sanger trace deconvolution (Uranga et al., 2023) using TIDE (http://shinyapps.datacurators.nl/tide-batch/) or ICE (https://ice.synthego.com/).

Chlorophyll quantification

Samples were collected from red mature tomato fruits. Aliquots of pericarp tissue (±100 mg) and seeds plus the surrounding locular parenchyma (±50 mg) were, respectively, mixed with 10 or 20 volumes (~1 ml) of aqueous 80% (v/v) acetone precooled to −20°C, homogenized using a ball mill (Star-Beater, VWR) for 5 minutes at 30 s^{-1}, and mixed (1 h, 250 rpm) at 4°C in the dark. Samples were centrifuged at 14,000×*g* (5 minutes) to remove cell debris; supernatants were collected and kept in the dark until analysis.

Fluorometric quantification of chlorophyll *a* (chl *a*) was done in a monochromator-based plate reader (Infinite M Plex, Tecan Group) as reported (Pasin et al., 2014; Welschmeyer, 1994), with minor modifications. Briefly, the above-prepared supernatant samples were mixed 1:1 with aqueous 80% (v/v) acetone or aqueous 80% (v/v) acetone, 0.02 N HCl, to obtain native (*N*) and acidified (*A*) extracts, respectively. Samples were aliquoted to a black 96-well plate (Nunc), and top reading measurements recorded using λ_{Ex} 436 nm and λ_{Em} 680 nm; the instrument gain value was manually adjusted to avoid signal saturation. Fluorescence intensity of *N* and *A* extracts was measured to obtain F_N and F_A, respectively; chl *a* amount was calculated as $F_N - F_A$ with background values subtracted.

Spectrophotometric quantification of chl *a* was done as reported (Lichtenthaler and Wellburn, 1983). Briefly, supernatant absorbance was measured at 646 nm (A_{646}) and 663 nm (A_{663}) against an aqueous 80% (v/v) acetone blank in a plate reader (Infinite M Plex, Tecan Group), and chl *a* quantified using the equation:

Chl a=12.21*A663−2.81*A646

Statistics

Student's *t* test was used for two-group comparisons; significance levels of *p* values are indicated in the figures. Pearson correlation coefficient was calculated to estimate the linear relationship between the two variables.

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Data availability

Supplementary data accompany this work. The pLX-PVX vector is available at Addgene with catalog number 213040 (https:// www.addgene.org/213040/). The sequence information of tomato *PDS* (Solyc03g123760) and *SGR1* (Solyc08g080090) are available at Solanaceae Genomics Network (https://solgenomics.net/); GB2234 sequence is available at the GoldenBraid database (https://gbcloning.upv.es/feature/GB_UA_2578/).

Supplementary Data

Supplementary data is available at the end of this chapter.

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Supporting information

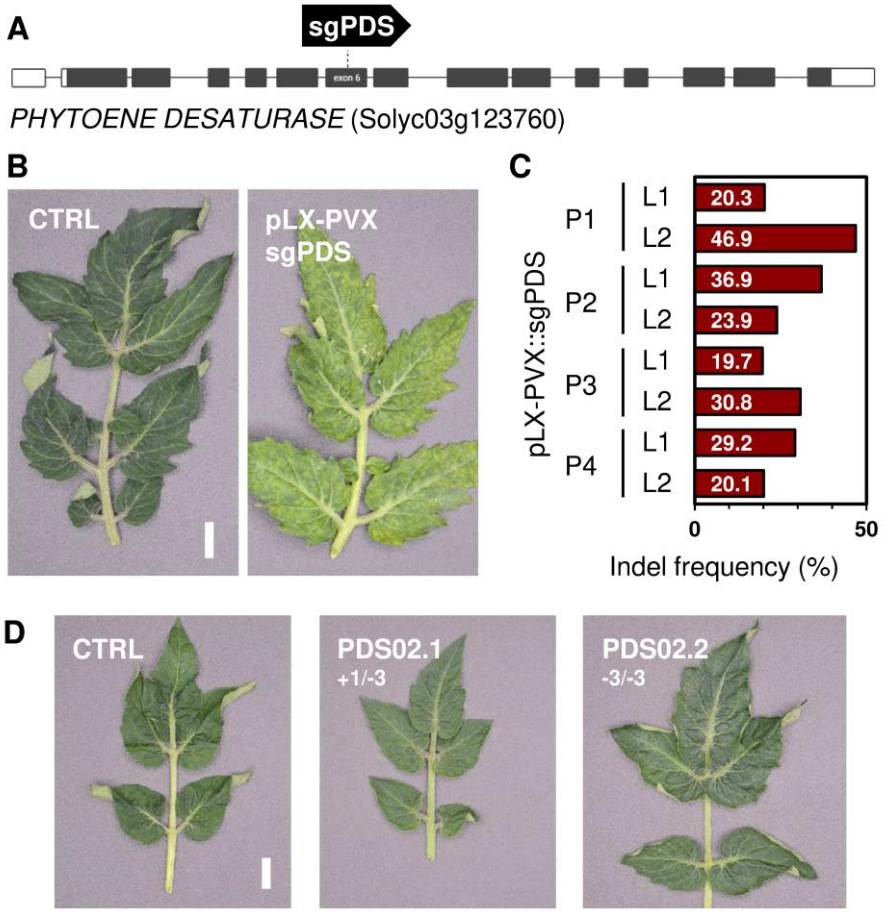


Figure S1. Virus-induced genome editing (VIGE) of tomato *PHYTOENE DESATURASE* (*PDS*). (**A**) Genetic structure of the tomato *PDS* (Solyc03g123760) and the pLX-PVX::sgPDS target region in exon 6. (**B**) Phenotype of a Cas9-expressing tomato cv. Micro-Tom (MT-Cas9) plant after 6 weeks of agroinoculation with pLX-PVX::sgPDS; CTRL, untreated plant; scale = 1 cm. (**C**) VIGE of *PDS* in tomato somatic cells. MT-Cas9 plants were agroinoculated with pLX-PVX::sgPDS; after 21 days, VIGE was assessed in upper uninoculated leaf samples by PCR of a *PDS* genomic fragment and Sanger trace deconvolution. Indel frequency percentages are shown for four plants (P1 – P4) and two leaves each (L1 – L2). (**D**) Pictures show the phenotypes of PDS02.1 and PDS02.2, progeny lines of a single plant (PDS02) regenerated from MT-Cas9 agroinoculated with pLX-PVX::sgPDS; see **Table S2** for genotyping results.

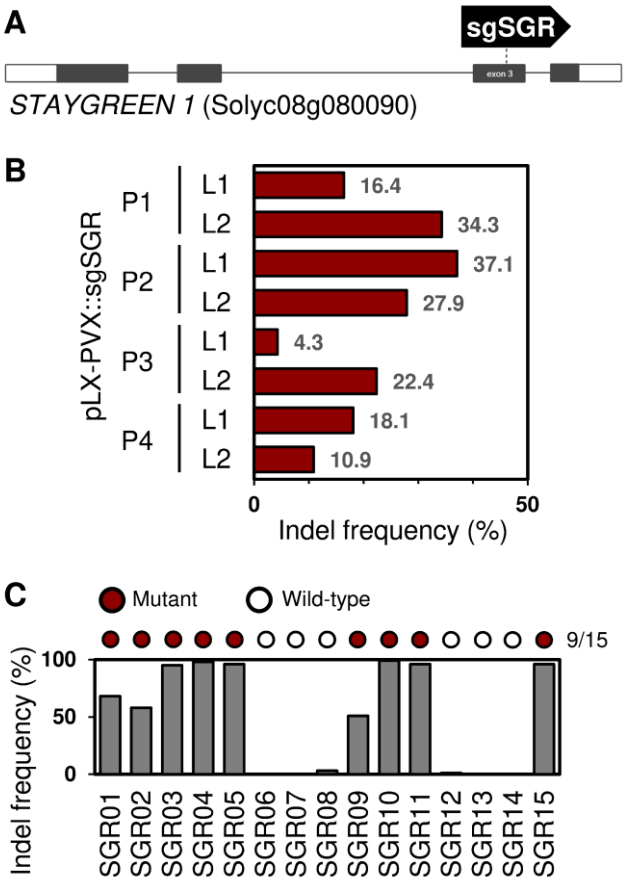


Figure S2. VIGE of tomato *STAYGREEN 1* (*SGR1*). (**A**) Genetic structure of the tomato *SGR1* (Solyc08g080090) and the pLX-PVX::sgSGR target region in exon 3. (**B**) VIGE of *SGR1* in tomato somatic cells. MT-Cas9 plants were agroinoculated with pLX-PVX::sgSGR; after 21 days, VIGE was assessed in upper uninoculated leaf samples by PCR of an *SGR1* genomic fragment and Sanger trace deconvolution. Indel frequency percentages are shown for four plants (P1 – P4) and two leaves each (L1 – L2). (**C**) VIGE of *SGR1* in regenerated tomato plants. Indel frequency percentages and genotypic classification of 15 regenerated plants are shown.

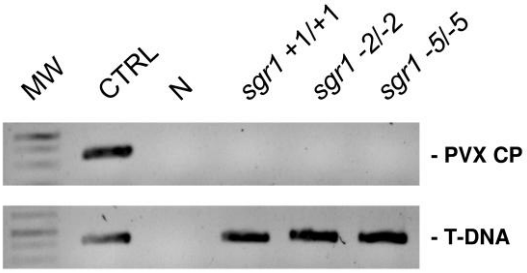


Figure S3. PVX detection in *SGR1* mutant lines. Top, RT–PCR detection of a PVX genomic fragment (CP) from total RNA samples; bottom, PCR detection of a T-DNA fragment from genomic DNA samples; MW, DNA size standards; CTRL, positive control; N, negative control.

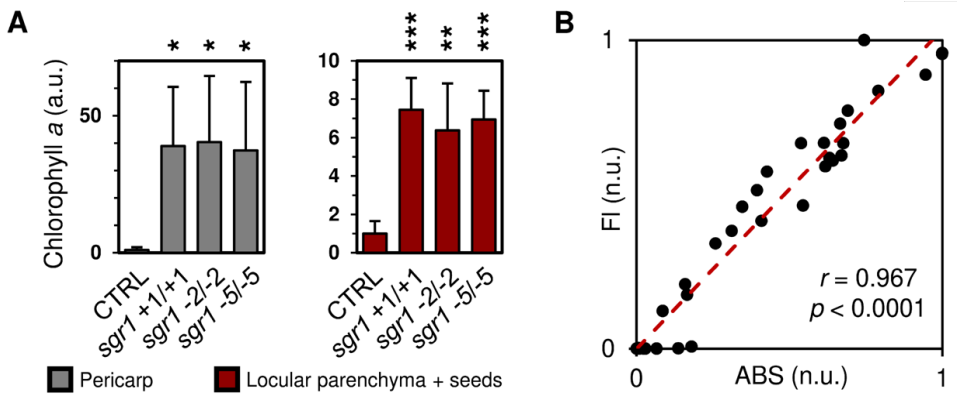


Figure S4. Chlorophyll spectrophotometric quantification in fruits of *SGR1* mutant lines. (**A**) Chlorophyll spectrophotometric quantification. Normalized absorbance-derived amounts of chlorophyll *a* in fruit samples are plotted (mean ± SD, *n* = 4); samples from the unedited MT-Cas9 line were used as the control condition (CTRL); significance levels versus CTRL as per Student's *t*-test are shown; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. (**B**) Linear relationship between chlorophyll *a* normalized units (n.u.) determined by spectrophotometric (ABS) and fluorometric (FI) analyses (*n* = 32); Person's *r* and *p* value are indicated.

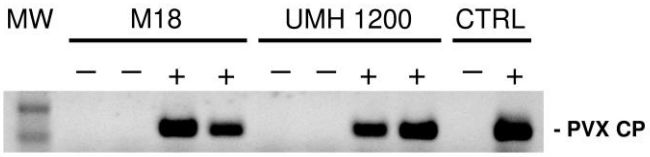


Figure S5. PVX infection of cultivated tomato varieties. PVX was inoculated to plants of the tomato lines Muchamiel 18 (M18) and UMH 1200, an M18 derived line (García-Martínez et al., 2011) with the introgressed viral resistance *Tm-2*^{2}, *Sw-5*, and *Ty-1* loci. After 3 weeks, total RNA samples were obtained from upper uninoculated leaves and RT–PCR detection of a PVX genomic fragment (CP) is shown. Each lane is from a single plant sample of the untreated (–) or PVX-inoculated (+) condition; MW, DNA size standards; CTRL, samples from control plants.

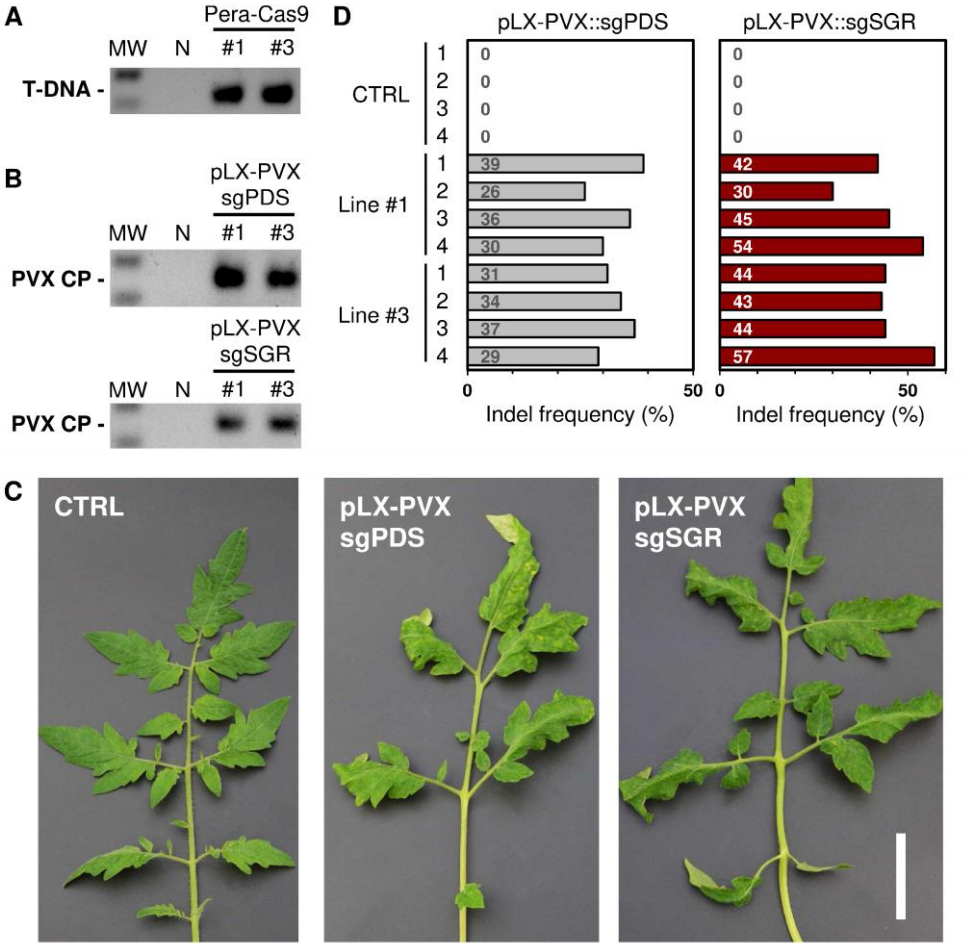


Figure S6. Somatic gene editing of Pera, a cultivated traditional tomato. (**A**) Generation of the transgenic Cas9-expressing lines #1 and #3 of the traditional cultivar Pera (Pera-Cas9). Gel shows PCR detection of a T-DNA fragment from genomic DNA samples; MW, DNA size standards; N, negative control. (**B**-**C**) Agroinoculation of the Pera-Cas9 lines #1 and #3 with pLX-PVX vectors for sgRNA expression. T_1 seedlings were agroinoculated with pLX-PVX::sgPDS and pLX-PVX::sgSGR. After 3 weeks, total RNA samples were obtained from upper uninoculated leaves and RT–PCR detection of a PVX genomic fragment (CP) is shown in **B**; MW, DNA size standards; N, negative control. Phenotype pictures are shown in **C**; scale bar = 5 cm. (**D**) VIGE of *PDS* and *SGR1* in somatic cells of Pera. Three weeks after inoculation, VIGE was assessed in upper uninoculated leaf samples by PCR of *PDS* and *SGR1* genomic fragments and Sanger trace deconvolution. Indel frequency percentages are shown for the Pera-Cas9 lines #1

and #3, and four plants per condition (1-4); CTRL, uninoculated Pera-Cas9 samples.

Table S1. Single guide RNAs used in the study.

sgRNA	Target gene	Target gene accession number	Spacer sequence	Reference
sgPDS	*PHYTOENE DESATURASE*	Solyc03g123760	GGACTCTTGCCAG CAATGCT	(Pan et al., 2016)
sgSGR	*STAYGREEN 1*	Solyc08g080090	GTCCATTGCCACA TTAGTGG	(Gianoglio et al., 2022)

Table S2. Heritability of *PHYTOENE DESATURASE* (*PDS*) mutations.

Line[a]	Allele	Allele sequence[b]	Allele frequency[c]
Control	wild-type	GGACTCTTGCCAGCAATGCTTGG	-
PDS02	+1	GGACTCTTGCCAGCAATTGCTTGG	47%
	-3	GGACTCTTGCCAGC---GCTTGG	44%
PDS02.1	+1	GGACTCTTGCCAGCAATTGCTTGG	48%
	-3	GGACTCTTGCCAGC---GCTTGG	46%
PDS02.2	-3	GGACTCTTGCCAGC---GCTTGG	74%
PDS02.3	+1	GGACTCTTGCCAGCAATTGCTTGG	97%

[a] The *in vitro* regenerated line PDS02 and its progeny lines PDS02.1, PDS02.2, and PDS02.3 are shown. [b] sgPDS target sequence with indels in mutant alleles shown in red; the protospacer adjacent motif (PAM) is underlined. [c] Frequencies by Sanger trace deconvolution.

Table S3. Primers used in the study.

Primer*	Primer sequence (5'→3')	Amplification	Purpose
D4747 (F)	GAGGTCAGCACCAGCTAGCAGGA CTCTTGCCAGCAATGCTGTTTTAGAGC	pPVX::sgXT2B-tFT fragment (238 bp) using D3898 as reverse primer	sgPDS cloning
D4748 (F)	GAGGTCAGCACCAGCTAGCAGTC CATTGCCACATTAGTGGGTTTTAGAGC		sgSGR cloning
D3898 (R)	GGGAAACTTAACAAACCCTATTG GCCATAAGTAACCTTTAG		
D2409 (R)	ATTTATATTATTCATACAATCAAACC		PVX cDNA synthesis
D3436 (F)	ATGTCAGGCCTGTTCACTATCC	PVX coat protein fragment (627 bp)	PVX detection
D2410 (R)	TGGTGGTGGTAGAGTGACAAC		
D3663 (F)	ATGATTGAACAAGATGGATTGC	*npt*II fragment (792 pb)	T-DNA detection
D3664 (R)	GAAGAACTCGTCAAGAAGGCGA		
D4762 (F)	TCTGTATTTTGTCTAGCTTCTCCT	*PDS* fragment (864 bp)	Genotyping the target site of sgPDS
D4763 (R)	TGCACTGCATTGAAAGTTCGTC		
D4766 (F)	TCCGTGCCGTCTATTGTTCT	*SGR* fragment (642 bp)	Genotyping the target site of sgSGR
D4767 (R)	ACCCAAACTAAAGCTTCTTGTAACT		

*F, forward primer; R, reverse primer.

Chapter II

Virus-induced gene editing using potyviral vectors in LbCas12a expressing plants

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Author contributions:
F.M. and J.A.D. conceived the work with input from the rest of the authors. A.G., U.R., I.Q.M. and F.M. performed the experiments. All authors analysed the data. F.M., A.G. and J.A.D. wrote the manuscript with input from the rest of the authors. All authors discussed and revised the manuscript.

Abstract

CRISPR/Cas systems are revolutionising precision genome editing and gene expression control in crop plants. While effective CRISPR-Cas applications traditionally rely on labour-intensive stable genetic transformation to deliver Cas nucleases and guide RNAs into plant cells, plant viruses are now emerging as a faster, more efficient alternative, a strategy known as virus-induced gene editing (VIGE). Cas12a, a Class 2 Type V CRISPR system, has become a vital alternative to Cas9 for plant genome engineering. Both nucleases offer precise editing, but Cas12a unique features make it particularly well-suited for VIGE. In this study, we first used the gold-standard tobacco rattle virus (TRV) vector to compare the editing efficiency of various target sequences and CRISPR RNA (crRNA) structures in Cas12a-expressing *Nicotiana benthamiana* plants, evaluating results in both infected tissue and seeds. Next, we developed a tobacco etch virus (genus *Potyvirus*)-derived vector designed for self-replication and systemic movement, efficiently delivering crRNAs throughout the plant. This approach enabled us to generate plants with all four edited alleles in the allotetraploid *N. benthamiana* through *in vitro* regeneration from infected leaves, and also to produce edited not-infected progeny. We then demonstrated the successful application of the potyviral vector for VIGE in agronomically important crops, such as tomato or cultivated tobacco. Finally, we replicated this design using two other potyviral vectors, turnip mosaic virus and lettuce mosaic virus. Given the conserved biological properties among potyviruses, we believe these findings are broadly applicable to this large genus, significantly expanding the host range of the VIGE technology.

Keywords: CRISPR/Cas, virus-induced gene editing, LbCas12a, *Potyvirus*, heritable editing.

Introduction

Technologies derived from the clustered regularly interspaced short palindromic repeat (CRISPR) defensive systems from bacteria and archaea are revolutionizing all aspects of life sciences. Among these, agricultural biotechnology stands as a field with exceptional potential for advancement through CRISPR-derived applications (Li et al., 2024). The escalating demands of a growing human population, compounded by global warming and the intrusion into natural ecosystems, mandate the development of improved crop varieties at an unprecedented rate. Such varieties are required to demonstrate increased productivity, enhanced nutritional content, and superior resilience to challenging environmental conditions. A particularly salient challenge is presented by the global proliferation of agricultural pests and pathogens. The dynamics of global commerce, combined with the aforementioned climate warming and human disturbance of wild ecosystems, are contributing to the dissemination of these threats on a scale previously unencountered by agricultural production. Despite the substantial contributions of conventional breeding to developing new varieties that counteract these challenges, the prolonged timelines associated with successive generations and the inherent limitations of available genetic resources strongly indicate the critical necessity for the rapid introduction of CRISPR technologies into agricultural systems.

CRISPR technologies have extensively been applied to plants since more than a decade ago (Feng et al., 2013). However, some aspects of plant biology, particularly the presence of a thick cell wall highly impermeable to large molecules pose some difficulties to the delivery of CRISPR reaction components. The most straight forward strategy to express CRISPR reaction components in plants is stable transformation. Although effective, this is a labour-intensive and time-consuming process that, in addition, cannot be generally applied to all crops and varieties. These characteristics make it difficult to use CRISPR technology in species that are recalcitrant to genetic transformation and regeneration through *in vitro* tissue culture. Alternative strategies to express the CRISPR reaction components in plant tissues are being intensively researched (Laforest and Nadakuduti., 2022). Among them, the use of viral vectors is gaining attention among plant biotechnologists, based on

simplicity and speed, and the promise of bypassing cumbersome tissue culture (Steinberger and Voytas, 2025; Khakhar and Voytas, 2021).

Focusing on plant virus-derived vectors able to move systemically through the plant, the so called virus-induced gene editing (VIGE) was first demonstrated in *Nicotiana benthamiana* plants transformed to constitutively express CRISPR associated nuclease 9 from *Streptococcus pyogenes* (SpCas9) using a tobacco rattle virus (TRV) vector to express the single guide RNA (sgRNA) (Ali et al., 2015). CRISPR edits were even detected in the progeny, although at a very low rate. Heritability was substantially improved by using an augmented sgRNA that incorporated a fragment of *Arabidopsis thaliana Flowering locus T* (*FT*) mRNA that was previously shown to favour RNA systemic movement, including the trafficking to the germline cells of the meristems, a tissue that is particularly surveilled to avoid viral infection (Ellison et al., 2020; Hoffmann and Incarbone, 2024; Nagalakshmi, et al., 2022). While expression of sgRNAs in transgenic lines that stably express Cas nucleases is an important step forward, VIGE aims to co-express both the sgRNA and the Cas nuclease. This aim is particularly difficult due to cargo limitation of plant virus-derived vectors, although remarkable progress has been recently done using negative-strand RNA viruses, as well as intensively researched miniature Cas nucleases (Liu et al., 2023; Yoshida et al., 2024; Weiss et al., 2025).

Cas12a (originally named Cpf1) is a Class 2 Type V CRISPR nuclease that has emerged as the most robust alternative to the widely adopted Cas9 for genome engineering. While both nucleases enable precise genome editing, Cas12a possesses several distinct features that make it particularly well-suited for CRISPR applications (Bernabé-Orts et al., 2019; Bandyopadhyay et al., 2020; Paul and Montoya, 2020; Zetsche et al., 2015; Yang et al., 2023). Unlike Cas9, which typically recognizes a guanine-rich PAM sequence, Cas12a recognizes thymine-rich PAM sequences. This significantly expands the range of genomic sites that can be targeted, especially in adenine and thymine-rich (AT) regions, such as promoters and introns, where Cas9 might not be suitable. Moreover, Cas12a has intrinsic endoribonuclease activity that allows it to process its own precursor crRNAs (pre-crRNAs) into mature crRNAs. This means it does not require a trans-activating crRNA (tracrRNA) or host RNase III, both of which are

needed for Cas9. Finally, Cas12a introduces staggered double-strand breaks in the target DNA with a 5' overhang of 4 or 5 nucleotides, unlike the blunt ends produced by Cas9. These staggered ends have been reported to favor the introduction of larger deletions as well as promoting gene insertion events by inducing homology-directed repair (HDR). It is noteworthy that, to date, few publications describe the implementation of VIGE in plant systems engineered to express Cas12a (Liu et al., 2023; Uranga et al., 2021).

Despite recent progress, VIGE must achieve substantial improvements to genuinely become a real alternative to stable plant transformation. A key challenge lies in host range: no universal viral vector exists. Each vector has a specific host range, and even with the same vector, CRISPR outcomes can vary drastically across different hosts. Specific host-virus interactions restrict the use of viral vectors. These restrictions are usually caused by the interaction of plant resistance (R) genes and viral proteins that act as elicitors of resistance (analogous to Avirulence (*Avr*) factors in the bacteria-plant pathosystem). Some R genes conferring resistance to specific viruses are the *N* gene, that confers resistance to tobacco mosaic virus (TMV); the potato *Rx1* gene, conferring resistance to potato virus X (PVX) and the *Arabidopsis HRT* gene, that confers resistance to turnip crinkle virus (TCV) (Marathe et al., 2002). While various viral vectors have proven useful for CRISPR applications, the VIGE toolbox still needs to expand. Therefore, our aim in this work was to extend VIGE capabilities to vectors within the genus *Potyvirus*, the largest group of plant RNA viruses. Collectively, potyviruses infect a broad spectrum of host plant species, many of which are of significant agronomical interest (Wylie et al., 2017).

In this study, we initially employed the well-established TRV vector for VIGE to investigate critical aspects of genome editing in Cas12a-expressing *N. benthamiana* plants (Bernabé-Orts et al., 2019). Specifically, we evaluated the efficiency of various crRNA sequences, the systemic spread of edits, and their heritability. Building on these findings, we then adapted the VIGE technology to utilize tobacco etch virus (TEV; genus *Potyvirus*) as a vector. Subsequently, we developed new tomato (*Solanum lycopersicum* 'Moneymaker') and tobacco (*N. tabacum*) lines constitutively expressing *Lachnospiraceae bacterium* ND2006

(LbCas12a), and successfully demonstrated the TEV vector functionality. Finally, we expanded VIGE to other potyvirus vectors, highlighting the broad applicability of our findings.

Results

Optimization of a *CHLI* reporter system to evaluate VIGE using a TRV-derived vector

VIGE is gaining impact in the world of plant biotechnology due to its great versatility and relative speed compared to more conventional techniques based on stable transformation. However, the properties of each viral vector are unique and can vary even within cultivars in a single host species, meaning that not all viruses can be efficiently used to express gRNAs in all host plants. The first objective of this work was to develop a robust VIGE system in *N. benthamiana* plants stably expressing the LbCas12a nuclease, as an alternative to SpCas9. To this end, we implemented a reporter system targeting the two homeologs of the *Magnesium chelatase subunit I* (*CHLI*), a component of an enzyme involved in chlorophyll biosynthesis (**Figure 1a**, left), which is frequently used as a reporter gene in CRISPR/Cas assays, due to the particularly visible yellow or whitish phenotype conferred by its impairment (Eid et al., 2021).

To efficiently knockout *NbCHLI*, we targeted the two homeologous loci present in *N. benthamiana* genome, which we called for simplicity *CHLI-A* (located on chromosome 10) and *CHLI-B* (located on chromosome 5). Using the CRISPOR online tool, we designed three different crRNAs (named as crRNA-1, crRNA-2 and crRNA-3), which simultaneously targeted exon 3 of both homeologs (**Figure 1a**, right). The crRNAs were composed of the Cas12 direct repeat scaffold (DR) followed by the 23-nt spacers (SP) CHLI1, CHLI2 and CHLI3, respectively. SP selection was based on the specificity scores given by the CRISPOR tool, trying to avoid guides presenting off-targets at other genomic loci different from that targeted. To test the efficiency of the selected crRNAs *in vivo*, we cloned them into a TRV gold-standard vector, well-known to be functional in VIGE (Ellison et al., 2020). In this vector crRNAs were expressed under the control of the pea

early browning virus (PEBV) promoter, and were cloned at the 3´ end of the coat protein (CP) coding region, in the TRV RNA2 segment, after removal of the non-structural proteins P2b and P2c (**Figure 1b**).

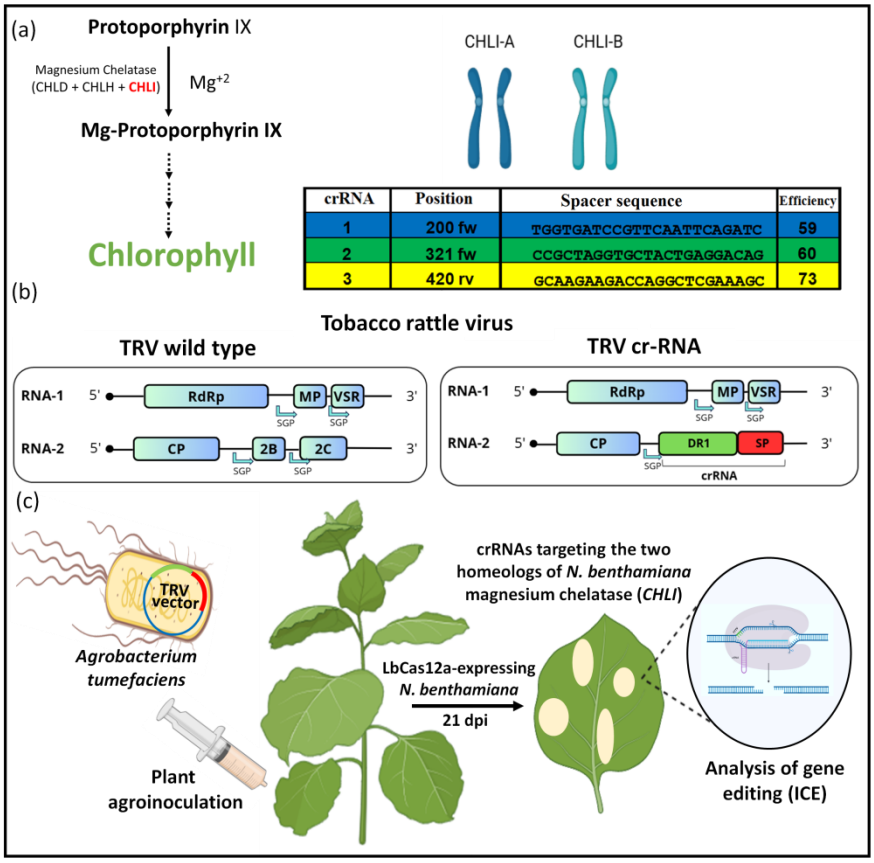


Figure1. Schematic representation of the guide selection procedure and workflow followed to evaluate our *CHLI* reporter system. (**a**) Metabolic pathway of chlorophyll biosynthesis, in which *CHLI* is involved. Three different crRNAs were selected that could simultaneously target both homeologs of *NbCHLI*, referred to in this article as *CHLI-A* and *CHLI-B*. Targets selection was based on the specificity scores given by CRISPOR tool. (**b**) The crRNA was formed by the LbCas12a direct repeat (DR) scaffold, and the 23-nt spacer (SP) located in its 3' end. The three crRNAs were cloned into a TRV gold-standard vector, and their expression was driven by the PEBV promoter. (**c**) *N. benthamiana* plants were agroinoculated with the three constructs, and gene editing was analysed at 21 dpi in systemic non inoculated leaves displaying photobleaching phenotype.

Viral constructs were agroinoculated in transgenic *N. benthamiana* plants expressing LbCas12a. Samples were taken from upper non inoculated leaves 21 days-post inoculation (dpi). Genomic DNA was purified, and the regions targeted by each crRNA were amplified by PCR, using primers specific of each homeolog. The Synthego ICE online tool was used to compare Sanger sequencing results with plants inoculated with the TRV empty vector, representing unedited control plants (**Figure 1c**).

Pictures of whole plants were taken at 30 dpi. The expected whitish-photobleached phenotype in upper non inoculated leaves was observed in plants inoculated with TRV-crRNA-2 and -crRNA-3, but not in plants inoculated with TRV-crRNA-1 (**Figure 2a**). Supporting these visual results, ICE analysis confirmed indel percentages in systemic leaves of around 40% in both homeologs of plants inoculated with TRV-crRNA-2 and -crRNA-3, while negligible genome editing was found (less than 5%) in plants inoculated with TRV-crRNA-1. We wondered whether these results would be consistent among time, and we analysed the same plants at 60 dpi. Again, both analysed homeologs displayed very similar ICE percentages for each crRNA, and average results had increased compared to the values obtained 30 days in advance (**Figure 2b**).

In order to confirm that, in the case of TRV-crRNA-1, absence of editing was related with the target sequence and not with a problem with viral replication, RT-PCR analysis was applied to detect the presence of TRV in the different samples. TRV detection in all the analysed samples supports the notion that crRNA-1 is not an efficient guide RNA for this technology. Representative sequencing electropherograms clearly showing gene editing in the expected targets sites of crRNA-2 and crRNA-3 are shown in **Figure 2c**. We realised that there was no significant difference between the average indel percentages obtained by analyzing the different homeologs separately, which suggests that the capacity of these two crRNAs to target both loci is the same. To deeper evaluate this idea, we individually plot the ICE values of both homeologs from 12 plants inoculated with each TRV-crRNA-2 and -crRNA-3. Results showed comparable indel levels between both homeologs in all the samples, confirming that these crRNAs are equally functional against both genomic loci (**Figure 2d**).

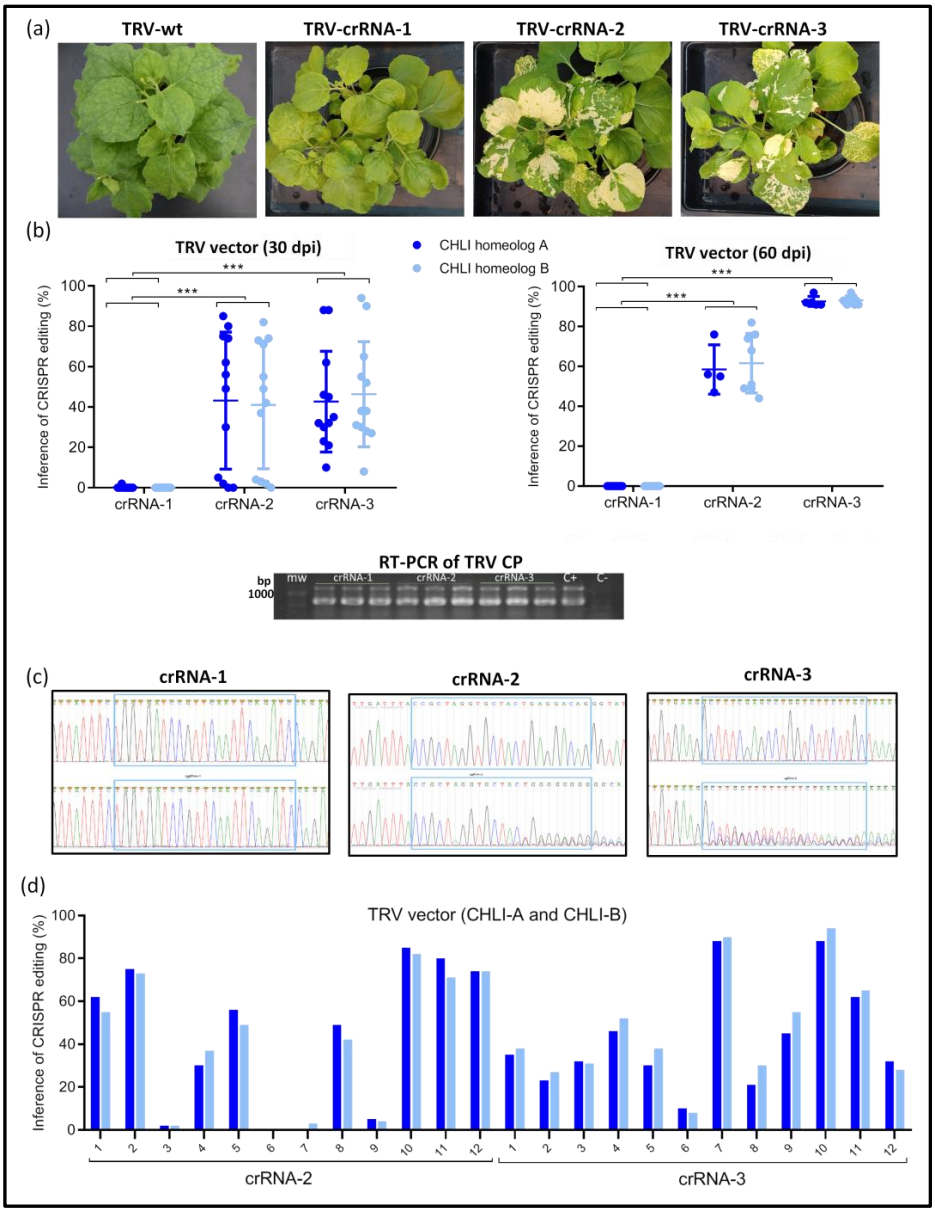


Figure 2. Comparison of the editing efficiency for three different crRNAs. (**a**) Pictures of representative whole plants agroinoculated with TRV vectors expressing the three selected crRNAs. A plant agroinoculated with a wild type TRV vector is displayed on the left. (**b**) Comparison of the editing efficiency in both *NbCHLI* homeologs using the three selected crRNAs at 30 dpi (left) and 60 dpi (right). Statistical analysis was performed using the GraphPad Prism 8

software. Data were analysed by two-way ANOVA with Tukey's posttest (***, P<0.001). Gel electrophoresis shows the results of an RT-PCR detecting the CP of TRV in all agroinoculated plants at 60 dpi. mw: molecular weight marker; c+: positive control; c-: negative control. (**c**) Sequencing chromatograms displaying representative Sanger sequences at target sites of the three crRNAs analysed. PAMs are highlighted by a black line. Electropherograms of control (unedited) plants are shown on top. (**d**) Analysis of the editing efficiency of crRNA-2 and crRNA-3 in the two *NbCHLI* homeologs from 12 individual samples per guide.

Analysis of the crRNA architecture in the TRV vector

To achieve heritable gene editing, we first tested whether our crRNAs structure could be more efficient when making any structural change. Based on previous studies supporting the use of two direct repeats flanking the 23-nt spacer (Bernabé-Orts et al., 2019), we generated a modified TRV-crRNA-2, which also presented a second direct repeat located at the 3´ end of the crRNA (**Figure 3a**). The architecture of this new crRNA was named as DR1-CHLI2-DR2. *N. benthamiana* LbCas12a plants were agroinoculated with TRV vectors expressing the crRNAs expressing CHLI2 spacer, with both architectures DR1-CHLI2 and DR1-CHLI2-DR2. Upper non inoculated leaves displayed at 21 dpi the expected photobleaching phenotype in both cases (**Figure 3a**). These visual symptoms correlated with the ICE percentages, in which values, although slightly higher for plants inoculated with the DR1-CHLI2-DR2 structure, did not present statistically significant differences (**Figure 3a**).

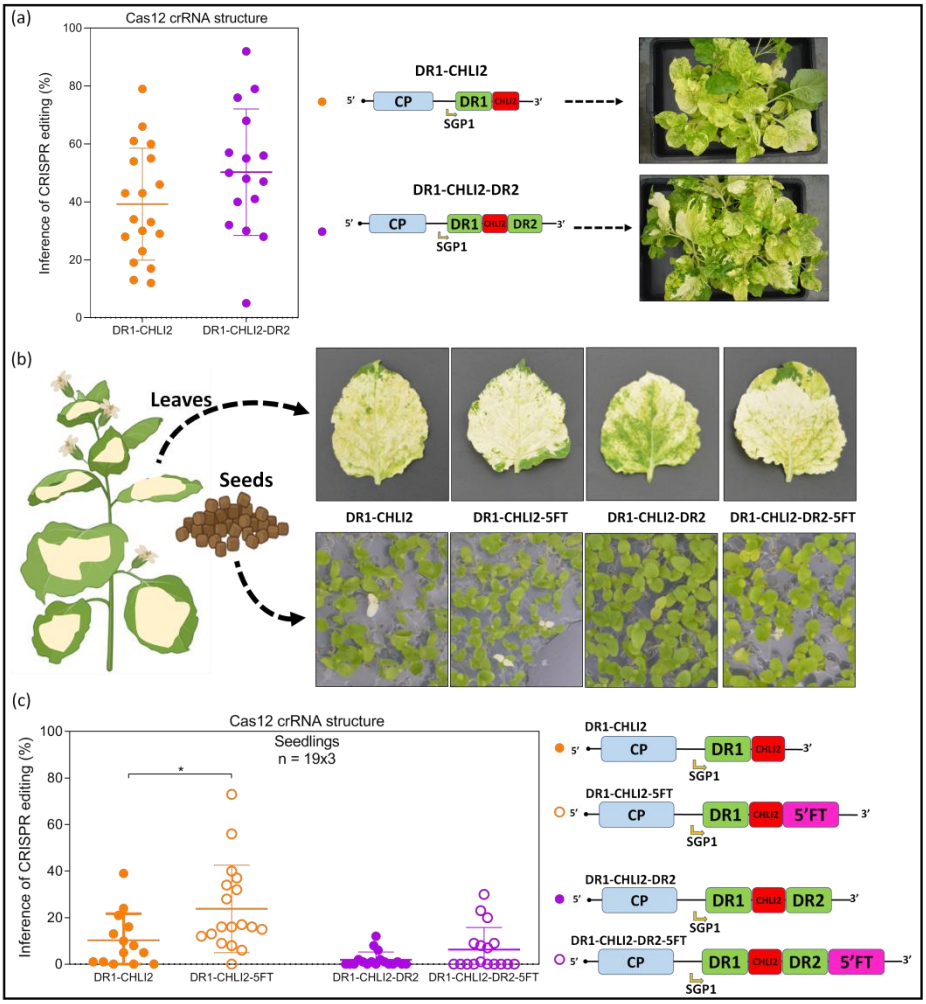


Figure 3. Heritable gene editing using our TRV vector. (**a**) A new crRNA-2 structure with an additional direct repeat (DR) located at the 3' end of the spacer (CHLI2, in red) was developed. Dot plot of the indel percentages of *N. benthamiana* plants inoculated with TRV vectors expressing the different crRNA-2 structures (DR1-CHLI2 and DR1-CHLI2-DR2). Pictures of representative whole plants agroinoculated with TRV vectors expressing the two different structures of crRNA-2 are displayed on the right. (**b**) Four different crRNAs structures, differing on the direct repeats flanking the spacer (CHLI2) and the presence or absence of the *A. thaliana Flowering Locus T* (5FT), were expressed by our TRV vector (DR1-CHLI2, DR1-CHLI2-DR2, DR1-CHLI2-5FT and DR1-CHLI2-

DR2-5FT). Leaves displaying white phenotype suggested the knockout of *NbCHLI* homeologs (top). Seeds from infected plants were collected and sown *in vitro* in sterile Petri dishes supplemented with sucrose (bottom). (**c**) Seedlings coming from three different plants for each vector were grouped in pools of three and total DNA was extracted and analysed. ICE values obtained are displayed on the left.

Heritable gene editing using the TRV and the *CHLI* reporter system

Obtaining an edited progeny after plant selfing, and therefore avoiding tissue culture regeneration, is one of the main goals of the VIGE strategy. For this purpose, our next milestone was to induce editing of the germline of agroinoculated plants. Different studies suggested that fusing mobile RNA motifs to guide RNAs can increase their mobility in plants, even reaching the germline (Ellison et al., 2020; Yang et al., 2023). To achieve this goal, we generated two new constructs based on the different TRV-crRNA-2 vectors (DR1-CHLI2 and DR1-CHLI2-DR2). We fused the 5' fragment of the *Flowering locus T* (5FT) from *Arabidopsis thaliana* to the 3´end of each crRNA, giving rise to two new architectures, named DR1-CHLI2-5FT and DR1-CHLI2-DR2-5FT (**Figure 3c**, right). These new constructs, along with the parentals with no 5FT, were agroinoculated in *N. benthamiana* LbCas12a-expressing plants. After 30 days, all agroinoculated plants displayed clear symptoms of *NbCHLI* inactivation in upper non-inoculated leaves (**Figure 3b**, upper row). Seeds from different flowers of three different infected plants (for each construct) were collected. After sterilisation, seeds were germinated *in vitro* on agar plates supplemented with sucrose. Completely white seedlings, indicating gene editing in the two alleles of each homolog were found in the progeny of plants inoculated with all the constructs but DR1-CHLI2-DR2 (**Figure 3b**, lower row). We further analysed the ICE percentages obtained in seedlings grouped in pools of three, showing that best heritable editing was obtained when using a single direct repeat and a 5FT fragment flanking the spacer (DR1-CHLI2-5FT) in the crRNA construct (**Figure 3c**, left).

Implementation of a potyviral-derived vector for VIGE using LbCas12a

Extending the VIGE technology to potyviruses is of great interest due to the large number of species in the genus and a broad collective host range. Although presenting a big cargo capacity, the ability to express heterologous sequences is restricted due to their replication strategy, which relies on the expression of large polyproteins. Thus, when expressing exogenous sequences within a potyviral vector, it is essential to keep the reading frame and not to introduce any stop codon in the coding sequence, which would impair viral replication and accumulation. To evaluate this, we selected the crRNA-3 instead of the crRNA-2 because the former had no stop codons in its sequence. The crRNA-3 was then cloned in between the nuclear inclusion protein b (NIb) and the coat protein (CP) cistrons of a vector derived from tobacco etch virus (TEV) (**Figure 4a**). *N. benthamiana* plants were agroinoculated, and ICE was evaluated as previously reported. A TRV vector expressing the same crRNA-3 was used as a control. At 21 dpi, plants showed the expected whitish phenotypes, and both homeologs of the *NbCHLI* gene were edited in the same proportion, when guides were delivered by TRV or TEV vectors (**Figure 4b**). However, TEV-infected plants presented necrotic symptoms and died after 30 dpi (**Figure 4b**). To overcome this situation, we designed four single-point mutations in the TEV HC-Pro cistron, that can attenuate symptom development in infected plants (Torres-Barceló et al., 2008). The four mutations were named AS20a, CLA2, CLA5 and AS13a. When inoculated with these virus variants, plants showed a better morphological development compared to the wild type version of our TEV-crRNA-3. However, only CLA2 and AS13a allowed plant survival and developing of functional flowers. Genome editing evaluation in upper non-inoculated tissues, showed comparable results in plants inoculated with the TEV-wt, the CLA2 and AS13a variants. Average indel percentage decreased in plants inoculated with the CLA5 variant, and was higher in plants inoculated with the AS20 variant (**Figure 4c**).

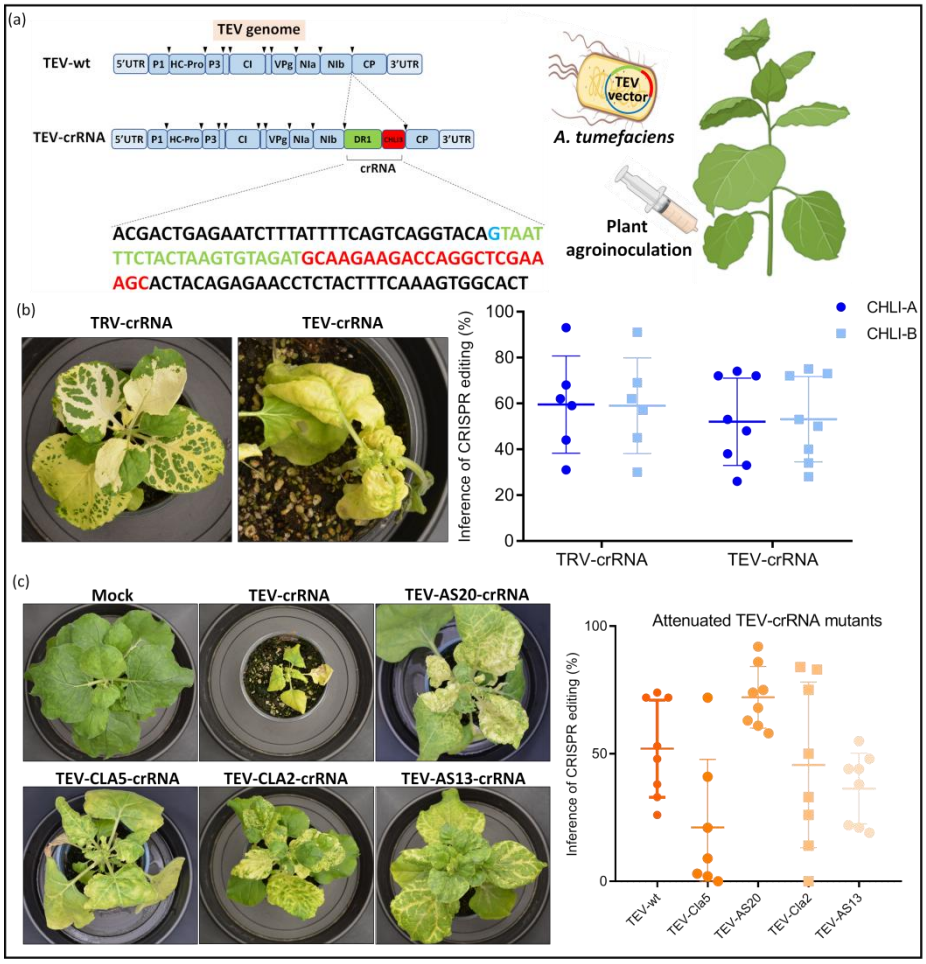


Figure 4. Developing a potyviral vector for crRNA delivery. (**a**) crRNA-3 was selected and cloned in between the NIb and CP cistrons of a TEV vector. A single DR (green) located at 5' of the spacer (red) was used. An additional "G" nucleotide (light blue) was added on the 5' end to maintain the ORF translation pattern (left). *N. benthamiana* plants were agroinoculated and systemic tissue was evaluated at 21 dpi. (**b**) Both TRV and TEV vector infected plants presented the expected whitish phenotype, but TEV killed the plants after 30 dpi. When analyzing ICE scores, both homeologs resulted to be edited at similar proportions, using the two viral vectors expressing crRNA-3. (**c**) Plants were inoculated with four attenuated versions of TEV: CLA2, CLA5, AS13a and AS20a (AS13 and AS20 for simplification). A considerable symptom reduction was observed (left). Inference of CRISPR edits was analysed in the four different variants developed, compared to the wild type TEV vector (TEV-crRNA) (right).

In order to obtain solid mutants, and thus generate individuals with the same mutation in all the cells, we decided to regenerate complete plants from infected tissue. At 60 dpi, tissues from plants inoculated with TEV-CLA2-crRNA-3 and TEV-AS13a-crRNA-3 presenting white patches were surface sterilised and used for tissue culture regeneration. Several fully white plants were obtained. Light green plants were also obtained in the case of the AS13a vector (**Figure 5a**). ICE analysis of both *NbCHLI* homeologs of four white plants regenerated from a TEV-CLA2-crRNA3 infected plant showed that all the alleles were edited, supporting complete inactivation of the reporter gene, and thus explaining the typical white phenotype. In the case of white plants regenerated from a TEV-AS13a-crRNA3 infected plant, the same outcome was observed, while light green plants presented only two or three edited alleles (**Figure 5b**). The presence of TEV was confirmed in all regenerated plants by RT-PCR (**Figure 5c**).

We finally wanted to evaluate the heritability of the genome edits, which were caused from attenuated TEV versions (TEV-CLA2-crRNA-3 and TEV-AS13a-crRNA-3). Seeds were collected from different flower buds close to white tissues, and they were germinated *in vitro* (72 seedlings per construct) (**Figure 5d**). ICE analysis of the obtained progeny revealed that 1 of the seedlings from TEV-CLA2-crRNA-3 infected plants presented an edited allele. However, no edited seedlings were found in the case of TEV-AS13a-crRNA-3 infected plants progeny (**Figure 5e**). Notably, TEV was not detectable by RT-PCR in the edited seedling. Taken together, these results suggest that the TEV attenuated vector (TEV-CLA2) can be implemented for VIGE, and edited plants can be obtained after tissue culture regenerations, as well as after plant selfing.

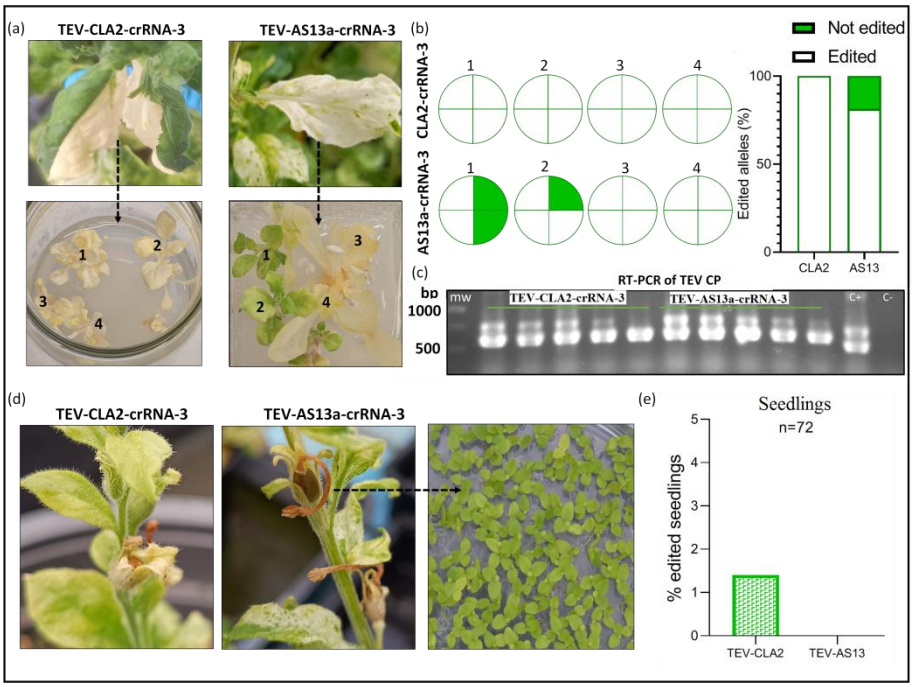


Figure 5. *In vitro* regeneration of edited plants from TEV-agroinoculated tissue and heritable gene editing. (**a**) Plant leaves displaying white patches were collected and served as explants for *in vitro* regeneration in agar plates supplemented with sucrose. Complete white plants were obtained after regeneration from plants infected with both TEV attenuated variants (TEV-CLA2-crRNA-3 and TEV-AS13a-crRNA-3). (**b**) ICE analysis was conducted on 4 plants from each condition. Results showed that most of the plants were completely edited. The proportion of edited (white) and non-edited (green) alleles is displayed for individual plants (left) and in the complete population (right). (**c**) Gel electrophoresis showing the results of a RT-PCR detecting the CP of TEV in regenerated plants. mw: molecular weight marker; c+: positive control; c-: negative control. (**d**) Seeds from plants agroinoculated with TEV-CLA2-crRNA-3 and TEV-AS13a-crRNA-3 were harvested and sown in sterile Petri dishes supplemented with sucrose. (**e**) The presence of edited alleles in both homeologs was evaluated by ICE in grown seedlings derived from mother plants infected either with TEV-CLA2-crRNA-3 or TEV-AS13a-crRNA-3.

VIGE with potyviral vector approach in species of agronomical interest

Encouraged by the validation of our TEV-based VIGE system in the model plant *N. benthamiana*, we set out to verify the transferability of the results to plants

of agronomical interest, such as cultivated tobacco and tomato. To do so, the first step was to obtain transgenic *Nicotiana tabacum* (cv. Ottawa) and *Solanum lycopersicum* (cv. Moneymaker) plants that expressed the LbCas12a nuclease. We first designed a construct (pLBHig-Cas12att) to transform the plants to stably express a temperature-tolerant version of LbCas12a (named LbCas12att), harboring the single mutation D156R. This nuclease is more efficient than LbCas12a when expressed at similar temperatures to those required for plant growth (Schindele and Puchta, 2020; Liu et al., 2019). The expression of the Cas nuclease was driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminator. Plants regenerated in selective medium were tested by PCR to verify the presence of the T-DNA. Positive plants were selfed to generate the lines that were later used in the analyses. Transformed tobacco and tomato plants expressing LbCas12att nuclease were inoculated with both TRV and TEV vectors expressing crRNA-3. The 23-nt sequence targeted by these vectors is conserved in *NtCHLI* homeologs, while presenting a single mismatch at position 21 in the case of *SlCHLI*. At 30 dpi plants displayed the expected photobleaching phenotype in upper non inoculated leaves, especially in tobacco (**Figure 6a**). Samples were taken from these tissues, and ICE analysis was performed. The results showed a robust gene editing in systemic tissue of both cultivars, with a comparable outcome when using the TRV or TEV vectors (**Figure 6b**).

Aiming to obtain solid mutants of *N. tabacum* plants, we repeated the same approach used with edited *N. benthamiana* plants. Adult plants were regenerated by tissue culture techniques from infected tissue collected 2 months post-agroinoculation. Gene editing in *NtCHLI* homeologs was verified in the explant leaf source, prior to plant regeneration. As in the case of *N. benthamiana*, several fully white plants were obtained, suggesting a complete inactivation of both *NtCHLI* homeologs. Similarly, light green plants were also obtained after regeneration (**Figure 6c**). ICE analysis of 8 different fully white plants proved the presence of CRISPR edits in all the four alleles of *NtCHLI* (**Figure 6d**). Light green plants presented either 1, 2 or 3 edited alleles. This showed that the presence of at least one allele, coding for a functional protein, leads to a chlorophyll level visible to the naked eye in *N. tabacum*. Considering the total amount of alleles analysed, more than 80% of them were classified as edited. Notably, TEV diagnosis in the regenerated plants by RT-PCR showed

that one of the plants, carrying one edited allele, was apparently free of virus (**Figure 6e**).

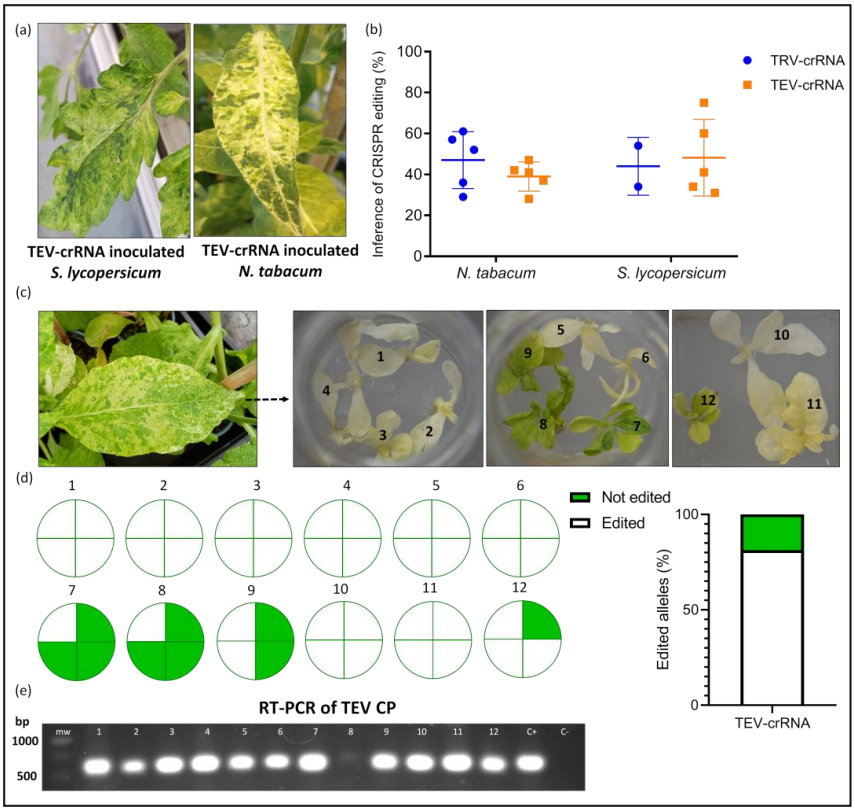


Figure 6. Implementation of our VIGE approach in species of agronomical interest. (**a**) *N. tabacum* plants and *S. lycopersicum* LbCas12att-expressing plants agroinoculated with TEV vectors displayed whitish and yellowish phenotypes at 30 dpi. (**b**) Both TRV and TEV vectors were able to induce similar CRISPR editing in inoculated plants. (**c**) As performed with *N. benthamiana*, *N. tabacum* plant leaves displaying white patches were collected and served as explants for *in vitro* regeneration in agar plates supplemented with sucrose (left). Complete white shoots, and light green plants were regenerated from *N. tabacum* plants infected with TEV-crRNA-3. (**d**) ICE analysis was conducted on 8 fully white plants, and 4 light green plants. The proportion of edited (white) and non-edited (green) alleles in individual plants (left) and in the complete population (right) is presented. (**e**) Gel electrophoresis showing the results of a RT-PCR detecting the CP of TEV in regenerated plants. mw: molecular weight marker; c+: positive control; c-: negative control.

Transferring the potyvirus-based VIGE approach to other virus species

The positive results obtained when expressing crRNAs using our TEV viral vector, led us to evaluate the possibility of using other potyviral vectors with the same purposes. This would increase the number of hosts that would benefit from our developed VIGE strategy. We therefore developed two vectors, derived from lettuce mosaic virus (LMV) and turnip mosaic virus (TuMV), which expressed crRNA-3 in the same position as previously described for our TEV vector. LbCas12a-expressing *N. benthamiana* plants were agroinoculated with the two viral vectors, and plants were analysed at 30 dpi. Upper non inoculated leaves displayed infection symptoms for both vectors (**Figure 7a**). ICE analysis supported this visual symptom evaluation. When guides were delivered by TuMV, 11 out of 12 analysed samples were positively edited, while for LMV, 50% of analysed samples presented genome editing in the target alleles (**Figure 7b**). Positively edited samples were plotted individually based on their ICE value, showing a higher editing efficiency of TuMV compared to LMV (**Figure 7c**). These results indicate that vectors based on different potyvirus species can be extensively applied for VIGE.

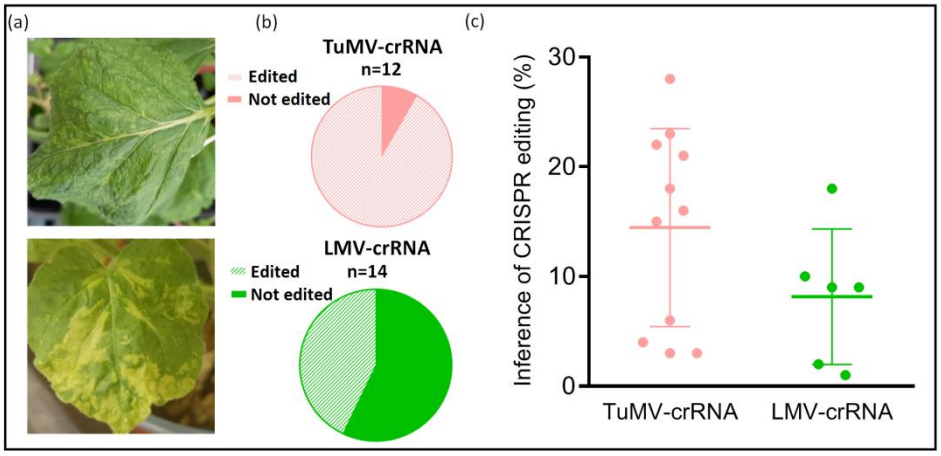


Figure 7. Transferring our VIGE approach to other potyviral systems. (**a**) LbCas12a-expressing *N. benthamiana* plants were agroinoculated with TuMV (top) and LMV (bottom) vectors expressing crRNA-3, and plants were analysed at 30 dpi. (**b**) Pie charts represent the proportion of samples that presented detectable editing in the *CHLI-A* homeolog, according to the ICE analysis performed. (**c**) Plot of ICE percentages from positive edited samples.

Discussion

In recent years, we have witnessed the emergence of CRISPR-Cas systems as highly effective platforms for precision genome editing and targeted control of gene expression. This technology holds significant appeal for crop science, offering the potential to develop new cultivars that are more productive, nutritious, resistant to pests and pathogens, and better adapted to evolving environmental conditions. However, plant genome editing has traditionally depended on stable genetic transformation and *in vitro* tissue culture regeneration. This lengthy and complex process involves *A. tumefaciens*-mediated genetic transformation to express CRISPR-Cas reaction components, followed by *in vitro* culture to regenerate whole plants. Consequently, the genomes of many plant species are difficult to edit due to low transformation efficiency or poor *in vitro* regeneration (Steinberger and Voytas, 2025). To overcome this limitation, VIGE has emerged as a faster innovative alternative, which also includes in some modalities a tissue culture-free process. VIGE utilizes plant viruses natural ability to replicate and spread systemically, employing them as vectors to deliver CRISPR-Cas reaction components directly to adult plants, ideally including the germline cells for heritable genome editing (Zhang et al., 2022; Uranga and Daròs, 2023).

The majority of VIGE-based research to date has utilized plants that express the SpCas9 nuclease. However, the Cas12a system could offer several key advantages over Cas9, broadening its utility in genome editing (Bernabé-Orts et al., 2019; Bandyopadhyay et al., 2020; Paul and Montoya, 2020; Zetsche et al., 2015; Yang et al., 2023). Unlike Cas9, Cas12a nucleases recognize thymine-rich PAM sequences (e.g., 5'-TTTN-3'), thereby expanding the range of targetable genomic sites, especially within adenine and thymine-rich regions like promoters and introns. Cas12a nucleases possess intrinsic endoribonuclease activity, allowing them to process their own crRNAs without the need for tracrRNA, which streamlines gene editing tool design and facilitates multiplex editing. Furthermore, Cas12a nucleases produce staggered double-strand breaks with 5' overhangs, which have been reported to favour gene insertion events and potentially promote homology-directed repair (HDR), unlike the blunt ends produced by Cas9. Their cleavage sites are distal to the PAM,

preserving the spacer sequence for potential subsequent rounds of cleavage, a benefit not seen with Cas9 which cleaves near the PAM. Notably, some Cas12a variants are smaller than SpCas9, and its gRNA is shorter, simplifying delivery, particularly via viral vectors. Studies also suggest that Cas12a may exhibit lower off-target effects and a greater propensity to induce larger deletions compared to Cas9. In addition, the thermotolerant LbCas12a engineered variant offers improved efficiency at typical plant growing temperatures (Schindele and Puchta, 2020).

TRV has become one of the most widely used viral vectors for genome editing and its ability to systemically spread through plant tissues has been used as an effective tool for delivering sgRNAs into transgenic plants that express a Cas9 nuclease (Ellison et al., 2020; Nagalakshmi et al., 2022). The first objective of our work was to develop a robust VIGE system in *N. benthamiana* plants stably expressing the LbCas12a nuclease. Various studies have demonstrated TRV effectiveness in inducing visible somatic gene edits by delivering sgRNAs targeting the *CHLI* gene. This gene is involved in the biosynthesis of chlorophyll, serving as an effective visual marker (Sun et al., 2023). In particular, *N. benthamiana* possesses two homeologous genes that encode this subunit, named in this work *CHLI-A* and *CHLI-B*. The expression of both must be interrupted to observe a visually detectable white phenotype (Huang and Li, 2009). Three distinct crRNAs were computationally designed using the CRISPOR online tool, aiming for simultaneous targeting of exon 3 in both homeologous genes (**Figure 1**). Each crRNA was structured with the Cas12a direct repeat scaffold preceding a 23-nt spacer. Spacer selection was rigorously guided by specificity scores provided by the CRISPOR tool, with a strong emphasis on avoiding off-target interactions at non-target genomic loci. Indel percentages, quantified via ICE analysis in systemic leaves, demonstrated approximately 40% editing efficiency in both homeologs following inoculation with viruses expressing crRNA-2 and crRNA-3. Conversely, plants treated with crRNA-1 showed negligible evidence of genome editing (**Figure 2**). These quantitative results increased with time, suggesting that VIGE efficiency can intensify over time in inoculated plants, probably due to the accumulative effect of editing complex. Notably, TRV was detected by RT-PCR in all the analysed samples, supporting the idea that crRNA1 is not an efficient guide RNA for this

technology. Although predicted efficiencies of the three selected crRNAs were very similar based on CRISPOR results, the results based on *in vivo* assays showed that *in silico* results are not completely transferrable (Concordet and Haeussler, 2018). A complementary analysis revealed that the designed crRNAs effectively and consistently targeted both homeologous genes, achieving similar editing levels across both. Consequently, evaluating the editing outcome of just one homeolog can serve as a reliable indicator of overall functional efficacy in future comparative studies, significantly reducing sequencing needs.

In model organisms like *N. benthamiana* and *A. thaliana*, VIGE's capacity to induce heritable modifications has been demonstrated when sgRNAs reach germline cells of SpCas9 expressing plants (Ellison et al., 2020; Nagalakshmi et al., 2022). Different studies have shown that fusing mobile RNA motifs to transcripts can increase their mobility in plants, and even direct them to the germline (Ellison et al., 2020; Yang et al., 2023). To achieve this goal, we compared four different designs based on the TRV-crRNA-2 vector. We obtained seedlings with at least one edited allele with all the assayed vectors, although the higher level of inheritance was achieved with the crRNA presenting a unique direct repeat at the 5´ end and a 102-nt fragment of the *A. thaliana FT* fused to the 3´end, which is in accordance with previous works (Ellison et al., 2020; Nagalakshmi et al., 2022). Notably, adding a second direct repeat at 3´ end, and therefore promoting a clean end of the crRNA after processing, did not increase significantly the editing efficiency neither in adult plants nor in seedlings (**Figure 3**). This strategy, however, could be used in a multiplexing approach, as Cas12a ability to process multimeric crRNAs containing two or more spacer sequences separated by direct repeats has already been described (Bernabé-Orts et al., 2019; Zetsche et al., 2017). Taken together, our results demonstrate the feasibility of using the TRV-vector to obtain edited seedlings from *N. benthamiana* plants expressing LbCas12a and therefore bypassing tissue culture regeneration.

Despite recent advancements, VIGE still necessitates substantial improvements to genuinely become as a robust alternative to stable plant transformation. A primary impediment is the restricted host range of viral vectors. Each vector exhibits a specific host range, and even with the same vector, CRISPR outcomes

can vary drastically across different hosts. While diverse viral vectors have demonstrated utility for CRISPR applications, the VIGE toolbox remains limited. Consequently, a key objective of this work was to broaden VIGE capabilities by incorporating vectors from the genus *Potyvirus*. Potyvirus-based viral vectors are particularly appealing because they constitute the largest group of RNA viruses infecting plants and, collectively, exhibit a broad host range, including major crops. They possess a positive-sense, single-stranded RNA genome of approximately 10 kb and are characterized by a gene expression strategy based on production of large polyproteins that are specifically processed by virus-encoded proteases (Revers and García, 2015). Given that their replication mechanism hinges on this polyprotein processing, any engineered clones must preserve the correct translational reading frame to ensure proper processing and accurate protein formation. Therefore, when expressing exogenous sequences within a potyviral vector, it is crucial to avoid introducing any premature stop codons in the coding sequence, as this would impair viral replication. For this reason, we selected the crRNA-3, instead of the crRNA-2, because the former had no stop codons in its sequence. The crRNA-3 was then cloned in between the NIb and CP cistrons of a vector derived from TEV (**Figure 4**).

Despite both homeologs of *NbCHLI* gene resulted edited in the same proportion when guides were delivered by TRV or TEV vector, TEV-infected plants presented necrotic symptoms and died after 30 dpi. To overcome this situation, we designed four single-point mutations in the HC-Pro cistron, which were previously described to attenuate symptom development in infected plants (Torres-Barceló et al., 2008). TEV CLA2 and AS13a mutants were successfully generated, exhibiting survival and functional flower development. We then explored the feasibility of obtaining stable mutants from the inoculated plants (**Figure 5**). Through tissue culture regeneration, we successfully recovered multiple fully white plants. ICE analysis confirmed that all alleles of both *NbCHLI* homeologs were edited, consistent with the complete inactivation of the reporter gene and the observed white phenotype. TEV presence was confirmed in all these regenerated plants. Intriguingly, when seeds from agroinoculated plants were germinated *in vitro*, one seedling from TEV-CLA2-crRNA-3 infected plants showed an edited allele, even though TEV was no longer detectable by

RT-PCR. These compelling results indicate that the attenuated TEV vector is a suitable tool for VIGE, offering paths to obtain edited plants both through tissue culture regeneration and, significantly, through seed propagation where the virus could be segregated.

We aimed to replicate these experiments in agronomically important crops like tobacco and tomato. For this, we generated transgenic tobacco and tomato plants expressing a temperature-tolerant LbCas12a nuclease (LbCas12att, specifically harbouring the D156R mutant previously reported) (Schindele and Puchta, 2020), and agroinoculated these transformed lines with TRV and TEV vectors carrying crRNA-3. Both vectors achieved 40-50% ICE levels in both species (**Figure 6**). Notably, the TEV vector did not require attenuation, as in the case of *N. benthamiana*, for these hosts to reach the flowering stage. Next, we evaluated the capacity to regenerate stable tobacco mutants from infected leaves showing editing symptoms. Similar to our *N. benthamiana* results, we successfully grew white plants on agar plates, all of which had all four of their alleles edited. Interestingly, one of the regenerated plants carrying one edited allele showed no detectable TEV via RT-PCR. Adding antiviral treatment to the regeneration media, as previously described, could further increase the number of regenerated edited, non-infected plants (Liu et al., 2023). Finally, to further enhance the versatility of our VIGE strategy, we successfully demonstrated the implementation of additional potyviral vectors, such as TuMV and LMV (**Figure 7**). This expands the potential number of plant hosts that could benefit from our gene-editing approach. While these vectors proved effective when tested in Cas12a-expressing *N. benthamiana* plants, future research should focus on evaluating their performance in their natural hosts, brassica and lettuce, following the development of the necessary transformed lines.

While expression of crRNAs in transgenic lines that stably express Cas nucleases is an important step forward, the major aim of VIGE is to co-express both the gRNA and the Cas nuclease. This would make it possible to avoid the transformation process, thereby avoiding future regulatory problems when entering the market. Expressing Cas nucleases with a viral vector is a particularly difficult aim due to cargo limitation of plant virus-derived vectors. However, significant advances have been recently made in this regard. CRISPR

edits were obtained in *N. benthamiana* wild-type plants when LbCas12a nuclease was expressed by means of a TEV vector lacking NIb protein, which was supplied by a potato virus X (PVX; genus *Potexvirus*), which also expressed the crRNA (Uranga et al., 2021). Moreover, using a vector derived from tomato spotted wilt virus (TSWV; genus *Orthotospovirus*), to express CRISPR/Cas12a reaction components, CRISPR edits were obtained not only in *N. benthamiana* plants, but also in crop plants such as tobacco, tomato, pepper and peanut (Liu et al., 2023). In addition, miniature variants less than half the size of Cas12a such as Cas12j and Cas12f have been developed and demonstrated functionality in editing of soybean and rice (Bai et al., 2025; Sukegawa et al., 2023). Among these, the AsCas12f variant (from *Acidibacillus sulfuroxidans*) stands out due to its compactness, which has allowed for its successful integration into viral vectors like PVX, achieving efficient gene editing in both inoculated and systemic leaves of *N. benthamiana* (Ishibashi et al., 2024). Future research should focus to incorporate novel miniature Cas nucleases into potyviral vectors. This approach seeks to directly produce DNA-free, non-transgenic edited seeds from wild-type inoculated plants, streamlining the editing process (Bai et al., 2025; Ishibashi et al., 2024). Ultimately, VIGE represents a substantial advance in plant biotechnology, capable of accelerating biological discoveries and offering a powerful, accessible, and potentially regulatory-compliant tool for precise genome engineering across a broad spectrum of economically vital crops and model plants.

Materials and methods

Design of crRNAs and vector construction

Both homeologs of *Nicotiana benthamiana Magnensium chlatase subunit I* (*CHLI*) gene (*CHLI-A* and *CHLI-B*), were selected as targets for our VIGE LbCas12a system. crRNAs were designed using the CRISPR-P online tool (http://crispor.tefor.net/) (Bernabé-Orts et al., 2019), specifically selecting sequences within exon 3 of both homeologs based on the specificity scores and trying to avoid guides with potential off-targets at other genomic loci different from the target ones. Genomic analysis determined that the region targeted by

crRNA-3, was conserved also in both homologs of *N. tabacum CHLI* (*NtCHLI*) gene.

Plasmid pLX-TRV2 cDNA contains a full-length tobacco rattle virus (TRV) cDNA, whose expression is driven by a cauliflower mosaic virus (CaMV) 35S promoter and terminator, and presents a hepatitis delta virus-derived ribozyme (Aragonés et al., 2022). This vector also incorporates the pea early browning virus (PEBV) coat protein (CP) promoter to drive *in planta* crRNA expression. The plasmid pGTEVa (Bedoya et al., 2012) contains the cDNA of an infectious tobacco etch virus (TEV) variant (GenBank DQ986288, with G273A, A1119G mutations), whose expression is driven by a CaMV 35S promoter and terminator within a binary vector derived from pCLEAN-G181 (Thole et al., 2007). In TEV-based recombinant vectors, the crRNA was strategically inserted at the 5' end of the TEV CP cistron. The first three codons of TEV CP, including silent mutations, were duplicated to mediate NIaPro proteolytic processing.

Vector developed for *N. tabacum* and *S. lycopersicum* stable transformation, pLBHig-Cas12att, derives from minibinary vector pLX-B2 (Pasin et al., 20017), which contains the broad host-range pBBR1 origin for plasmid replication suitable for both *E. coli* and *A. tumefaciens*. Transformation plasmid T-DNA expresses the *hygromycin phosphotransferase* (*hpt*) gene as a selection marker, under the control of the NoS promoter and terminator of *A. tumefaciens*. pLBHig-Cas12att expresses a temperature-tolerant version harbouring the single mutation D156R of a LbCas12 nuclease previously reported (Schindele and Puchta, 2020; Liu et al., 2019). This nuclease was under the control of a dual CaMV 35S promoter and a 35S terminator. 5´ and 3´untranslated regions (UTR) of cowpea mosaic virus (CPMV), which served as stabilizing elements, flanked the CDS of the nuclease. The nuclease was fused to two nuclear localization signals (NLS), of nucleoplasmin and simian virus 40 (SV40) in their carboxyl terminus.

All recombinant plasmids were constructed using standard molecular biology techniques. These included PCR amplification of crRNAs with high-fidelity Phusion DNA polymerase (Thermo Scientific), Gibson DNA assembly utilizing the NEBuilder HiFi DNA assembly master mix (New England Biolabs) or

restriction and ligation cloning with T4 DNA ligase (Thermo Scientific). The sequence of all constructs is presented in **Figure S1**. The sequence of the construct used for plant transformation (pLBHig-Cas12att) is presented in **Figure S2**.

Plant growth conditions and inoculation

Transgenic LbCas12a-expressing *N. benthamiana*, *N. tabacum* and *S. lycopersicum* plants were grown under controlled conditions at 25°C and a 16-h day/8-h night cycle. In the case of tobacco and *N. benthamiana*, fully expanded upper leaves from 4- to 6-week-old plants were used for inoculation with viral vectors carrying crRNA constructs. For tomato plants, the first true leaf and one cotyledon were inoculated.

Agrobacterium tumefaciens C58C1 electrocompetent cells carrying the pLX-TRV1 plasmid were electroporated with pLX-TRV2 derivatives and selected on Luria Bertani (LB) plates supplemented with rifampicin (50 µg/ml), gentamicin (20 µg/ml), and kanamycin (50 µg/ml). The same *A. tumefaciens* strain, previously transformed with the pCLEAN-S48 helper plasmid (Thole et al., 2007) was used for TEV-derivatives expression. Transformed bacteria were selected on LB plates supplemented with rifampicin (50 µg/ml), kanamycin (50 µg/ml) and tetracycline (7.5 µg/ml).

Individual colonies were selected and grown for 24 h at 28°C in liquid LB medium supplemented with 50 µg/ml kanamycin in the case of TEV clones. For TRV clones, liquid medium was also supplemented with 20 µg/ml of gentamicin. When cultures reached an optical density at 600 nm (OD600) of 1-2, they were pelleted by centrifugation and resuspended to an OD600 of 0.5 in inoculation buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH pH 5.6), 10 mM $MgCl_2$, supplemented with 150 µM acetosyringone (Bedoya et al., 2010). Bacterial cultures were incubated in dark for 2 h at 28°C. The abaxial side of selected leaves or cotyledons were infiltrated using a needle-less 1 ml syringe. After infiltration, plants were transferred to a growth chamber under a 12-h day/12-h night cycle at 25°C. Three weeks after inoculation, systemic non

inoculated leaves were sampled (1 cm^2 approximately), immediately frozen in liquid nitrogen and stored at -80°C until they were processed.

Analysis of Cas12-mediated gene editing

Plant tissue was ground in 2 ml Eppendorf tubes containing a 4-mm diameter stainless steel ball, and using a VWR Star-Beater mill, for 1 min at 30 sec^{-1}. Extraction buffer (4 M guanidinium thiocyanate, 0.1 M sodium acetate, 10 mM EDTA and 0.1 M 2-mercaptoethanol, pH 5.5) was previously added to each tube. After homogenization, samples were centrifuged at 15,000g for 5 min. An aliquot of the supernatant (0.7 ml) was transferred to a silica-gel column (Zymo Research). After centrifugation at 15000g for 1 min, the column was washed twice with 0.5 ml of washing buffer (70% ethanol, 10 mM sodium acetate, pH 5.5). The DNA was eluted from the column in 10 µl of 20 mM Tris-HCl, pH 8.5.

N. benthamiana CHLI-A and *CHLI-B* target fragments corresponding to both homologs were individually amplified by PCR using high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific) and primers 5'-TTACGATAGGAAGTATATTT-3' and 5'-AACTCGAAGATCATGATCTA-3' (for *NbCHLI-A*) or 5'-TTACGACACGAAGTATATAG-3' and 5'-AACTCGAAGATCATGAGCAG-3' (for *NbCHLI-B)*. In the case of *N. tabacum* samples, a primer pair amplifying both homeologs at the same time was used (5'-GGAGGAGAGCCAGAGACCGGTG-3' and 5'-TTAGTTCTGCACAGACCTTAG-3'). To amplify the target region of *SlCHLI*, primer pair 5'-ACAGCCAGAGACCGGTGTATCC-3' and 5'-CTTGGGCATGCATTCCAAATCG-3' was used. PCR products were separated by agarose gel electrophoresis, purified from the gel, and subjected to Sanger sequencing. The presence of sequence modifications was analysed using the inference of CRISPR edits (ICE) software by the EditCo Bio (https://ice.editco.bio/).

RNA purification and analysis of virus infectivity

To verify the presence of TEV in *N. benthamiana* and *N. tabacum* seedlings and plants regenerated from tissue culture, a RT-PCR was performed. Total RNA was purified from leaf tissue using silica-gel columns (Zymo Research). The protocol followed was the same as previously described for DNA isolation, with an additional step. Before loading into the column, the supernatant was mixed

with 0.65 volumes of 96% ethanol and centrifuged at 15,000g for 1 min. Aliquots of 1 µl of the purified RNA were used for reverse transcription (RT) with RevertAid reverse transcriptase (Thermo Scientific), using 5'-TCATAACCCAAGTTCCGTTC-3' primer. 1 µl of the obtained cDNA was used for PCR amplification with primers 5'-CATCTGTGCATCAATGATCGAA-3' and 5'-GTGTGGCTCGAGCATTTGACAA-3'. PCR products were analysed by electrophoresis in 1% agarose gels in TAE buffer, and gels were stained with ethidium bromide.

Plant stable transformation

Transgenic LbCas12a-expressing *N. benthamiana* plants were obtained as previously described by Bernabé-Orts et al.,2019. *N. tabacum* transformation was performed with *A. tumefaciens* strain LBA4404. Transformation and regeneration were done following a modification of transformation and *in vitro* regeneration standard procedure (Fisher et al., 1995). *In vitro* regeneration steps were carried out under light, humidity and temperature-controlled conditions (16-h light/8-h dark; 26 ± 2°C, 40% relative humidity during the light period; 22 ± 2°C, 70% relative humidity during the dark period). Selective medium for plant regeneration was supplemented with 5 µg/ml of hygromycin.

S. lycopersicum transformation experiments were developed as previously described using *A. tumefaciens* strain LBA4404 (Ellul et al., 2003). Light, humidity and temperature conditions for plant regeneration were the same a used in tobacco regeneration. Selective medium for plant regeneration was supplemented with 5 µg/ml of hygromycin. For both species, T-DNA detection in regenerated plants was performed by means of PCR, using primers 5'-ATAAGGAGACAGACTATCGGGC-3´ and 5´-TGATCTGTCCGTGATTGTTCTC-3´ that amplify a region of ttLbCas12a nuclease.

***In vitro* regeneration of edited *N. benthamiana* and *N. tabacum* plants**

Upper leaves showing symptoms of viral infection were removed and submerged in water for 30 min to remove dirt and dust present on the surface. After that, leaves were surface-sterilised by submersion in 50% commercial bleach solution for 10 minutes, and subsequently washed three times in sterile water. Sterilised leaves were cut into pieces of approximately 1 cm^2 using a

scalpel, and were transferred to an organogenesis medium, composed of 4.3 g/l Murashige and Skoog (MS) with vitamins, 30 g/l sucrose, 8 g/l bacteriological agar, 1 µg/ml of 6-benzylaminopurine (BAP), and 0.1 µg/ml 1-naphthaleneacetic acid (NAA), pH 5.8. Leaf explants were transferred to fresh plates every 2 weeks until shoots emerged. Shoots were cut and transferred to shoot elongation medium, composed of 4.3 g/l MS with vitamins, 30 g/l sucrose, 8 g/l bacteriological agar, 0.1 µg/ml BAP, and 0.1 µg/ml NAA, pH 5.8. Elongated shoots were transferred to root induction medium, composed of 4.3 g/l MS with vitamins, 30 g/l sucrose, 7 g/l bacteriological agar, and 0.2 µg/ml NAA, pH 5.8. Regenerated plantlets with roots were analysed for editing of the target genes in the case of *N. benthamiana*. In the case of *N. tabacum*, elongated unrooted shoots were analysed.

Seed germination of *N. benthamiana* and *N. tabacum*

N. benthamiana seeds were collected from infected plants. Seeds from different flowers were pooled in the same Eppendorf tube, and they were sterilised with 50% bleach and 0.1% Nonidet P-40 and washed with sterile water three times before sowing on LB plates with germination medium (4.3 g/l MS, 0.1 g/l MES, 20 g/l sucrose, 7.5 g/l agar). Once germinated, seedlings were collected for genome extraction and ICE analysis.

In the case of *N. tabacum* seeds, the collection method was the same as described above. Seeds were directly sown on soil mixture composed of one part of perlite and two parts of potting substrate. Pots were located in a growth chamber set at 16 h-light/8 h-dark and 25°C until cotyledons emerged and samples were taken.

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Supplementary data

Supplementary data is available at the end of this chapter.

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Supporting information

Figure S1. Nucleotide sequences of the viral vectors used for plant agroinoculation.

>TRV2 (derived from GenBank: OM372496)

GATAAAACATTGCACCTATGGTGTTGCCCTGGCTGGGGTATGTCAGTGATCGCA
GTAGAATGTACTAATTGACAAGTTGGAGAATACGGTAGAACGTCCTTATCCAAC
ACAGCCTTTATCCCTCTCCCTGACGAGGTTTTTGTCAGTGTAATATTTCTTTTT
GAACTATCCAGCTTAGTACCGTACGGGAAAGTGACTGGTGTGCTTATCTTTGAA
ATGTTACTTTGGGTTTCGGTTCTTTAGGTTAGTAAGAAAGCACTTGTCTTCTCA
TACAAAGGAAAACCTGAGACGTATCGCTTACGAAAGTAGCAATGAAAGAAAGGT
GGTGGTTTTAATCGCTACCGCAAAAACGATGGGGTCGTTTTAATTAACTTCTCC
TACGCAAGCGTCTAAACGGACGTTGGGGTTTTGCTAGTTTCTTTAGAGAAAACT
AGCTAAGTCTTTAATGTTATCATTAGAGATGGCATAAATATAATACTTGTGTCT
GCTGATAAGATCATTTTAATTTGGACGATTAGACTTGTTGAACTACAGGTTACT
GAATCACTTGCGCTAATCAAC**ATG**GGAGATATGTACGATGAATCATTTGACAAG
TCGGGCGGTCCTGCTGACTTGATGGACGATTCTTGGGTGGAATCAGTTTCGTGG
AAAGATCTGTTGAAGAAGTTACACAGCATAAAATTTGCACTACAGTCTGGTAGA
GATGAGATCACTGGGTTACTAGCGGCACTGAATAGACAGTGTCCTTATTCACCA
TATGAGCAGTTTCCAGATAAGAAGGTGTATTTCCTTTTAGACTCACGGGCTAAC
AGTGCTCTTGGTGTGATTCAGAACGCTTCAGCGTTCAAGAGACGAGCTGATGAG
AAGAATGCAGTGGCGGGTGTTACAAATATTCCTGCGAATCCAAACACAACGGTT
ACGACGAACCAAGGGAGTACTACTACTACCAAGGCGAACACTGGCTCGACTTTG
GAAGAAGACTTGTACACTTATTACAAATTCGATGATGCCTCTACAGCTTTCCAC
AAATCTCTAACTTCGTTAGAGAACATGGAGTTGAAGAGTTATTACCGAAGGAAC
TTTGAGAAAGTATTCGGGATTAAGTTTGGTGGAGCAGCTGCTAGTTCATCTGCA
CCGCCTCCAGCGAGTGGAGGTCCGATACGTCCTAATCCC**TAG**GGATTTAAGGAC
GTGAACTCTGTTGAGATCTCTGTGAAATTCAGAGGGTGGGTGATACCATATTCA
CTGATGCCATTAGCGACATCTAAATAGGGCTAATTGTGACTAATTTGAGGGAAT
TTCCTTTACCATTGACGTCAGTGTCGTTGGTAGCATTTGAGTTTCGCAATGCAC
GAATTACTTAGGAAGTGGCTTGACGACACTAATGTGTTATTGTTAGATAATGGT
TTGGTGGTCAAGGTACGTAGTAGAGTCCCACATATTCGCACGTATGAAGTAATT
GGAAAGTTGTCAGTTTTTGATAATTCACTGGGAGATGATACGCTGTTTGAGGGA
AAAGTAGAGAACGTATTTGTTTTTATGTTCAGGCGGTTCTTGTGTGTCAACAAA
GATGGACATTGTTACTCAAGGAAGCACGATGAGCTTTATTATTACGGACGAGTG
GACTTAGATTCTGTGAGTAAGGTTACCGAATTC**TGCACACAAAGTTAAAAACGC**
TGTAGTAATACATGCGCAAGAACAGGCTGAGCATCTTGTCCTGGGGTTTCACAC
TATCTTTAGAGAAAGTGTTAAGTTAATTAAGTTATCTTAATTAAGAGCATAATT
ATACTGATTTGTCTCTCGTTGATAGAGTCTATCATTCTGTTACTAAAAAATTTG
ACAACTCGGTTTGCTGACCTACTGGTTACTGTATCACTTACCCGAGTTAACGCC
ATGTCCCGAAGACATTAAACTACGGTTCTTTAAGTAGATCCGTGTCTGAAGTTT
TAGGTTCAATTTAAACCTACGAGATTGACATTCTCGACTGATCTTGATTGATCG

GTAAGTCTTTTGTAATTTAATTTTCTTTTTGATTTTATTTTAAATTGTTATCTG
TTTCTGTGTATAGACTGTTTGAGATCGGCGTTTGGCCGACTCATTGTCTTACCA
TAGGGGAACGGACTTTGTTTGTGTTGTTATTTTATTTGTATTTTATTAAAATTC
TCAACGATCTGAAAAAGCCTCGCGGCTAAGAGATTGTTGGGGGGTGAGTAAGTA
CTTTTAAAGTGATGATGGTTACAAAGGCAAAAGGGGTAAAACCCCTCGCCTACG
TAAGCGTTATTACGCCC

EBV subgenomic promoter (SGP) is denoted in blue.

>TRV-crRNA-1 (insert at 3´position of EBV SGP)
TAATTTCTACTAAGTGTAGATTGGTGATCCGTTCAATTCAGATC

>TRV-crRNA-2 or DR1-CHLI2 (insert at 3´position of EBV SGP)
TAATTTCTACTAAGTGTAGATCCGCTAGGTGCTACTGAGGACAG

>TRV-crRNA-3 (insert at 3´position of EBV SGP)
TAATTTCTACTAAGTGTAGATGCAAGAAGACCAGGCTCGAAAGC

>TRV- DR1-CHLI2-DR2 (insert at 3´position of EBV SGP)
TAATTTCTACTAAGTGTAGATCCGCTAGGTGCTACTGAGGACAGTAATTTCTAC
TAAGTGTAGAT

>TRV-DR1-CHLI2-5FT (insert at 3´position of EBV SGP)
TAATTTCTACTAAGTGTAGATCCGCTAGGTGCTACTGAGGACAGATGTCTATAA
ATATAAGAGACCCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATCCGT
TTAATAGATCAATCACTCTAAAGGTTACTTATGGCCAA

>TRV-DR1-CHLI2-DR2-5FT (insert at 3´position of EBV SGP)
TAATTTCTACTAAGTGTAGATCCGCTAGGTGCTACTGAGGACAGTAATTTCTAC
TAAGTGTAGATATGTCTATAAATATAAGAGACCCTCTTATAGTAAGCAGAGTTG
TTGGAGACGTTCTTGATCCGTTTAATAGATCAATCACTCTAAAGGTTACTTATG
GCCAA

Cas12 direct repeats are highlighted in yellow. Spacers are highlighted in green. *Flowering Locus T* of *A. thaliana* denoted in blue.

>TEV (derived from GenBank DQ986288, G273A, A1119G)
GAAAATAACAAATCTCAACACAACATATACAAAACAAACGAATCTCAAGCAATC
AAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCA
ATTTTCTGAAAATTTTCACCATTTACGAACGATAGCC**ATG**GCACTCATCTTTGG
CACAGTCAACGCTAACATCCTGAAGGAAGTGTTCGGTGGAGCTCGTATGGCTTG

CGTTACCAGCGCACATATGGCTGGAGCGAATGGAAGCATTTTGAAGAAGGCAGA
AGAAACCTCTCGTGCAATCATGCACAAACCAGTGATCTTCGGAGAAGACTACAT
TACCGAGGCAGACTTGCCTTACACACCACTCCATTTAGAGGTCGATGCTGAAAT
GGAGCGGATGTATTATCTTGGTCGTCGCGCGCTCACCCATGGCAAGAGACGCAA
AGTTTCTGTGAATAACAAGAGGAACAGGAGAAGGAAAGTGGCCAAAACGTACGT
GGGGCGTGATTCCATTGTTGAGAAGATTGTAGTGCCCCACACCGAGAGAAAGGT
TGATACCACAGCAGCAGTGGAAGACATTTGCAATGAAGCTACCACTCAACTTGT
GCATAATAGTATGCCAAAGCGTAAGAAGCAGAAAAACTTCTTGCCCGCCACTTC
ACTAAGTAACGTGTATGCCCAAACTTGGAGCATAGTGCGCAAACGCCATATGCA
GGTGGAGATCATTAGCAAGAAGAGCGTCCGAGCGAGGGTCAAGAGATTTGAGGG
CTCGGTGCAATTGTTCGCAAGTGTGCGTCACATGTATGGCGAGAGGAAAAGGGT
GGACTTACGTATTGACAACTGGCAGCAAGAGACACTTCTAGACCTTGCTAAAAG
ATTTAAGAATGAGAGAGTGGATCAATCGAAGCTCACTTTTGGTTCAAGTGGCCT
AGTTTTGAGGCAAGGCTCGTACGGACCTGCGCATTGGTATCGACATGGTATGTT
CATTGTACGCGGTCGGTCGGATGGGATGTTGGTGGATGCTCGTGCGAAGGTAAC
GTTCGCTGTTTGTCACTCAATGACACATTATAGCGACAAATCAATCTCTGAGGC
ATTCTTCATACCATACTCTAAGAAATTCTTGGAGTTGAGGCCAGATGGAATCTC
CCATGAGTGTACAAGAGGAGTATCAGTTGAGCGGTGCGGTGAGGTGGCTGCAAT
CCTGACACAAGCACTTTCACCGTGTGGTAAGATCACATGCAAACGTTGCATGGT
TGAAACACCTGACATTGTTGAGGGTGAGTCGGGAGACAGTGTCACCAACCAAGG
TAAGCTCCTAGCAATGCTGAAAGAACAGTATCCAGATTTCCCAATGGCCGAGAA
ACTACTCACAAGGTTTTTGCAACAGAAATCACTAGTAAATACAAATTTGACAGC
CTGCGTGAGCGTCAAACAACTCATTGGTGACCGCAAACAAGCTCCATTCACACA
CGTACTGGCTGTCAGCGAAATTCTGTTTAAAGGCAATAAACTAACAGGGGCCGA
TCTCGAAGAGGCAAGCACACATATGCTTGAAATAGCAAGGTTCTTGAACAATCG
CACTGAAAATATGCGCATTGGCCACCTTGGTTCTTTCAGAAATAAAATCTCATC
GAAGGCCCATGTGAATAACGCACTCATGTGTGATAATCAACTTGATCAGAATGG
GAATTTTATTTGGGGACTAAGGGGTGCACACGCAAAGAGGTTTCTTAAAGGATT
TTTCACTGAGATTGACCCAAATGAAGGATACGATAAGTATGTTATCAGGAAACA
TATCAGGGGTAGCAGAAAGCTAGCAATTGGCAATTTGATAATGTCAACTGACTT
CCAGACGCTCAGGCAACAAATTCAAGGCGAAACTATTGAGCGTAAAGAAATTGG
GAATCACTGCATTTCAATGCGGAATGGTAATTACGTGTACCCATGTTGTTGTGT
TACTCTTGAAGATGGTAAGGCTCAATATTCGGATCTAAAGCATCCAACGAAGAG
ACATCTGGTCATTGGCAACTCTGGCGATTCAAAGTACCTAGACCTTCCAGTTCT
CAATGAAGAGAAAATGTATATAGCTAATGAAGGTTATTGCTACATGAACATTTT
CTTTGCTCTACTAGTGAATGTCAAGGAAGAGGATGCAAAGGACTTCACCAAGTT
TATAAGGGACACAATTGTTCCAAAGCTTGGAGCGTGGCCAACAATGCAAGATGT
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GCTAATTGAATTCGTTCATTCAGGTTTGGAATCCGAAATGAAAACTTACAATGT
TGGAGGGATGAACCGAGATATGGTCACACAAGGTGCAATTGAGATGTTGATCAA
GTCCATATACAAACCACATCTCATGAAGCAGTTACTTGAGGAGGAGCCATACAT
AATTGTCCTGGCAATAGTCTCCCCTTCAATTTTAATTGCCATGTACAACTCTGG

AACTTTTGAGCAGGCGTTACAAATGTGGTTGCCAAATACAATGAGGTTAGCTAA
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CGTCCAGCAGCGTAATTTGATTAATGAGTATGCGCAGGTAATTTTGGACAATCT
GATTGACGGTGTCAGGGTTAACCATTCGCTATCCCTAGCAATGGAAATTGTTAC
TATTAAGCTGGCCACCCAAGAGATGGACATGGCGTTGAGGGAAGGTGGCTATGC
TGTGACCTCTGAAAAGGTGCATGAAATGTTGGAAAAAAACTATGTAAAGGCTTT
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GGAACTCCTGAAGGGAACCATCTCAAGAGCCGTAAATGGTGGTGCAAGAAAGGT
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GCTTCCTGACGTCTACAAGTTTATCACAGTCTCGAGTGTCCTTTCCTTGTTGTT
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TGCTGCACAGTTGCAGAAAGAGAGCGAGTGGGACAATATCATCAATAGAACTTT
CCAGTATTCTAAGCTTGAAAATCCTATTGGCTATCGCTCTACAGCGGAGGAAAG
ACTCCAATCAGAACACCCCGAGGCTTTCGAGTACTACAAGTTTTGCATTGGAAA
GGAAGACCTCGTTGAACAGGCAAAACAACCGGAGATAGCATACTTTGAAAAGAT
TATAGCTTTCATCACACTTGTATTAATGGCTTTTGACGCTGAGCGGAGTGATGG
AGTGTTCAAGATACTCAATAAGTTCAAAGGAATACTGAGCTCAACGGAGAGGGA
GATCATCTACACGCAGAGTTTGGATGATTACGTTACAACCTTTGATGACAATAT
GACAATCAACCTCGAGTTGAATATGGATGAACTCCACAAGACGAGCCTTCCTGG
AGTCACTTTTAAGCAATGGTGGAACAACCAAATCAGCCGAGGCAACGTGAAGCC
ACATTATAGAACTGAGGGGCACTTCATGGAGTTTACCAGAGATACTGCGGCATC
GGTTGCCAGCGAGATATCACACTCACCCGCAAGAGATTTTCTTGTGAGAGGTGC
TGTTGGATCTGGAAAATCCACAGGACTTCCATACCATTTATCAAAGAGAGGGAG
AGTGTTAATGCTTGAGCCTACCAGACCACTCACAGATAACGTGCACAAGCAACT
GAGAAGTGAACCATTTAACTGCTTCCCAACTTTGAGGATGAGAGGGAAGTCAAC
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TGCACGAAACATAGCTGAGGTAAAAACATACGATTTTGTCATAATTGATGAATG
TCATGTGAATGATGCTTCTGCTATAGCGTTTAGGAATCTACTGTTTGAACATGA
ATTTGAAGGAAAAGTCCTCAAAGTGTCAGCCACACCACCAGGTAGAGAAGTTGA
ATTCACAACTCAGTTTCCCGTGAAACTCAAGATAGAAGAGGCTCTTAGCTTTCA
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GCTCCTTGTGCAAAAGGGATACAAAGTGTCGAAGATTGATGGAAGAACAATGAA
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GGATTTTGGGACTAAGGTTGTACCAGTTTTGGATGTGGACAATAGAGCGGTGCA
GTACAACAAAACTGTGGTGAGTTATGGGGAGCGCATCCAAAGACTCGGTAGAGT
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GCGCTTTAAGCTACACACTTGTGAGACATTCCTCAATAAGTTGGCGATCCCAAA
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GAGAGAGGCGCGTGGGGCTAGAGGGCAATATGAGGTTGCAGCGGAGCCAGAGGC
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CACGAGAGGAATGGGTGCAAAGTCTCGGAAATTCATAAACATGTATGGGTTTGA
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TGATGAGTCCACAAACGCACCTATTGATTTAGTGCAGCATGAGTTTGGAAAGGT
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CACCACAATCCATGCTTATTTGGTGAATAGTGGCACGAAGAAAGTTCTTAAGGT
TGATTTAACACCACACTCGTCGCTACGTGCGAGTGAGAAATCAACAGCAATAAT
GGGATTTCCTGAAAGGGAGAATGAATTGCGTCAAACCGGCATGGCAGTGCCAGT
GGCTTATGATCAATTGCCACCAAAGAGTGAGGACTTGACGTTTGAAGGAGAAAG
CTTGTTTAAGGGACCACGTGATTACAACCCGATATCGAGCACCATTTGTCACTT
GACGAATGAATCTGATGGGCACACAACATCGTTGTATGGTATTGGATTTGGTCC
CTTCATCATTACAAACAAGCACTTGTTTAGAAGAAATAATGGAACACTGTTGGT
CCAATCACTACATGGTGTATTCAAGGTCAAGAACACCACGACTTTGCAACAACA
CCTCATTGATGGGAGGGACATGATAATTATTCGCATGCCTAAGGATTTCCCACC
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TGTGACAACCAACTTCCAAACTAAGAGCATGTCTAGCATGGTGTCAGACACTAG
TTGCACATTCCCTTCATCTGATGGCATATTCTGGAAGCATTGGATTCAAACCAA
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GAAAAACTTCATGGAATTGTTGACAAATCAGGAGGCGCAGCAGTGGGTTAGTGG
TTGGCGATTAAATGCTGACTCAGTATTGTGGGGGGGCCATAAAGTTTTCATGAG
CAAACCTGAAGAGCCTTTTCAGCCAGTTAAGGAAGCGACTCAACTCATGAGTGA
ATTGGTGTACTCGCAAGGGGAGAAGAGGAAATGGGTCGTGGAAGCACTGTCAGG
GAACTTGAGGCCAGTGGCTGAGTGTCCCAGTCAGTTAGTCACAAAGCATGTGGT
TAAAGGAAAGTGTCCCCTCTTTGAGCTCTACTTGCAGTTGAATCCAGAAAAGGA
AGCATATTTTAAACCGATGATGGGAGCATATAAGCCAAGTCGACTTAATAGAGA
GGCGTTCCTCAAGGACATTCTAAAATATGCTAGTGAAATTGAGATTGGGAATGT
GGATTGTGACTTGCTGGAGCTTGCAATAAGCATGCTCATCACAAAGCTCAAGGC
GTTAGGATTCCCAACTGTGAACTACATCACTGACCCAGAGGAAATTTTTAGTGC

ATTGAATATGAAAGCAGCTATGGGAGCACTATACAAAGGCAAGAAGAAAGAAGC
TCTCAGCGAGCTCACACTAGATGAGCAGGAGGCAATGCTCAAAGCAAGTTGCCT
GCGACTGTATACGGGAAAGCTGGGAATTTGGAATGGCTCATTGAAAGCAGAGTT
GCGTCCAATTGAGAAGGTTGAAAACAACAAAACGCGAACTTTCACAGCAGCACC
AATAGACACTCTTCTTGCTGGTAAAGTTTGCGTGGATGATTTCAACAATCAATT
TTATGATCTCAACATAAAGGCACCATGGACAGTTGGTATGACTAAGTTTTATCA
GGGGTGGAATGAATTGATGGAGGCTTTACCAAGTGGGTGGGTGTATTGTGACGC
TGATGGTTCGCAATTCGACAGTTCCTTGACTCCATTCCTCATTAATGCTGTATT
GAAAGTGCGACTTGCCTTCATGGAGGAATGGGATATTGGTGAGCAAATGCTGCG
AAATTTGTACACTGAGATAGTGTATACACCAATCCTCACACCGGATGGTACTAT
CATTAAGAAGCATAAAGGCAACAATAGCGGGCAACCTTCAACAGTGGTGGACAA
CACACTCATGGTCATTATTGCAATGTTATACACATGTGAGAAGTGTGGAATCAA
CAAGGAAGAGATTGTGTATTACGTCAATGGCGATGACCTATTGATTGCCATTCA
CCCAGATAAAGCTGAGAGGTTGAGTGGATTCAAAGAATCTTTCGGAGAGTTGGG
CCTGAAATATGAATTTGACTGCACCACCAGGGACAAGACACAGTTGTGGTTCAT
GTCACACAGGGCTTTGGAGAGGGATGGCATGTATATACCAAAGCTAGAAGAAGA
AAGGATTGTTTCTATTTTGGAATGGGACAGATCCAAAGAGCCGTCACATAGGCT
TGAAGCCATCTGTGCATCAATGATCGAAGCATGGGGTTATGACAAGCTGGTTGA
AGAAATCCGCAATTTCTATGCATGGGTTTTGGAACAAGCGCCGTATTCACAGCT
TGCAGAAGAAGGAAAGGCGCCATATCTGGCTGAGACTGCGCTTAAGTTTTTGTA
CACATCTCAGCACGGAACAAACTCTGAGATAGAAGAGTATTTAAAAGTGTTGTA
TGATTACGATATTCCA**ACGACTGAGAATCTTTATTTTCAGTCAGGTACA**ACTAC
AGAGAACCTCTACTTTCAAAGTGGCACTGTGGGTGCTGGTGTTGACGCTGGTAA
GAAGAAAGATCAAAAGGATGATAAAGTCGCTGAGCAGGCTTCAAAGGATAGGGA
TGTTAATGCTGGAACTTCAGGAACATTCTCAGTTCCACGAATAAATGCTATGGC
CACAAAACTTCAATATCCAAGGATGAGGGGAGAGGTGGTTGTAAACTTGAATCA
CCTTTTAGGATACAAGCCACAGCAAATTGATTTGTCAAATGCTCGAGCCACACA
TGAGCAGTTTGCCGCGTGGCATCAGGCAGTGATGACAGCCTATGGAGTGAATGA
AGAGCAAATGAAAATATTGCTAAATGGATTTATGGTGTGGTGCATAGAAAATGG
GACTTCCCCAAATTTGAACGGAACTTGGGTTATGATGGATGGTGAGGAGCAAGT
TTCATACCCGCTGAAACCAATGGTTGAAAACGCGCAGCCAACACTGAGGCAAAT
TATGACACACTTCAGTGACCTGGCTGAAGCGTATATTGAGATGAGGAATAGGGA
GCGACCATACATGCCTAGGTATGGTCTACAGAGAAACATTACAGACATGAGTTT
GTCACGCTATGCGTTCGACTTCTATGAGCTAACTTCAAAAACACCTGTTAGAGC
GAGGGAGGCGCATATGCAAATGAAAGCTGCTGCAGTACGAAACAGTGGAACTAG
GTTATTTGGTCTTGATGGCAACGTGGGTACTGCAGAGGAAGACACTGAACGGCA
CACAGCGCACGATGTGAACCGTAACATGCACACACTATTAGGGGTCCGCCAG**TG**
ATAGTTTCTGCGTGTCTTTGCTTTCCGCTTTTAAGCTTATTGTAATATATATGA
ATAGCTATTCACAGTGGGACTTGGTCTTGTGTTGAATGGTATCTTATATGTTTT
AATATGTCTTATTAGTCTCATTACTTAGGCGAACGACAAAGTGAGGTCACCTCG
GTCTAATTCTCCTATGTAGTGCGAGAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAA

NiaPro site 1 is denoted in orange **,** **NiaPro site 2** is denoted in green.

>TEV-crRNA-3 (insert between NiaPro site 1 and NiaPro site 2)

GTAATTTCTACTAAGTGTAGATGCAAGAAGACCAGGCTCGAAAGC

G extra nucleotide added to maintain the ORF. **Cas12 direct repeat** is highlighted in yellow. **Spacer** is highlighted in green.

Figure S2. Nucleotide sequence of the plasmid used for *N. tabacum* and *S. lycopersicum* transformation.

>pLBHig-Cas12att

ACTAGTGCGGCGGCGGCCGCGATTCCATTGCCCAGCTATCTGTCACTTTATTGT
GAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGG
AAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCC
ACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCA
AGTGGATTGATGTGATAGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAA
GATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAA
GGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACC
CACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGT
GGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCC
TTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGTATTA
AAATCTTAATAGGTTTTGATAAAAGCGAACGTGGGGAAACCCGAACCAAACCTT
CTTCTAAACTCTCTCTCATCTCTCTTAAAGCAAACTTCTCTCTTGTCTTTCTTG
CGTGAGCGATCTTCAACGTTGTCAGATCGTGCTTCGGCACCAGTACAACGTTTT
CTTTCACTGAAGCGAAATCAAAGATCTCTTTGTGGACACGTAGTGCGGCGCCAT
TAAATAACGTGTACTTGTCCTATTCTTGTCGGTGTGGTCTTGGGAAAAGAAAGC
TTGCTGGAGGCTGCTGTTCAGCCCCATACATTACTTGTTACGATTCTGCTGACT
TTCGGCGGGTGCAATATCTCTACTTCTGCTTGACGAGGTATTGTTGCCTGTACT
TCTTTCTTCTTCTTCTTGCTGATTGGTTCTATAAGAAATCTAGTATTTTCTTTG
AAACAGAGTTTTCCCGTGGTTTTCGAACTTGGAGAAAGATTGTTAAGCTTCTGT
ATATTCTGCCCAAATTTGAAATGAGCAAGCTGGAGAAGTTTACAAACTGCTACT
CCCTGTCTAAGACCCTGAGGTTCAAGGCCATCCCTGTGGGCAAGACCCAGGAGA
ACATCGACAATAAGCGGCTGCTGGTGGAGGACGAGAAGAGAGCCGAGGATTATA
AGGGCGTGAAGAAGCTGCTGGATCGCTACTATCTGTCTTTTATCAACGACGTGC
TGCACAGCATCAAGCTGAAGAATCTGAACAATTACATCAGCCTGTTCCGGAAGA
AAACCAGAACCGAGAAGGAGAATAAGGAGCTGGAGAACCTGGAGATCAATCTGC
GGAAGGAGATCGCCAAGGCCTTCAAGGGCAACGAGGGCTACAAGTCCCTGTTTA
AGAAGGATATCATCGAGACAATCCTGCCAGAGTTCCTGGACGATAAGGACGAGA
TCGCCCTGGTGAACAGCTTCAATGGCTTTACCACAGCCTTCACCGGCTTCTTTC
GTAACAGAGAGAATATGTTTTCCGAGGAGGCCAAGAGCACATCCATCGCCTTCA
GGTGTATCAACGAGAATCTGACCCGCTACATCTCTAATATGGACATCTTCGAGA
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TGAACAGCGACTATGATGTGGAGGATTTCTTTGAGGGCGAGTTCTTTAACTTTG
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CCGAGAGCGGCGAGAAGATCAAGGGCCTGAACGAGTACATCAACCTGTATAATC
AGAAAACCAAGCAGAAGCTGCCTAAGTTTAAGCCACTGTATAAGCAGGTGCTGA
GCGATCGGGAGTCTCTGAGCTTCTACGGCGAGGGCTATACATCCGATGAGGAGG
TGCTGGAGGTGTTTAGAAACACCCTGAACAAGAACAGCGAGATCTTCAGCTCCA
TCAAGAAGCTGGAGAAGCTGTTCAAGAATTTTGACGAGTACTCTAGCGCCGGCA
TCTTTGTGAAGAACGGCCCCGCCATCAGCACAATCTCCAAGGATATCTTCGGCG
AGTGGAACGTGATCCGGGACAAGTGGAATGCCGAGTATGACGATATCCACCTGA

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AGAAGATCGGCTCCTTTTCTCTGGAGCAGCTGCAGGAGTACGCCGACGCCGATC
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AGAGCCTGAAGAAGAACGACGCCGTGGTGGCCATCATGAAGGACCTGCTGGATT
CTGTGAAGAGCTTCGAGAATTACATCAAGGCCTTCTTTGGCGAGGGCAAGGAGA
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GGGACAAGGATAAGGAGACAGACTATCGGGCCACCATCCTGAGATACGGCTCCA
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GCATCTCCCGGTATCCAAAGTGGTCCAATGCCTACGATTTCAACTTTTCTGAGA
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ATCACGGACAGATCAGGCTGAGCGGAGGAGCAGAGCTGTTCATGAGGCGCGCCT
CCCTGAAGAAGGAGGAGCTGGTGGTGCACCCAGCCAACTCCCCTATCGCCAACA
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AGTGCCCCAAGAACATCTTCAAGATCAATACAGAGGTGCGCGTGCTGCTGAAGC
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GCGAGCTGGTGGAGAAGTACGATGCCGTGATCGCCCTGGAGGACCTGAACTCTG
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AATTCCTGCAGGCTAGCTCGCATCCTTGTCCCGTCTCCTGAC

Hygromycin resistance cassette in green (ORF under the control of the *A. tumefaciens* NoS **promoter** and **terminator**) flanked by unique (5') **SpeI**, **ApaI**, **NotI**, and (3') **MluI** sites in the T-DNA of pLX-B2 [U22]. Double **LB** and **RB** of the T-DNA. **kanamycin** resistance for plasmid selection is denoted in light grey. **LbCas12a** coding sequence in black followed by double NLS (**nucleoplasmin**, **GS** spacers and and **SV40)**. Dual (CaMV) 35S promoter (**2X35S Promoter** in garnet and red) underlining +1 nucleotide of transcription. **35S terminator** in fuchsia with the processing and polyadenylation site underlined. Sequences of **5' and 3' UTR of RNA-2 ofCPMV** are denoted in green. CG mutation (D156R) is marked in fuchsia.

Chapter III

Development of a CRISPR/Cas system to edit melon genome combining stable transformation to express nucleases and viral vectors to express guide RNAs

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Author contributions:
All authors participated in the work conception and design. A.G., V.A., B.G.-S. and B.P. performed the experiments. All authors participated in the result and data analyses. A.G. wrote the manuscript with input from the rest of the authors. All authors discussed and revised the manuscript.

Abstract

CRISPR/Cas technologies promise manipulation of the genomes of crop plants in an unprecedented manner. To obtain genetically edited plants, CRISPR/Cas usually goes hand in hand with transformation and regeneration techniques. Although plant transformation has broadly been achieved for years, there are certain families of plants, such as cucurbits, that show some recalcitrance to *in vitro* regeneration, thus hampering application of CRISPR/Cas technologies. In this work, we developed a strategy that combines the *Agrobacterium tumefaciens*-mediated transformation of different melon cultivars, to obtain lines that stably express SpCas9, LbCas12a, and ttLbCas12a (thermotolerant) nucleases, with the expression of guide RNAs by means of viral vectors. As a proof-of-concept, we first tired to edit the reporter genes *Phytoene desaturase* (*PDS*) and *Magnesium chelatase subunit I* (*CHLI*), expressing the gRNAs by means of a watermelon mosaic virus (WMV) vector. Our final goal is to obtain melon cultivars knocked-out in susceptibility (S) genes that mediate cucurbit infection by the tomato leaf curl New Delhi virus (ToLCNDV) and the powdery mildew *Podosphaera xanthii*.

Keywords: CRISPR/Cas, virus-induced gene editing, melon, watermelon mosaic virus, (S) genes.

Introduction

The use of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated (Cas; CRISPR/Cas) nucleases for plant biotechnology purposes has radically changed the way we understand the concept of plant breeding. This technology relies on the use of a Cas nuclease that generates a double stranded break in a targeted DNA position. To specifically target a particular DNA sequence, a RNA joining the endonuclease protein is required (Borrelli et al., 2018). The use of this technology for genome editing purposes in different plants has extensively been investigated during the last decade, since the first reports of its application in plants (Feng et al., 2013). Since then, many advances have been made, and there are several examples that validate its application in model plants, such as *Arabidopsis thaliana* and *Nicotiana benthamiana* (Jansing et al., 2019; Liu et al., 2015; Uetz et al., 2022), even developing platforms capable of editing 8 genes at the same time (multiplexing) (Stuttmann et al., 2021). The application of this technology has also been implemented in crops of agronomical interest such as tomato, rice, grape vine, potato or sugar cane (Bernabé-Orts et al., 2019; Karlsson et al., 2024; Laksana et al., 2024; Lv et al., 2024; Moffa et al., 2024; Nekrasov et al., 2017; Ren et al., 2023; Távora et al., 2022; Vu et al., 2021).

The most common way in which the different CIRSPR/Cas components are delivered to plant cells is mediated by *Agrobacterium tumefaciens,* leading to what is usually called "stable transformation". In addition, Cas nucleases and guide RNAs can also be expressed in plants by viral vectors, what is usually named as virus-induced genome editing (VIGE). One of the main advantages when using plant viruses as delivery vectors is the possibility to obtain transgene-free events in a single generation, without the need of tissue culture regeneration, and thus reducing the time required for each breeding program (Mikhaylova, 2025). On the other hand, the limited cargo capacity of viral vectors, makes difficult the possibility of expressing large Cas nucleases. This is the reason why most of the examples found in the literature, refer to the use of viruses to express the short guide RNAs in plants stably expressing Cas nucleases (Ali et al., 2015; Ellison et al., 2020; Lee at al., 2025; Liu et al*.,* 2024). However, different negative-strand RNA viruses have also been employed to express Cas nucleases in wild type plants, something possible due to a greater

cargo capacity and unprecedented genome stability (Gao et al., 2019; Liu et al., 2023; Ma et al., 2020).

Regarding agronomic importance, Cucurbitaceae family stands out for its worldwide distribution and for its relevance in Spanish economy. Some of the crops belonging to this family are cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), summer squash and zucchini (*Cucurbita pepo*), pumpkins and squash (*Cucurbita maxima*) (Chen et al., 2024). In this group of crops, *C. melo* (2n=24) is a species with a particular morphological diversity as well as nutritional properties, such as a richness in vitamins and minerals, that make it a common food in the diets of developed and developing countries (Manchali et al., 2021). According to the Food and Agriculture Organization (FAO) of the United Nations, more than 29 million metric tons of melon were produced worldwide in 2023. The main producer was China, followed by India and Turkey. In Europe, Spain was the second-largest producer, with more than 16 million hectares of cultivated land, following Italy (FAOSTAT 2025).

Powdery mildew in cucurbits is caused by three different biotrophic fungal species, *Leveillula taurica*, *Golovinomyces orontii* and *Podosphaera xanthii*, *P. xanthii* being the most common. This disease strongly affects melon production, both in open field and greenhouse (Cui et al., 2022; Li et al., 2017). Although reported worldwide, high temperature and humidity conditions favor fungal development. The way powdery mildew spreads is mostly asexually. When a spore penetrates the plant, the feeding haustorium is formed, and it grows as a white powder on the surface reducing the photosynthetic area of the plant. This cycle results in the release of more spores. A less common sexual cycle involves the fusion of hyphae and the formation of chasmothecia (Cui et al., 2022). Resistance to powdery mildew has been achieved through the loss of function of genes belonging to the *MLO* (*Mildew Resistance Locus O*) gene family, considered as susceptibility factors, and which are present in a large number of plants including barley, wheat, rice and tomato (Jacott et al., 2021; Zhang et al., 2023).

Another important pathogen affecting melon production is tomato leaf curl New Delhi virus (ToLCNDV; genus *Begomovirus*). This virus presents a bipartite DNA genome of single-stranded DNA, formed of two segments named as DNA-A and DNA-B (Hanley-Bowdoin et al., 2013; Padidam et al., 1985). This virus is transmitted in nature by the whitefly *Bemisia tabaci*. Different species of this insect have been involved in the dispersion of this pathogen across several countries (Moriones et al., 2017). In addition to this natural transmission, some ToLCNDV isolates can be transmitted mechanically. An example is the Spanish isolate ToLCNDV-ES, whose mechanical transmission has been confirmed in different species of the genera *Citrullus*, *Cucumis*, and *Cucurbita* (López et al., 2015).

Disease caused by ToLCNDV leads to important yield and economic losses in melon fields. Symptoms observed in cucurbits include intense curling and spooning of young leaves, often accompanied by growth arrest. Late-infected plants often show curled and spoon-shaped young leaves, with severe mosaics and short internodes. The disease also causes symptoms in fruits, such as soft wrinkling of the skin (Juárez et al., 2014; Ruiz et al., 2015). The study of genetic resistance to ToLCNDV has mainly been done by QTL analysis using resistant melon cultivars, and candidate regions have been successfully mapped (Pérez-Moro et al., 2024; Sáez et al., 2017).

Melon breeding has been carried out for decades using classical techniques, such as crossbreeding and subsequent selection. Efforts have been directed towards developing hybrids with improved storage capacity and shelf life, as well as obtaining lines resistant to biotic and abiotic stress (Kesh and Kaushik, 2021). Despite the identification of a few resistance genes, as well as QTLs involved in disease resistance (Sáez et al., 2017; C.S. Wang et al., 2024), there is still a widespread lack of resistant varieties on the market (Demirci et al., 2020). In general, many breeders agree that powdery mildew and *Fusarium* resistance are strongly needed requirements for modern varieties (Branham et al., 2018).

The publication of an annotated melon reference genome in 2012 (Garcia-Mas et al., 2012) and the economic affordability of next generation sequencing, mainly genotyping by sequencing and restriction site associated (RAD) DNA

sequencing have made it possible to apply new CRISPR/Cas genome editing techniques to melon breeding (Kesh and Kaushik, 2021). However, it should be mentioned the considerable difficulty to stably transform melon plants (recalcitrance) (Chovelon et al., 2011). Although this characteristic is influenced by genotype, it affects all melon varieties in a general manner (Wan et al., 2023). However, although at low efficiencies compared to other crops such as tomato, gene editing in melon has been successfully achieved through stable transformation, (Hooghvorst et al., 2019; Z. Wang et al., 2024), although to our knowledge, there is no report supporting VIGE in cucurbits.

In this work, we tried to develop a VIGE strategy in melon plants. For this purpose, we combined the *A*. *tumefaciens*-mediated stable transformation of five different melon genotypes: 'Meló Groc d'Ontinyent' (MGO, 'Amarillo' type); 'Védrantais' ('Cantaloupe' type); 'Pipa de Oro' (PO, 'Piel de Sapo' type); CH5 ('Piel de Sapo' type) and 'Charentais' ('Cantaloupe type'), to obtain lines that stably express SpCas9, LbCas12a, and ttLbCas12a (a thermotolerant variant of the former) nucleases, with the expression of guide RNAs by means of viral vectors. For this second purpose, we used a vector derived from watermelon mosaic virus (WMV; genus *Potyvirus*) (GenBank accession number MH469650), that could transiently express the different guide RNAs in inoculated plants. Our final goal is to obtain knock out melon lines in some susceptibility (S) genes that mediate melon infection by the ToLCNDV and the powdery mildew *Podosphaera xanthii*. To try to validate this VIGE platform, two reporter genes were also targeted: *Phytoene desaturase* (*PDS*) MELO3C017772.2 and *Magnesium chelatase subunit I* (*CHLI*) MELO3C007233.2. Some advance has been done towards these goals, although no VIGE-based genome editing could be detected in cucurbits yet.

Results

Vector design and development

The designed platform developed for genome editing is a two-component system that requires the expression of a specific nuclease (either SpCas9, LbCas12a or ttLbCas12a) and a gRNA in the same target cell. To achieve this, the first step was to obtain melon plants that stably express the different

nucleases, by means of *A. tumefaciens* transformation. For this purpose, three different constructs were designed and developed, derived from the minibinary vector pLX-B2 (Pasin et al., 2017). In these vectors, Cas nucleases are under the control of a cauliflower mosaic virus (CaMV) 35S promoter and terminator. Nucleases are fused to two nuclear localization signals (NLS), of nucleoplasmin and simian virus 40 (SV40) in their carboxyl terminus. Two different versions of a construct expressing LbCas12a from *Lachnospiraceae* bacterium were developed. The first construct was named as pLBHig-Cas12a, which expresses a nuclease previously reported (Liu et al., 2019). The second construct, named as pLBHig-Cas12att expresses a temperature-tolerant version which harbours the single mutation D156R. This nuclease has been proven to be more efficient at similar temperatures to those required for plant growth (Schindele and Puchta, 2020). The third construct was named pLBHig-Cas9, and expressed a human codon-optimized version of *Streptococcus pyogenes* Cas9, which exhibited improved Cas9 activity in plants (Yin et al., 2015). The *hygromycin phosphotransferase* (*hpt*) gene was used as a selection marker, under the control of the nopaline synthase (NOS) promoter and terminator from *A. tumefaciens*. An additional version of pLBHig-Cas12att was developed, in which the *neomycin phosphotransferase II* gene (*npt*II), conferring resistance to kanamycin was used as a selection marker, pLBKan-Cas12att (**Figure 1**). The sequences of all vectors used for plant stable transformation are shown in **Figure S1**.

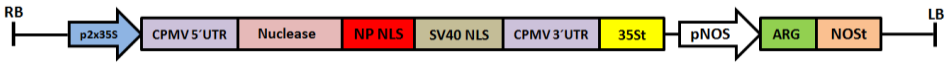


Figure 1. Schematic representation of pLB plasmids used for stable melon transformation (not at scale). From left to right: dual CaMV 35S promoter; 5´ untranslated region (UTR) of cowpea mosaic virus (CPMV); genome editing nuclease (either SpCas9, LbCas12a or ttLbCas12a); double NLS of nucleoplasmin and SV40; 3´UTR of CPMV; 35S terminator; NOS promoter; antibiotic resistance gene (either *hpt* or *npt*II); NOS terminator.

Plant transformation and regeneration

The difficulty to stably transform melon plants is broadly known, and it is considered a recalcitrant species for this purpose (Chovelon et al., 2011). This

characteristic affects in a general manner to every melon variety, although there is also a genotype-dependent transformation efficiency (Wan et al., 2023). Generally, 'Védrantais' melon, a 'Cantaloupe' type cultivar, is considered one of the best transformable genotypes, and it presents a relatively high regeneration efficiency compared to other cultivars (ranging from 12 to 15%) (Z. Wang et al., 2024). However, its transformation efficiency presents percentages close to 3%. 'Piel de Sapo', which is an important commercial type of melon presents a really low transformation efficiency, which can be estimated around 0.1% (Castelblanque et al., 2008). In this study, 7 different plant genetic transformations were performed, considered 6 of them as successful. Five different melon genotypes of agronomic interest were selected for the genetic transformations: 'Meló Groc d'Ontinyent' (MGO); 'Védrantais'; 'Pipa de Oro' (PO); CH5 and 'Charentais'. A total of 43 transgenic plants were obtained after regeneration in selective media, containing hygromycin or kanamycin (depending on the construct used), which all derived from 9 different organogenic calluses (**Figure 2**). The combined transformation efficiency of these experiments was calculated to be 0.95% (amount of organogenic calluses that gave rise to shoots that tested positive by PCR / total amount of explants). Specific data of each transformation are summarised in **Table 1.**

Table 1. Summary of the different genetic transformations performed in this study.

Vector	Genotype	Efficiency	Plants	Explant	Strain	Selective agent
pLBHig-Cas9	Védrantais	0.63%	0	Cotyledon	LBA 4404	Hygromycin
	CH5	1.25%	12			
pLBHig-Cas12a	MGO	1.89 %	14			
pLBHig-Cas12att	PO	0.78%	10			
pLBKan-Cas12att	Charentais	0.48%	4		AGL1	Kanamycin
		1.75%	2			
		0.56%	1	Leaf		

After regeneration, explants (mainly leaves and hypocotyls) were subjected to an antibiotic resistance test, to verify the T-DNA integration, and thus the optimal growth in selective medium (**Figure 3**). In addition, the presence of the T-DNA was checked in all 43 transformed plants by means of PCR. Specific primers were designed to amplify specific parts of the developed constructs

(either the different nucleases or the antibiotic resistance genes) (**Figure S2**; **Table S1**). Regenerated plants were selfed, and T_1 was obtained from individuals transformed with pLBHig-Cas9 and pLBHig-Cas12a constructs. To further verify the correct and complete integration of the desired construct in T_1 plants the whole T-DNA of two randomly selected lines was sequenced. Plants were named as 2.17 (T_1 MGO plant obtained after selfing a mother plant transformed with pLBHig-Cas12a) and 2.1 (T_1 CH5 plant obtained after selfing a mother plant transformed with pLBHig-Cas9). For this purpose, the full T-DNA sequence was split in three fragments because of its long size (6915 bp for pLBHig-Cas12a and 7341 for pLBHig-Cas9). Six primer pairs were designed to amplify 3 PCR products per T-DNA, containing the full sequence comprised in between the right border and the double left border of the T-DNA (**Table S1**). These products were later cloned and sequenced. The otucome revealed a a sequence identity of 100% compared to that expected in both cases.

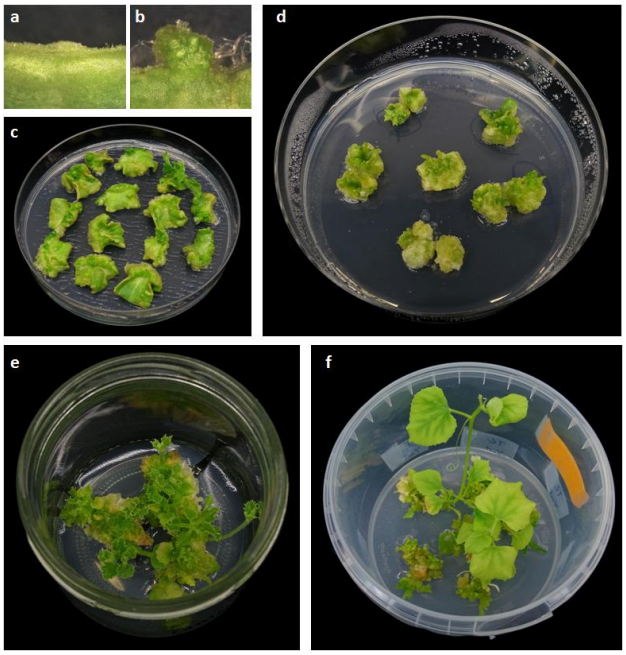


Figure Figure 2. Summarised representation of the regeneration process of transformed plants. Cotyledons were plated in callus induction medium, with the selective agent; (**a**) control untransformed explants did not develop the disrupted callus mass, that appeared in transformed explants (**b**); (**c**) once the disrupted callus mass reached a considerable size the rest of the cotyledon was

scised, and the callus was transferred to fresh medium (**d**); (**e**) grown calluses were transferred to shoot induction medium; (**f**) emerged shoots developed leaves that were later subjected to an antibiotic resistance test (**Figure 3**), once the plant had rooted.



Figure 3. Antibiotic resistance test. Plate containing callus induction medium, supplemented with kanamycin (100 µg/ml). Left, leaf fragments from untransformed plants unable to form callus; right, leaf fragments from transformed plants that can grow and form callus in selective medium.

Guide selection and vector development for plant inoculation

Once transgenic plants were obtained, the second step was to transiently express the gRNAs needed for CRISPR/Cas genome editing. For this purpose, we decided to clone different guides previously selected, in our vector derived from WMV (WMV-Vera; GenBank accession number MH469650), which was proved to efficiently infect cucurbits, and was used in reverse genetic analyses in melon (Aragonés et al., 2019; Houhou et al., 2021). In this vector, the expression of the viral cDNA was driven by CaMV 35S promoter and terminator. Inserts (direct repeat and spacer in the case of Cas12; spacer and scaffold in the case of Cas9) were cloned in between the nuclear inclusion b (NIb) and the coat protein (CP) cistrons (**Figure 4**).

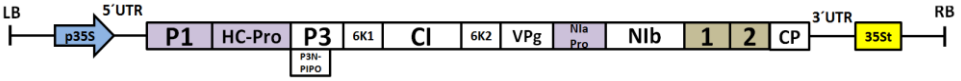


Figure 4. Schematic representation of WMV-Vera clone used for gRNA delivery to transgenic melon plants (not at scale). From left to right, CaMV 35S promoter; viral 5´UTR*;* viral cistrons P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIaPro, NIb and CP*;* viral 3´UTR; 35S terminator. Cloning positions are represented by brown rectangles. Cas12a RNA direct repeat (1) locates at 5´ position of the spacer (2). Cas9 spacer (1) locates at 5´ position of the RNA scaffold (2).

Guide RNA selection was based on the specificity scores given by the online tool CRISPOR (http://crispor.tefor.net/), and trying to avoid guides presenting off-targets at other genomic loci different from the target. One of the main requirements when selecting the different spacers was that they target exonic regions close to the 5´ UTR (the first or one of the firsts exons of the gene). So a hypothetical genome editing event would lead to an aberrant protein, and thus to a knockout event. Gene models were based on genome assembly v4.0 available at Melonomics website (http://www.melonomics.net/melonomics.html). To try to validate the functionality of the different nucleases, 17 different gRNAs were selected, and cloned in WMV vector (**Table S2**). Four different endogenous genes were selected based on previous studies and analysis (**Figure S3**). Two reporter genes were firstly selected: the gene *CHLI* MELO3C007233.2; and the gene *PDS* MELO3C017772.2. Both genes are directly or indirectly involved in chlorophyll biosynthesis and accumulation. Thus, an impair in *PDS* gene could lead to an albino phenotype, while inactivating *CHLI* gene could lead to a light green to yellow leaf phenotype, that can even turn whitish (Eid et al., 2021; Qin et al., 2007). Additionally, the candidate susceptibility gene (S gene) conferring resistance to powdery mildew (*Mildew locus O 1*; *MLO1*) MELO3C012438.2 and the candidate S gene conferring resistance to ToLCNDV, *DNA primase large subunit* (named in this work as ND1) MELO3C022319.2 were also targeted.

Twelve out of the 17 constructs expressed 23-nt spacers, suitable for Cas12a genome editing. Potyviral replication strategy makes it essential not to

introduce any stop codon in the coding sequence. This feature significantly reduces the number of gRNAs able to be cloned in the WMV vector. The long RNA scaffold (76 nt) required for efficient Cas9 genome editing presents at least one stop codon in all the six open reading frames (ORFs), which makes it impossible to be expressed by a potyviral vector. Because of this, an in-depth analysis has been carried out by members of our laboratory, resulting in the design of a more compact scaffold (67 nt) without stop codons, which also works efficiently and allows gene editing mediated by SpCas9 nuclease (Merwaiss et al., 2025, in preparation). All the five constructs that expressed Cas9 sgRNAs that were developed in this study, presented the so mentioned compact scaffold. Constructs #13b and #14b also expressed a fragment of *Flowering Locus T* (*FT*) of *Arabidopsis thaliana* directly fused to the 3' end of the RNA scaffold (**Table S2**). This strategy facilitates germline editing (Ellison et al., 2020), and constructs were built in parallel to #13 and #14 versions, which lacked the mentioned sequence. The sequences of all vectors used for guide delivery are shown in **Figure S4**.

Plant evaluation and genotyping

From the 17 WMV constructs, 5 were successfully tested. Constructs #1, #2 and #3 were tested in T_1 MGO plants derived from a mother plant transformed with LbCas12a nuclease and the hygromycin marker (from pLBHig-Cas12a plasmid). On the other hand, constructs #13b and #14b were tested on T_1 CH5 plants transformed with SpCas9 and the hygromycin marker (from pLBHig-Cas9 plasmid). For each experimental condition, between 7 and 9 plants were inoculated ($7 \leq n \leq 9$). That is, each of the 5 constructs was tested on at least 7 independent plants. The presence of each of the nucleases in inoculated plants was previously verified by PCR, as was done with T_0. Viral infection was confirmed by visual symptom evaluation and by RT-PCR of upper non inoculated leaf samples, 3 weeks post inoculation (**Figure S5**). Infected plants (values around 80% of the total amount of inoculated plants) were subjected to a genotype analysis by Sanger sequencing. It is worth mentioning that none of the plants infected with the WMV clones targeting the reporter genes *CmPDS* and *CmCHLI* presented the expected whitish or photobleaching phenotype. PCR products from targeted sequences of *CmPDS*, *CmCHLI* and *DNA primase* were purified and Sanger sequenced. No genome editing was detected in any of the

analysed samples. Sample collection was repeated 5 weeks after inoculation, leading to the same results. Certain studies suggest that subjecting transgenic plants to heat pulses (around 37°C) can increase gene editing when using both SpCas9 and LbCas12a nucleases (Blomme et al., 2022; LeBlanc et al., 2018). We tried to replicate this approach in our inoculated transgenic SpCas9 and LbCas12a melon lines, by subjecting the plants to 15 minutes pulses of 37°C, followed by 15 minutes at 25°C. However, we could not find any editing events.

Discussion

In this study, we aimed to develop a VIGE strategy for melon plants, combining the *Agrobacterium tumefaciens*-mediated stable transformation of five different cultivars with the expression of gRNAs by means of a viral vector derived from WMV. For this purpose, we selected three different nucleases, SpCas9, LbCas12a and ttLbCas12a. Our final goal was to obtain melon lines knocked-out in some S genes related to infection by powdery mildew and ToLCNDV. In addition, we also selected two reporter genes: *PDS* and *CHLI*, whose knock out would lead to a photobleaching phenotype easily distinguishable by the naked eye. However, we have not been able to find any editing event in the analysed plants so far.

When using CRISPR/Cas technology to edit plant endogenous genes, the main purpose is obtaining solid mutants (plants that present the same mutation in all the cells). When using a VIGE approach, the ultimate desire is editing the germline of the plant, leading to an edited plant in the next generation, after seed collection. There are various reports validating this approach not only in model plants (Ellison et al., 2020; Uranga et al., 2021), but also in crops of agronomical interest (Lee et al., 2025). The study of RNA molecule migration in plants is a complicated subject, with many aspects still unknown. However, it has been verified that the fusion of RNA motifs to different transcripts increases the mobility of these RNA molecules in plants (Yang et al., 2023). If not possible to edit the germline, the frequent alternative is regenerating complete plants from infected and edited tissue. Although time consuming due

to the requirement of *in vitro* assays, it has been proved to be an effective alternative to obtain solid mutant plants (Kang et al., 2025; Ma et al., 2020; Qiao et al., 2025). In any of both cases, the first milestone to achieve is obtaining gene editing either at inoculated tissue or at systemic non inoculated tissue, something that we failed to get.

There are different reasons that could explain why gene editing was not detected in transgenic melon plants using our VIGE system. Complete T-DNA sequencing of two randomly selected lines served to prove the correct and complete integration of the whole T-DNA, discarding the existence of a cassette deletion or aberrant inversions. However, although correct at a nucleotide level, the presence of a functional nuclease was not proved. Cauliflower mosaic virus (CaMV) 35S promoter controls the expression of the Cas nucleases in the transgenic plants used in this study. Although this promoter is thought to have species-specific activity in different tissues (Lee et al; 2025), its efficient use in melon transformation experiments has extensively been reported, and was found to be active in most tissues of transgenic melon plants (Dong et al., 1991; Wan et al., 2023). However, there may be another reason that triggers a lack of nuclease expression: T-DNA silencing in the transformed lines. In *A. thaliana*, a higher T-DNA copy number has been observed to be positively correlated with increased gene expression. When a certain expression threshold is exceeded, the RNA silencing machinery can be activated, resulting in a lack of transgene expression (Schubert et al., 2004). A similar effect has even been observed in tree species such as *Pinus strobus L*. (Tang et al., 2007). To verify the correct expression of the Cas nucleases, it would be convenient to perform a RT-qPCR analysis.

On the other hand, the translation of the three nucleases should be confirmed by western blot. Even if they are correctly expressed, there are some environmental factors that can significantly influence their efficiency and functionality. Several studies suggest that temperature changes or pulses can positively influence obtaining genetic editing mediated by a greater nuclease efficiency. This effect could be related to the optimal growth temperature of the bacteria from which Cas nucleases are obtained. *Streptococus pyogenes* presents an optimal growth temperature of 40°C (Le Blanc et al., 2018). In

addition, *Lachnospiraceae* bacterium is considered a mesophile with an growth range in between 30°C and 45°C (Zaplana et al., 2023). When transgenic SpCas9 *A. thaliana* plants were subjected to heat stress at 37°C, they showed higher frequencies of gene editing (LeBlanc et al., 2018). A very similar treatment presented comparable results in *A. thaliana* plants expressing LbCas12a nuclease (Blomme et al., 2022), suggesting that this protocol could be efficient for both nucleases. When we tried to replicate this approach in our inoculated transgenic SpCas9 and LbCas12a melon lines, we could not find any editing events. In addition, it should be mentioned that ttLbCas12a nuclease was proven to be more efficient at similar temperatures to those required for plant growth (Schindele and Puchta, 2020). So it would not be necessary to use these treatments in future assays with this nuclease. In terms of VIGE, there are studies that support the use of low temperatures (18°C) to boost genome editing (Liu et al., 2022), probably because low temperatures can counteract systemic silencing of viruses (Fu et al., 2006). Other studies claim that high temperature conditions (37°C) should be preferred when using this methodology (Lee et al., 2025). Further research in this topic should be done, and more trials with our transgenic melon lines should be required to determine whether increasing or reducing cultivation temperature could have a positive effect in genome editing.

When expressing the gRNAs by means of a viral vector, there are extra variables that should be taken into consideration. Viral vectors boost the expression of gRNAs due to their replication, and are able to deliver the transcripts at systemic non-inoculated leaves. However, long distance transport of plant virus varies among different viruses. In general, plant viruses are transported via phloem, and are passively transported within the source-to-sink flow of photoassimilates (Hipper et al., 2013). Most plant viruses exhibit pantropism (can infect multiple tissue types), and they can show differential host tropism (show different accumulation levels in different hosts within their range) (Núñez-Muñoz et al., 2025). Although replication machinery of potyviruses has been extensively studied, it is still unknown whether potyviruses move between cells as virions or as other movement complexes (Xue et al., 2023). Taking all of this into consideration, one may think that something as simple as the sampling area could significantly influence the

detection of edited tissue. In previous trials on tomato plants expressing the ttLbCas12a nuclease, in which the guides were transiently expressed using a vector derived from the tobacco rattle virus (TRV; genus *Tobravirus*), upper uninoculated leaves presented different and non-homogeneous indel percentages, even being 0% in certain leaves, while others surpassed a 20% of indels. This supports an irregular distribution of the virus in infected plants. Thus, it would be convenient to develop a WMV vector expressing a marker (such as GFP, for example) that can assist the sampling area to regions where the virus is replicating or has replicated, assuming that genomic editing will be found in those areas.

The lack of editing in our VIGE system may also be due to the low efficiency of the selected guides. In this work, five different gRNAs were evaluated, and no editing events were detected. When selecting gRNAs, the main criteria is to choose those with a higher efficiency score (http://crispor.tefor.net/). Based on the parameters followed, 'the higher the efficiency score, the more likely is cleavage at this position'. However, it should be taken into account that many factors can influence the way gRNAs are transcribed *in vivo*, and thus, gene editing efficiency. For example, the promoter used to control the expression of gRNAs could have a particular effect on guide expression (Haeussler et al., 2016). In addition, although the program can find present protospacers in the genomes of different organisms, the previous studies on which it relies to determine the efficiency of gRNAs are based on *in vitro* or animal models, so some information may change when transferred to plant models. Furthermore, in previous experiments done in our laboratory, with different species, we have observed that the character and efficiency of some guides change when transferring the results obtained *in silico* to plants, and guides with a low efficiency score are capable of generating high percentages of indels *in vivo*.

Although in different species, previous experiments in the laboratory validated the ability of our CRISPR/Cas constructs to generate genome editing. Transient agroinfiltration of our pLBHigCas9 vector into *N. benthamiana* plants in conjunction with sgRNAs targeting the *N. benthamiana Flowering locus T* gene (*NbFT*) revealed 16% editing in the agroinoculated tissue. Furthermore, lines of *Nicotiana tabacum* and *Solanum lycopersicum* transformed with our pLBHig-

Cas12att vector have been developed. Expressing guides against a reporter gene using a vector derived from tobacco etch virus (another potyvirus like WMV) we could achieve indel percentages of around 75% in both species. Thus, although the results cannot be totally extrapolated across different species, it could be hypothesized that a broader screening of guides could lead to the first editing event in our VIGE system in melon.

Materials and methods

Plant transformation and vector design

Vectors developed for plant stable transformation derive from the previously described minibinary vector pLX-B2 (Pasin et al., 2017). This small vector (3287 bp) presents the broad host-range pBBR1 origin for plasmid replication suitable for both *E. coli* and *Agrobacterium*. This cloning plasmid contains the *LacZα* reporter gene flanked by two BsaI recognition sites, and confers kanamycin resistance for bacterial selection. Obtaining the four plasmids used for plant transformation was done in a sequential manner. In summary, the three plasmids conferring resistance to hygromycin pLBHig-Cas9, pLBHig-Cas12a and pLBHig-Cas12att (**Figure S1**) came from the intermediate plasmid pLBHig3 (**Figure S1**), that derives from pLX-B2 and expresses the hygromycin resistance cassette under the control of the NoS promoter and terminator of *A. tumefaciens*. This cassette is flanked by ApaI and MluI recognition sites, which allow easy and fast transcriptional unit replacement. SpCas9 nuclease sequence was cloned into the pLBHig3 vector by ApaI/NotI restriction (Thermo Scientific) and T4 DNA ligase (Thermo Scientific) DNA ligation from the donor vector p235SpCas9-2xNLS. The same approach was followed to express LbCas12 and ttLbCas12a nucleases in the pLBHig3, in this case, using as a donor vector p235KLbCas12a and p235KttLbCas12a respectively, giving rise to pLBHig-Cas12 and pLBHig-Cas12att. For this purpose, the intermediate plasmid p235kLbCas12a was first mutagenized to obtain the thermotolerant version of the nuclease (D156R), leading to p235KLbCas12att vector. Finally, pLBKan-Cas12att (**Figure S1**) was obtained after cloning the corresponding nuclease in the intermediate plasmid pLBKan, an analog of pLBHig3, but expressing *npt*II instead of *hpt*. Every nuclease was under the control of a dual CaMV 35S

promoter and terminator. 5´ and 3´untranslated regions (UTR) of cowpea mosaic virus (CPMV) flanked the CDS of the nucleases. The nucleases were fused to two nuclear localization signals (NLS), of nucleoplasmin and simian virus 40 (SV40) in their carboxyl terminus. The four plasmids used for plant transformation and intermediate plasmids were built by standard molecular biology techniques, mainly restriction and ligation cloning; PCR amplification with high-fidelity Phusion DNA polymerase (Thermo Scientific) and Gibson DNA assembly with the NEBuilder HiFi DNA assembly master mix (New England Biolabs) (Gibson et al., 2009). Primers used for PCR amplification are listed in **Table S3**. Specific regions of developed plasmids were Sanger sequenced. To further evaluate the lack of punctual mutations, the whole plasmid were sequenced using Oxford Nanopore technology (Plasmidsaurus, https://plasmidsaurus.com/plasmid_sequencing).

Plant transformation and regeneration

Plant transformation experiments were done in Dr. Vicente Moreno´s lab. Most of the experiments were done and supervised by Dr. Begoña García-Sogo, with support of Dr. Benito Pineda. Seeds from 5 different melon genotypes of agronomic interest were selected as starting material for plant transformation: 'Meló Groc d'Ontinyent' (MGO, 'Amarillo' type); 'Védrantais' ('Cantaloupe' type); 'Pipa de Oro' (PO, 'Piel de Sapo' type); CH5 ('Piel de Sapo' type) and 'Charentais' ('Cantaloupe' type). Seeds were firstly introduced in a 10% hydrochloric acid solution and were later surface sterilised by immersion in 12.5% commercial bleach during 20 min followed by three rinses in sterile distilled water (5, 10 and 15 min, respectively). Seeds germinated in Petri dishes containing MS medium (Duchefa Biochemie), supplemented with sucrose (20 g/l) and bacteriological agar (8 g/l) (Sumilab). As explants sources, cotyledons and leaves were taken from axenic grown plants. Explants were treated and cut into pieces as described in Moreno et al., 1985. Two different *A. tumefaciens* strains were used for plant transformation (LBA4404 and AGL1). *A. tumefaciens* culture preparation and explant transformation was done as described in Albalat, 2016. The used protocol was previously described by Atarés et al., 2011. In the case of AGL1, solid and liquid selective medium for *Agrobacterium* growth also contained spectinomycin (50 µg/ml). Sliced explants were submerged in *Agrobacterium* culture for 10 min, and were later transferred to

coculture medium for 48 h at 28 °C (dark). To remove remaining bacteria, explants were transferred to a washing solution for 10 min, and were later located in a morphogenesis induction medium (IBZ), supplemented either with kanamycin (100 μg/ml) or hygromycin (5 μg/ml) depending on the specific transformation. Regenerated shoots were transferred to root inducing media. The composition of the used medium is described in Albalat, 2016. During clonal propagation stage, different explants from regenerated plants (leaves or hypocotyls) were subjected to an antibiotic resistance test (kanamycin 100 μg/ml or hygromycin 5 μg/ml). Regenerated plants were maintained *in vitro*, and later acclimated to greenhouse conditions, where they were selfed, and subsequent experiments were performed.

T-DNA detection and sequencing

The evaluation of the presence of the T-DNA in plants regenerated after transformation, and in T_1 generation was done by means of PCR amplification, using *Thermus thermophilus* (Tth) DNA polymerase (Biotools). Specific primers were designed to amplify concrete parts of the developed constructs (either the different nucleases or the antibiotic resistance genes) (**Table S1**). PCR conditions were as previously described (Bedoya et al., 2010). PCR products were separated by electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, and 1 mM EDTA, pH 7.2), and later stained with 1% ethidium bromide. Complete T-DNA integration was confirmed in two independent plants. For this purpose, the whole T-DNA sequence was split in three fragments of similar lengths. These fragments were PCR amplified using specific primers (**Table S1**) and Phusion DNA polymerase. PCR products were cloned in the EcoRV digested pBSΔE clonning vector (**Figure S6**), and the whole plasmid was sequenced using Oxford Nanopore technology (Plasmidsaurus).

Guide selection and vector development

Both Cas9 sgRNAs and Cas12 gRNAs were expressed in transgenic plants using a vector derived from watermelon mosaic virus (WMV), WMV-Vera (GenBank accession number MH469650), and named as pGWMV-Vera (Aragonés et al., 2019). The expression of the viral cDNA was driven by a 35S promoter and a terminator. Inserts were cloned in between the nuclear inclusion protein b (NIb) and the coat protein cistrons of WMV. Online tool CRISPOR

(http://crispor.tefor.net/), was used for guide RNA selection, ranking first guides with the highest specificity scores and lowest amount of off-targets. Guides leading to a stop codon in the WMV polyprotein were discarded, as it would impair viral replication. 17 different gRNAs were selected, and cloned in pGWMV vector (**Figure S4**, **Table S2**), targeting four different endogenous genes: *Magnesium chelatase subunit I* (*CHLI*) MELO3C007233.2; *Phytoene desaturase* (*PDS*) MELO3C017772.2; *Mildew locus O 1*; (*MLO1*) MELO3C012438.2 and *DNA primase large subunit* MELO3C022319.2. Gene models used for guide selection were based on genome assembly v4.0 available at Melonomics website (http://www.melonomics.net/melonomics.html). Every gRNA was cloned as a dsDNA product in the pGWMV-Vera digested with MluI (Thermo Scientific) by means of Gibson DNA assembly. For Cas12 gRNAs assembly, a PCR reaction was conducted with Phusion DNA polymerase, using a common forward primer for all constructs, serving as a donor of the Cas12 direct repeat (D4998). A specific reverse primer was designed for every gRNA, presenting a 15-nt overhang annealing to D4998 in its 3´end, allowing PCR product amplification in absence of DNA template. For Cas9 sgRNA cloning, a similar approach was followed. In this case, specific forward primers containing each spacer were designed. pGTEVΔEE sgCHLI1-5'FT Cas9 M3 plasmid served as a donor of a compact RNA scaffold lacking stop codons (amplification with D6299 as a reverse primer); or as a donor of the scaffold fused to the *Flowering Locus T* of *A. thaliana* in its 3´ end (amplification with D6300 as a reverse primer) (**Figure S7**). All the primers used for vector construction are listed in **Table S4**.

Plant inoculation

T_1 melon plants 'Meló Groc d'Ontinyent', and CH5 'Piel de Sapo' were grown under controlled conditions at 25°C and a 16-h day/8-h night cycle. Plants were watered three times/week. Two weeks old plants were agroinoculated in one cotyledon and the first true leaf with *A. tumefaciens* preparations harbouring the different mentioned constructs. C58C1 *A. tumefaciens* strain was previously transformed with pCLEAN-S48153 helper plasmid (Thole et al. 2007). Competent bacteria were electroporated with pGWMV derivatives , and transformed *A. tumefaciens* colonies were selected from Luria Bertani (LB) agar plates supplemented with 50 µg/ml rifampicin, 7.5 µg/ml tetracycline, and 50

µg/ml kanamycin and incubated at 28°C for four days. Individual colonies were selected and grown for 24 h in liquid LB medium supplemented with 50 µg/ml kanamycin. When cultures reached an optical density of 600 nm (OD600) 1-2, they were pelleted by centrifugation and resuspended to an OD600 of 1 in inoculation buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH pH 5.6), 10 mM $MgCl_2$, supplemented with 150 µM acetosyringone. The prepared inoculation mixture was incubated in dark for 2 h at 28°C. The abaxial side of one cotyledon and first true leaf were infiltrated using a needle-less 1 ml syringe. Plants were cultivated at the same conditions as previously described until sample collection.

RT-PCR analysis of inoculated plants

Three weeks after inoculation, systemic non inoculated leaves were sampled (1 cm^2 approximately). Plant tissue was ground in 2 ml Eppendorf tubes containing a 4-mm diameter stainless steel ball, and using a VWR Star-Beater machine, for 1 min at 30 s^{-1}. Extraction buffer (4 M guanidinium thiocyanate, 0.1 M sodium acetate, 10 mM EDTA and 0.1 M 2-mercaptoethanol, pH 5.5) was previously added to each tube. After homogenization, samples were centrifuged at 15,000g for 5 min and 0.6 ml of the supernatant was mixed with 0.39 ml of 96% ethanol. After centrifugation at 15,000g for 1 min, the upper face was transferred to a silica gel column (Zymo Research). The column was centrifuged at 15000g for 1 min, and was washed twice with 0.5 ml of washing buffer (70% ethanol, 10 mM sodium acetate, pH 5.5). WMV detection was done by means of reverse transcription (RT)-PCR amplification. Aliquots of 1 µl of the purified RNA were used for reverse transcription with RevertAid reverse transcriptase (Thermo Scientific), using D1553 primer in a 10-µl final volume reaction. 1 µl of the obtained cDNA was PCR amplified for 30 cycles with (Tth) DNA polymerase using D2380 and D3256 primers, amplifying a fragment of the coat protein of WMV, and following the previously mentioned conditions. PCR products were analysed by electrophoresis in 1% agarose gels in TAE buffer, and gels were stained with ethidium bromide. Primers used for RT-PCR are listed in **Table S5**.

Plant genotyping (editing analysis)

Infected plants were subjected to a genotype analysis by means of PCR amplification and later sequencing. For this, DNA was extracted from upper non inoculated leaves 3 weeks post inoculation. DNA purification protocol was the same as previously described for RNA isolation, but lacking ethanol addition. Aliquots (1 µl) of the purified DNA were subjected to PCR amplification using Phusion DNA polymerase and specific primers covering the target sites of the genes *CHLI,* MELO3C007233.2; *PDS*, MELO3C017772.2 and *DNA primase,* MELO3C022319.2. PCR products were separated by electrophoresis in 1% agarose gels, and they were purified using silica gel columns. Purified products were Sanger sequenced using the same primers employed for PCR amplification. Chromas software (https://technelysium.com.au/wp/chromas/) and Synthego ICE online tool (https://www.synthego.com/) were used to analyze and compare Sanger results, to uninoculated and thus unedited control plants. Primers used for plant genotyping are listed in **Table S5**.

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Supplementary data

Supplementary data is available at the end of this chapter.

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Supporting information

Figure S1. Full sequence of intermediate plasmids pLBHig3 and pLBKan, and the inserts required to obtain their derivative transformation plasmids: pLBHig-Cas9, pLBHig-Cas12a, pLBHig-Cas12att and pLBKan-Cas12att.

>pLBHig3

ACTAGTGCGGCGGCGGCCGCGCGGCGGGGCCCGATCATGAGCGGAGAATTAAGG
GAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGTTTGGA
ACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCGCGGGTTTCTGGAGTTT
AATGAGCTAAGCACATACGTCAGAAACCATTATTGCGCGTTCAAAAGTCGCCTA
AGGTCACTATCAGCTAGCAAATATTTCTTGTCAAAAATGCTCCACTGACGTTCC
ATAAATTCCCCTCGGTATCCAATTAGAGTCTCATATTCACTCTCAATCCAAATA
ATCTGCAATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGAT
CGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATC
TCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAG
CTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGC
CGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTTAGCGAGAGCCTGAC
CTATTGCATCTCCCGCCGTTCACAGGGTGTCACGTTGCAAGACCTGCCTGAAAC
CGAACTGCCCGCTGTTCTACAACCGGTCGCGGAGGCTATGGATGCGATCGCTGC
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TCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTA
TCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCT
CGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCA
CGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGT
CATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACAT
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GCGGAGGCATCCGGAGCTTGCAGGATCGCCACGACTCCGGGCGTATATGCTCCG
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CGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGT
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GAAATAAAGAAGGAGTGCGTCGAAGCAGATCGTTCAAACATTTGGCAATAAAGT
TTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTG
TTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATG
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AACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATG
TTACTAGATCGACGCGTTGAGACGGTTTCGACCAGGCTACTAGAGTACTTAATT
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CGGACATGAAGCCATTTACAATTGAATATATCCTGCCGCCGCTGCCGCTTTGCA
CCCGGTGGAGCTTGCATGTTGGTTTCTACGCAGAACTGAGCCGGTTTTGTTATC
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GATGATGGCCCCCTACGGGCTTGCTCTCCGGGCTTCGCCCTGCGCGGTCGCTGC

GCTCCCTTGCCAGCCCGTGGATATGTGGACGATGGCCGCGAGCGGCCACCGGCT
GGCTCGCTTCGCTCGGCCCGTGGACAACCCTGCTGGACAAGCTGATGGACAGGC
TGCGCCTGCCCACGAGCTTGACCACAGGGATTGCCCACCGGCTACCCAGCCTTC
GACCACATACCCACCGGCTCCAACTGCGCGGCCTGCGGCCTTGCCCCATCAATT
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CACTGGGTGCAAACGGAACGCAAAGCGCATGAGGCGTGGGCCGGGCTTATTGCG
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CAGAACGCCGTGGTGGTCAGCCAGAAGACACTTTCCAAGCTCATCGGACGTTCT
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TTGACCGAAACGGAGGAATGGGAACGGCGCGGGCAGCAGCGCCTGCCGATGCCC
GATGAGCCGTGTTTTCTGGACGATGGCGAGCCGTTGGAGCCGCCGACACGGGTA
ACGCTGCCGCGCCGGTAGAACCGCATAACCGCCAATCCGATCTTGTGTCTCAAA
ATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAAAC
TGTCTGCTTACATAAACAGTAATACAAGGGGTGTT**ATG**AGCCATATTCAGCGTG
AAACGAGCTGTAGCCGTCCGCGTCTGAACAGCAACATGGATGCGGATCTGTATG
GCTATAAATGGGCGCGTGATAACGTGGGTCAGAGCGGCGCGACCATTTATCGTC
TGTATGGCAAACCGGATGCGCCGGAACTGTTTCTGAAACATGGCAAAGGCAGCG
TGGCGAACGATGTGACCGATGAAATGGTGCGTCTGAACTGGCTGACCGAATTTA
TGCCGCTGCCGACCATTAAACATTTTATTCGCACCCCGGATGATGCGTGGCTGC
TGACCACCGCGATTCCGGGCAAAACCGCGTTTCAGGTGCTGGAAGAATATCCGG
ATAGCGGCGAAAACATTGTGGATGCGCTGGCCGTGTTTCTGCGTCGTCTGCATA
GCATTCCGGTGTGCAACTGCCCGTTTAACAGCGATCGTGTGTTTCGTCTGGCCC
AGGCGCAGAGCCGTATGAACAACGGCCTGGTGGATGCGAGCGATTTTGATGATG
AACGTAACGGCTGGCCGGTGGAACAGGTGTGGAAAGAAATGCATAAACTGCTGC
CGTTTAGCCCGGATAGCGTGGTGACCCACGGCGATTTTAGCCTGGATAACCTGA
TTTTCGATGAAGGCAAACTGATTGGCTGCATTGATGTGGGCCGTGTGGGCATTG
CGGATCGTTATCAGGATCTGGCCATTCTGTGGAACTGCCTGGGCGAATTTAGCC
CGAGCCTGCAAAAACGTCTGTTTCAGAAATATGGCATTGATAATCCGGATATGA

ACAAACTGCAATTTCATCTGATGCTGGATGAATTTTTCTAATAGTTGATTGCAA
AGGACCGCATGTCGCAAGTCGGCGCGCCACACTAACTAAAAACACCCTAACGGG
TGTTTTTTCTTTTCTTGTTCTTCCAAAAAAATTTACAACCGAACAAACAAACAA
ATACAGAAATTTAGACAATAAAAATGAATTACAACCGCATATATCCTGCCATCA
GTCATCACAAAATCAACAATTCCTGCAGGCTAGCTCGCATCCTTGTCCCGTCTC
CTGAC

Hygromycin resistance cassette in green (ORF under the control of the *A. tumefaciens* NoS **promoter** and **terminator**) flanked by unique (5') **SpeI**, **ApaI**, **NotI**, and (3') **MluI** sites in the T-DNA of pLX-B2 [U22]. Double **LB** and **RB** of the T-DNA. **kanamycin** resistance for plasmid selection is denoted in light grey.

>pLBKan

ACTAGTGCGGCG**GCGGCCGC**GCGGCG**GGGCCC**GATCATGAGCGGAGAATTAAGG
GAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGTTTGGA
ACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCGCGGGTTTCTGGAGTTT
AATGAGCTAAGCACATACGTCAGAAACCATTATTGCGCGTTCAAAAGTCGCCTA
AGGTCACTATCAGCTAGCAAATATTTCTTGTCAAAAATGCTCCACTGACGTTCC
ATAAATTCCCCTCGGTATCCAATTAGAGTCTCATATTCACTCTCAATCCAAATA
ATCTGCAATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGT
GGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGC
CGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGA
CCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCT
GGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGG
AAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCA
CCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCA
TACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGA
GCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGA
AGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCAT
GCCCGACGGCGATGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATAT
CATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGT
GGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCT
TGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGA
TTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTAAAGAAGGAG
TGCGTCGAAGCAGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAAT
CCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAG
CATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATG
ATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGC
GCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGACGC
GTTGAGACGGTTTCGACCAGGCTACTAGTACTTAATTAATCTTTTTCTCCATTG
TTTACACCACAATATATCCTGCCACCAGTAGATTTCCCGGACATGAAGCCATTT
ACAATTGAATATATCCTGCCGCCGCTGCCGCTTTGCACCCGGTGGAGCTTGCAT

GTTGGTTTCTACGCAGAACTGAGCCGGTTTTGTTATCAATAAAAAAGGCCGCGA
TTTGCGGCCTTATTGTTCGTCGGAAGACCACCGAACTGATGATGGCCCCCTACG
GGCTTGCTCTCCGGGCTTCGCCCTGCGCGGTCGCTGCGCTCCCTTGCCAGCCCG
TGGATATGTGGACGATGGCCGCGAGCGGCCACCGGCTGGCTCGCTTCGCTCGGC
CCGTGGACAACCCTGCTGGACAAGCTGATGGACAGGCTGCGCCTGCCCACGAGC
TTGACCACAGGGATTGCCCACCGGCTACCCAGCCTTCGACCACATACCCACCGG
CTCCAACTGCGCGGCCTGCGGCCTTGCCCCATCAATTTTTTTAATTTTCTCTGG
GGAAAAGCCTCCGGCCTGCGGCCTGCGCGCTTCGCTTGCCGGTTGGACACCAAG
TGGAAGGCGGGTCAAGGCTCGCGCAGCGACCGCGCAGCGGCTTGGCCTTGACGC
GCCTGGAACGACCCAAGCCTATGCGAGTGGGGGCAGTCGAAGGGCGAAGCCCGC
CCGCCTGCCCCCCGAGCCTCACGGCGGCGAGTGCGGGGGTTCCAAGGGGGCAGC
GCCACCTTGGGCAAGGCCGAAGGCCGCGCAGTCGATCAACAAGCCCCGGAGGGG
CCACTTTTTGCCGGAGGGGGAGCCGCGCCGAAGGCGTGGGGGAACCCCGCAGGG
GTGCCCTTCTTTGGGCACCAAAGAACTAGATATAGGGCGAAATGCGAAAGACTT
AAAAATCAACAACTTAAAAAAGGGGGGTACGCAACAGCTCATTGCGGCACCCCC
CGCAATAGCTCATTGCGTAGGTTAAAGAAAATCTGTAATTGACTGCCACTTTTA
CGCAACGCATAATTGTTGTCGCGCTGCCGAAAAGTTGCAGCTGATTGCGCATGG
TGCCGCAACCGTGCGGCACCCCTACCGCATGGAGATAAGCATGGCCACGCAGTC
CAGAGAAATCGGCATTCAAGCCAAGAACAAGCCCGGTCACTGGGTGCAAACGGA
ACGCAAAGCGCATGAGGCGTGGGCCGGGCTTATTGCGAGGAAACCCACGGCGGC
AATGCTGCTGCATCACCTCGTGGCGCAGATGGGCCACCAGAACGCCGTGGTGGT
CAGCCAGAAGACACTTTCCAAGCTCATCGGACGTTCTTTGCGGACGGTCCAATA
CGCAGTCAAGGACTTGGTGGCCGAGCGCTGGATCTCCGTCGTGAAGCTCAACGG
CCCCGGCACCGTGTCGGCCTACGTGGTCAATGACCGCGTGGCGTGGGGCCAGCC
CCGCGACCAGTTGCGCCTGTCGGTGTTCAGTGCCGCCGTGGTGGTTGATCACGA
CGACCAGGACGAATCGCTGTTGGGGCATGGCGACCTGCGCCGCATCCCGACCCT
GTATCCGGGCGAGCAGCAACTACCGACCGGCCCCGGCGAGGAGCCGCCCAGCCA
GCCCGGCATTCCGGGCATGGAACCAGACCTGCCAGCCTTGACCGAAACGGAGGA
ATGGGAACGGCGCGGGCAGCAGCGCCTGCCGATGCCCGATGAGCCGTGTTTTCT
GGACGATGGCGAGCCGTTGGAGCCGCCGACACGGGTAACGCTGCCGCGCCGGTA
GAACCGCATAACCGCCAATCCGATCTTGTGTCTCAAAATCTCTGATGTTACATT
GCACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAAC
AGTAATACAAGGGGTGTT**ATG**AGCCATATTCAGCGTGAAACGAGCTGTAGCCGT
CCGCGTCTGAACAGCAACATGGATGCGGATCTGTATGGCTATAAATGGGCGCGT
GATAACGTGGGTCAGAGCGGCGCGACCATTTATCGTCTGTATGGCAAACCGGAT
GCGCCGGAACTGTTTCTGAAACATGGCAAAGGCAGCGTGGCGAACGATGTGACC
GATGAAATGGTGCGTCTGAACTGGCTGACCGAATTTATGCCGCTGCCGACCATT
AAACATTTTATTCGCACCCCGGATGATGCGTGGCTGCTGACCACCGCGATTCCG
GGCAAAACCGCGTTTCAGGTGCTGGAAGAATATCCGGATAGCGGCGAAAACATT
GTGGATGCGCTGGCCGTGTTTCTGCGTCGTCTGCATAGCATTCCGGTGTGCAAC
TGCCCGTTTAACAGCGATCGTGTGTTTCGTCTGGCCCAGGCGCAGAGCCGTATG
AACAACGGCCTGGTGGATGCGAGCGATTTTGATGATGAACGTAACGGCTGGCCG
GTGGAACAGGTGTGGAAAGAAATGCATAAACTGCTGCCGTTTAGCCCGGATAGC
GTGGTGACCCACGGCGATTTTAGCCTGGATAACCTGATTTTCGATGAAGGCAAA

CTGATTGGCTGCATTGATGTGGGCCGTGTGGGCATTGCGGATCGTTATCAGGAT
CTGGCCATTCTGTGGAACTGCCTGGGCGAATTTAGCCCGAGCCTGCAAAAACGT
CTGTTTCAGAAATATGGCATTGATAATCCGGATATGAACAAACTGCAATTTCAT
CTGATGCTGGATGAATTTTTC**TAA**TAGTTGATTGCAAAGGACCGCATGTCGCAA
GTCGGCGCGCCACACTAACTAAAAACACCCTAACGGGTGTTTTTTCTTTTCTTG
TTCTTCCAAAAAAATTTACAACCGAACAAACAAACAAATACAGAAATTTAGACA
ATAAAAATGA**ATTACAACCGCATATATCCTGCCA**TCAGTCATCACAAAATCAAC
AATTCCTGCAGGCTAGCTCGCATCCTTGTCCCGTCTCCTGAC

Kanamycin resistance cassette in green (ORF under the control of the *A. tumefaciens* NoS **promoter** and **terminator**) flanked by unique (5') **SpeI**, **ApaI**, **NotI**, and (3') **MluI** sites in the T-DNA of pLX-B2 [U22]. Double **LB** and **RB** of the T-DNA. **kanamycin** resistance for plasmid selection is denoted in light grey.

>pLBHig-Cas9 (insert between C20 and G27 of pLBHig3; derived from p235SpCas9-2xNLS)

GATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAG
GTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATG
CCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGG
AAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAGAT
TCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTG
GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCT
CTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAA
AAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCA
CTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTA
TATAAGGAAGTTCATTTCATTTGGAGAGGTATTAAAATCTTAATAGGTTTTGAT
AAAAGCGAACGTGGGGAAACCCGAACCAAACCTTCTTCTAAACTCTCTCTCATC
TCTCTTAAAGCAAACTTCTCTCTTGTCTTTCTTGCGTGAGCGATCTTCAACGTT
GTCAGATCGTGCTTCGGCACCAGTACAACGTTTTCTTTCACTGAAGCGAAATCA
AAGATCTCTTTGTGGACACGTAGTGCGGCGCCATTAAATAACGTGTACTTGTCC
TATTCTTGTCGGTGTGGTCTTGGGAAAAGAAAGCTTGCTGGAGGCTGCTGTTCA
GCCCCATACATTACTTGTTACGATTCTGCTGACTTTCGGCGGGTGCAATATCTC
TACTTCTGCTTGACGAGGTATTGTTGCCTGTACTTCTTTCTTCTTCTTCTTGCT
GATTGGTTCTATAAGAAATCTAGTATTTTCTTTGAAACAGAGTTTTCCCGTGGT
TTTCGAACTTGGAGAAAGATTGTTAAGCTTCTGTATATTCTGCCCAAATTTGAA
ATGGACAAGAAGTACTCCATTGGGCTCGATATCGGCACAAACAGCGTCGGCTGG
GCCGTCATTACGGACGAGTACAAGGTGCCGAGCAAAAAATTCAAAGTTCTGGGC
AATACCGATCGCCACAGCATAAAGAAGAACCTCATTGGCGCCCTCCTGTTCGAC
TCCGGGGAAACGGCCGAAGCCACGCGGCTCAAAAGAACAGCACGGCGCAGATAT
ACCCGCAGAAAGAATCGGATCTGCTACCTGCAGGAGATCTTTAGTAATGAGATG
GCTAAGGTGGATGACTCTTTCTTCCATAGGCTGGAGGAGTCCTTTTTGGTGGAG
GAGGATAAAAAGCACGAGCGCCACCCAATCTTTGGCAATATCGTGGACGAGGTG

GCGTACCATGAAAAGTACCCAACCATATATCATCTGAGGAAGAAGCTTGTAGAC
AGTACTGATAAGGCTGACTTGCGGTTGATCTATCTCGCGCTGGCGCATATGATC
AAATTTCGGGGACACTTCCTCATCGAGGGGGACCTGAACCCAGACAACAGCGAT
GTCGACAAACTCTTTATCCAACTGGTTCAGACTTACAATCAGCTTTTCGAAGAG
AACCCGATCAACGCATCCGGAGTTGACGCCAAAGCAATCCTGAGCGCTAGGCTG
TCCAAATCCCGGCGGCTCGAAAACCTCATCGCACAGCTCCCTGGGGAGAAGAAG
AACGGCCTGTTTGGTAATCTTATCGCCCTGTCACTCGGGCTGACCCCCAACTTT
AAATCTAACTTCGACCTGGCCGAAGATGCCAAGCTTCAACTGAGCAAAGACACC
TACGATGATGATCTCGACAATCTGCTGGCCCAGATCGGCGACCAGTACGCAGAC
CTTTTTTTGGCGGCAAAGAACCTGTCAGACGCCATTCTGCTGAGTGATATTCTG
CGAGTGAACACGGAGATCACCAAAGCTCCGCTGAGCGCTAGTATGATCAAGCGC
TATGATGAGCACCACCAAGACTTGACTTTGCTGAAGGCCCTTGTCAGACAGCAA
CTGCCTGAGAAGTACAAGGAAATTTTCTTCGATCAGTCTAAAAATGGCTACGCC
GGATACATTGACGGCGGAGCAAGCCAGGAGGAATTTTACAAATTTATTAAGCCC
ATCTTGGAAAAAATGGACGGCACCGAGGAGCTGCTGGTAAAGCTTAACAGAGAA
GATCTGTTGCGCAAACAGCGCACTTTCGACAATGGAAGCATCCCCCACCAGATT
CACCTGGGCGAACTGCACGCTATCCTCAGGCGGCAAGAGGATTTCTACCCCTTT
TTGAAAGATAACAGGGAAAAGATTGAGAAAATCCTCACATTTCGGATACCCTAC
TATGTAGGCCCCCTCGCCCGGGGAAATTCCAGATTCGCGTGGATGACTCGCAAA
TCAGAAGAGACTATCACTCCCTGGAACTTCGAGGAAGTCGTGGATAAGGGGGCC
TCTGCCCAGTCCTTCATCGAAAGGATGACTAACTTTGATAAAAATCTGCCTAAC
GAAAAGGTGCTTCCTAAACACTCTCTGCTGTACGAGTACTTCACAGTTTATAAC
GAGCTCACCAAGGTCAAATACGTCACAGAAGGGATGAGAAAGCCAGCATTCCTG
TCTGGAGAGCAGAAGAAAGCTATCGTGGACCTCCTCTTCAAGACGAACCGGAAA
GTTACCGTGAAACAGCTCAAAGAAGATTATTTCAAAAAGATTGAATGTTTCGAC
TCTGTTGAAATCAGCGGAGTGGAGGATCGCTTCAACGCATCCCTGGGAACGTAT
CACGATCTCCTGAAAATCATTAAAGACAAGGACTTCCTGGACAATGAGGAGAAC
GAGGACATTCTTGAGGACATTGTCCTCACCCTTACGTTGTTTGAAGATAGGGAG
ATGATTGAAGAACGCTTGAAAACTTACGCTCATCTCTTCGACGACAAAGTCATG
AAACAGCTCAAGAGGCGCCGATATACAGGATGGGGGCGGCTGTCAAGAAAACTG
ATCAATGGGATCCGAGACAAGCAGAGTGGAAAGACAATCCTGGATTTTCTTAAG
TCCGATGGATTTGCCAACCGGAACTTCATGCAGTTGATCCATGATGACTCTCTC
ACCTTTAAGGAGGACATCCAGAAAGCACAAGTTTCTGGCCAGGGGGACAGTCTC
CACGAGCACATCGCTAATCTTGCAGGTAGCCCAGCTATCAAAAAGGGAATACTG
CAGACCGTTAAGGTCGTGGATGAACTCGTCAAAGTAATGGGAAGGCATAAGCCC
GAGAATATCGTTATCGAGATGGCCCGAGAGAACCAAACTACCCAGAAGGGACAG
AAGAACAGTAGGGAAAGGATGAAGAGGATTGAAGAGGGTATAAAAGAACTGGGG
TCCCAAATCCTTAAGGAACACCCAGTTGAAAACACCCAGCTTCAGAATGAGAAG
CTCTACCTGTACTACCTGCAGAACGGCAGGGACATGTACGTGGATCAGGAACTG
GACATCAATCGGCTCTCCGACTACGACGTGGATCATATCGTGCCCCAGTCTTTT
CTCAAAGATGATTCTATTGATAATAAAGTGTTGACAAGATCCGATAAAAATAGA
GGGAAGAGTGATAACGTCCCCTCAGAAGAAGTTGTCAAGAAAATGAAAAATTAT
TGGCGGCAGCTGCTGAACGCCAAACTGATCACACAACGGAAGTTCGATAATCTG
ACTAAGGCTGAACGAGGTGGCCTGTCTGAGTTGGATAAAGCCGGCTTCATCAAA

AGGCAGCTTGTTGAGACACGCCAGATCACCAAGCACGTGGCCCAAATTCTCGAT
TCACGCATGAACACCAAGTACGATGAAAATGACAAACTGATTCGAGAGGTGAAA
GTTATTACTCTGAAGTCTAAGCTGGTTTCAGATTTCAGAAAGGACTTTCAGTTT
TATAAGGTGAGAGAGATCAACAATTACCACCATGCGCATGATGCCTACCTGAAT
GCAGTGGTAGGCACTGCACTTATCAAAAAATATCCCAAGCTTGAATCTGAATTT
GTTTACGGAGACTATAAAGTGTACGATGTTAGGAAAATGATCGCAAAGTCTGAG
CAGGAAATAGGCAAGGCCACCGCTAAGTACTTCTTTTACAGCAATATTATGAAT
TTTTTCAAGACCGAGATTACACTGGCCAATGGAGAGATTCGGAAGCGACCACTT
ATCGAAACAAACGGAGAAACAGGAGAAATCGTGTGGGACAAGGGTAGGGATTTC
GCGACAGTCCGGAAGGTCCTGTCCATGCCGCAGGTGAACATCGTTAAAAAGACC
GAAGTACAGACCGGAGGCTTCTCCAAGGAAAGTATCCTCCCGAAAAGGAACAGC
GACAAGCTGATCGCACGCAAAAAAGATTGGGACCCCAAGAAATACGGCGGATTC
GATTCTCCTACAGTCGCTTACAGTGTACTGGTTGTGGCCAAAGTGGAGAAAGGG
AAGTCTAAAAAACTCAAAAGCGTCAAGGAACTGCTGGGCATCACAATCATGGAG
CGATCAAGCTTCGAAAAAAACCCCATCGACTTTCTCGAGGCGAAAGGATATAAA
GAGGTCAAAAAAGACCTCATCATTAAGCTTCCCAAGTACTCTCTCTTTGAGCTT
GAAAACGGCCGGAAACGAATGCTCGCTAGTGCGGGCGAGCTGCAGAAAGGTAAC
GAGCTGGCACTGCCCTCTAAATACGTTAATTTCTTGTATCTGGCCAGCCACTAT
GAAAAGCTCAAAGGATCTCCCGAAGATAATGAGCAGAAGCAGCTGTTCGTGGAA
CAACACAAACACTACCTTGATGAGATCATCGAGCAAATAAGCGAATTCTCCAAA
AGAGTGATCCTCGCCGACGCTAACCTCGATAAGGTGCTTTCTGCTTACAATAAG
CACAGGGATAAGCCCATCAGGGAGCAGGCAGAAAACATTATCCACTTGTTTACT
CTGACCAACTTGGGCGCGCCTGCAGCCTTCAAGTACTTCGACACCACCATAGAC
AGAAAGCGGTACACCTCTACAAAGGAGGTCCTGGACGCCACACTGATTCATCAG
TCAATTACGGGGCTCTATGAAACAAGAATCGACCTCTCTCAGCTCGGTGGAGAC
GGTTCAAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAAG
GGATCCCCCAAGAAGAAGAGGAAGGTGTAACTCTGGTTTCATTAAATTTTCTTT
AGTTTGAATTTACTGTTATTCGGTGTGCATTTCTATGTTTGGTGAGCGGTTTTC
TGTGCTCAGAGTGTGTTTATTTTATGTAATTTAATTTCTTTGTGAGCTCCTGTT
TAGCAGGTCGTCCCTTCAGCAAGGACACAAAAAGATTTTAATTTTATTCGCTGA
AATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTTTCTCCATAAATAAT
GTGTGAGTAGTTTCCCGATAAGGGAAATTAGGGTTCTTATAGGGTTTCGCTCAT
GTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCT
ATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAG

SpCas9 coding sequence in black (human optimized codon) followed by **GS** spacers and double NLS (**nucleoplasmin** and **SV40**). Dual (CaMV) 35S promoter (**2X35S Promoter** in garnet and red) underlining +1 nucleotide of transcription. **35S terminator** in fuchsia with the processing and polyadenylation site underlined. Sequences of **5' and 3' UTR of RNA-2 of CPMV** are denoted in green.

>pLBHig-Cas12 (insert between C20 and G27 of pLBHig3; derived from p235KLbCas12a)

GATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAG
GTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATG
CCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGG
AAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAGAT
TCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTG
GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCT
CTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAA
AAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCA
CTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTA
TATAAGGAAGTTCATTTCATTTGGAGAGGTATTAAAATCTTAATAGGTTTTGAT
AAAAGCGAACGTGGGGAAACCCGAACCAAACCTTCTTCTAAACTCTCTCTCATC
TCTCTTAAAGCAAACTTCTCTCTTGTCTTTCTTGCGTGAGCGATCTTCAACGTT
GTCAGATCGTGCTTCGGCACCAGTACAACGTTTTCTTTCACTGAAGCGAAATCA
AAGATCTCTTTGTGGACACGTAGTGCGGCGCCATTAAATAACGTGTACTTGTCC
TATTCTTGTCGGTGTGGTCTTGGGAAAAGAAAGCTTGCTGGAGGCTGCTGTTCA
GCCCCATACATTACTTGTTACGATTCTGCTGACTTTCGGCGGGTGCAATATCTC
TACTTCTGCTTGACGAGGTATTGTTGCCTGTACTTCTTTCTTCTTCTTCTTGCT
GATTGGTTCTATAAGAAATCTAGTATTTTCTTTGAAACAGAGTTTTCCCGTGGT
TTTCGAACTTGGAGAAAGATTGTTAAGCTTCTGTATATTCTGCCCAAATTTGAA
ATGAGCAAGCTGGAGAAGTTTACAAACTGCTACTCCCTGTCTAAGACCCTGAGG
TTCAAGGCCATCCCTGTGGGCAAGACCCAGGAGAACATCGACAATAAGCGGCTG
CTGGTGGAGGACGAGAAGAGAGCCGAGGATTATAAGGGCGTGAAGAAGCTGCTG
GATCGCTACTATCTGTCTTTTATCAACGACGTGCTGCACAGCATCAAGCTGAAG
AATCTGAACAATTACATCAGCCTGTTCCGGAAGAAAACCAGAACCGAGAAGGAG
AATAAGGAGCTGGAGAACCTGGAGATCAATCTGCGGAAGGAGATCGCCAAGGCC
TTCAAGGGCAACGAGGGCTACAAGTCCCTGTTTAAGAAGGATATCATCGAGACA
ATCCTGCCAGAGTTCCTGGACGATAAGGACGAGATCGCCCTGGTGAACAGCTTC
AATGGCTTTACCACAGCCTTCACCGGCTTCTTTGATAACAGAGAGAATATGTTT
TCCGAGGAGGCCAAGAGCACATCCATCGCCTTCAGGTGTATCAACGAGAATCTG
ACCCGCTACATCTCTAATATGGACATCTTCGAGAAGGTGGACGCCATCTTTGAT
AAGCACGAGGTGCAGGAGATCAAGGAGAAGATCCTGAACAGCGACTATGATGTG
GAGGATTTCTTTGAGGGCGAGTTCTTTAACTTTGTGCTGACACAGGAGGGCATC
GACGTGTATAACGCCATCATCGGCGGCTTCGTGACCGAGAGCGGCGAGAAGATC
AAGGGCCTGAACGAGTACATCAACCTGTATAATCAGAAAACCAAGCAGAAGCTG
CCTAAGTTTAAGCCACTGTATAAGCAGGTGCTGAGCGATCGGGAGTCTCTGAGC
TTCTACGGCGAGGGCTATACATCCGATGAGGAGGTGCTGGAGGTGTTTAGAAAC
ACCCTGAACAAGAACAGCGAGATCTTCAGCTCCATCAAGAAGCTGGAGAAGCTG
TTCAAGAATTTTGACGAGTACTCTAGCGCCGGCATCTTTGTGAAGAACGGCCCC
GCCATCAGCACAATCTCCAAGGATATCTTCGGCGAGTGGAACGTGATCCGGGAC
AAGTGGAATGCCGAGTATGACGATATCCACCTGAAGAAGAAGGCCGTGGTGACC
GAGAAGTACGAGGACGATCGGAGAAAGTCCTTCAAGAAGATCGGCTCCTTTTCT
CTGGAGCAGCTGCAGGAGTACGCCGACGCCGATCTGTCTGTGGTGGAGAAGCTG

AAGGAGATCATCATCCAGAAGGTGGATGAGATCTACAAGGTGTATGGCTCCTCT
GAGAAGCTGTTCGACGCCGATTTTGTGCTGGAGAAGAGCCTGAAGAAGAACGAC
GCCGTGGTGGCCATCATGAAGGACCTGCTGGATTCTGTGAAGAGCTTCGAGAAT
TACATCAAGGCCTTCTTTGGCGAGGGCAAGGAGACAAACAGGGACGAGTCCTTC
TATGGCGATTTTGTGCTGGCCTACGACATCCTGCTGAAGGTGGACCACATCTAC
GATGCCATCCGCAATTATGTGACCCAGAAGCCCTACTCTAAGGATAAGTTCAAG
CTGTATTTTCAGAACCCTCAGTTCATGGGCGGCTGGGACAAGGATAAGGAGACA
GACTATCGGGCCACCATCCTGAGATACGGCTCCAAGTACTATCTGGCCATCATG
GATAAGAAGTACGCCAAGTGCCTGCAGAAGATCGACAAGGACGATGTGAACGGC
AATTACGAGAAGATCAACTATAAGCTGCTGCCCGGCCCTAATAAGATGCTGCCA
AAGGTGTTCTTTTCTAAGAAGTGGATGGCCTACTATAACCCCAGCGAGGACATC
CAGAAGATCTACAAGAATGGCACATTCAAGAAGGGCGATATGTTTAACCTGAAT
GACTGTCACAAGCTGATCGACTTCTTTAAGGATAGCATCTCCCGGTATCCAAAG
TGGTCCAATGCCTACGATTTCAACTTTTCTGAGACAGAGAAGTATAAGGACATC
GCCGGCTTTTACAGAGAGGTGGAGGAGCAGGGCTATAAGGTGAGCTTCGAGTCT
GCCAGCAAGAAGGAGGTGGATAAGCTGGTGGAGGAGGGCAAGCTGTATATGTTC
CAGATCTATAACAAGGACTTTTCCGATAAGTCTCACGGCACACCCAATCTGCAC
ACCATGTACTTCAAGCTGCTGTTTGACGAGAACAATCACGGACAGATCAGGCTG
AGCGGAGGAGCAGAGCTGTTCATGAGGCGCGCCTCCCTGAAGAAGGAGGAGCTG
GTGGTGCACCCAGCCAACTCCCCTATCGCCAACAAGAATCCAGATAATCCCAAG
AAAACCACAACCCTGTCCTACGACGTGTATAAGGATAAGAGGTTTTCTGAGGAC
CAGTACGAGCTGCACATCCCAATCGCCATCAATAAGTGCCCCAAGAACATCTTC
AAGATCAATACAGAGGTGCGCGTGCTGCTGAAGCACGACGATAACCCCTATGTG
ATCGGCATCGATAGGGGCGAGCGCAATCTGCTGTATATCGTGGTGGTGGACGGC
AAGGGCAACATCGTGGAGCAGTATTCCCTGAACGAGATCATCAACAACTTCAAC
GGCATCAGGATCAAGACAGATTACCACTCTCTGCTGGACAAGAAGGAGAAGGAG
AGGTTCGAGGCCCGCCAGAACTGGACCTCCATCGAGAATATCAAGGAGCTGAAG
GCCGGCTATATCTCTCAGGTGGTGCACAAGATCTGCGAGCTGGTGGAGAAGTAC
GATGCCGTGATCGCCCTGGAGGACCTGAACTCTGGCTTTAAGAATAGCCGCGTG
AAGGTGGAGAAGCAGGTGTATCAGAAGTTCGAGAAGATGCTGATCGATAAGCTG
AACTACATGGTGGACAAGAAGTCTAATCCTTGTGCAACAGGCGGCGCCCTGAAG
GGCTATCAGATCACCAATAAGTTCGAGAGCTTTAAGTCCATGTCTACCCAGAAC
GGCTTCATCTTTTACATCCCTGCCTGGCTGACATCCAAGATCGATCCATCTACC
GGCTTTGTGAACCTGCTGAAAACCAAGTATACCAGCATCGCCGATTCCAAGAAG
TTCATCAGCTCCTTTGACAGGATCATGTACGTGCCCGAGGAGGATCTGTTCGAG
TTTGCCCTGGACTATAAGAACTTCTCTCGCACAGACGCCGATTACATCAAGAAG
TGGAAGCTGTACTCCTACGGCAACCGGATCAGAATCTTCCGGAATCCTAAGAAG
AACAACGTGTTCGACTGGGAGGAGGTGTGCCTGACCAGCGCCTATAAGGAGCTG
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AATAATGTGTGAGTAGTTTCCCGATAAGGGAAATTAGGGTTCTTATAGGGTTTC
GCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAAT
ACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAG

LbCas12a coding sequence in black followed by double NLS (**nucleoplasmin**, **GS** spacers and **SV40)**. Dual (CaMV) 35S promoter (**2X35S Promoter** in garnet and red) underlining +1 nucleotide of transcription. **35S terminator** in fuchsia with the processing and polyadenylation site underlined. Sequences of **5' and 3' UTR of RNA-2 of CPMV** are denoted in green. GA->CG mutation is marked in yellow, to obtain the D156R amino acid change, leading to pLBHig-Cas12tt vector. In the case of pLBKan-Cas12att vector, this same insert coming from p235KttLbCas12a was cloned in pLBKan (between C20 and G27 positions).

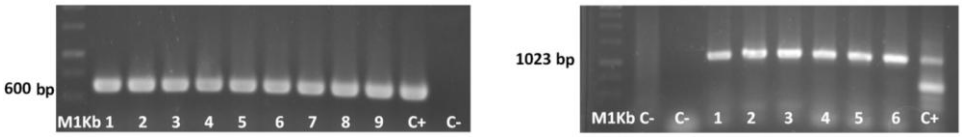


Figure S2. Evaluation of the presence of the T-DNA in transformed plants. Left, gel electrophoresis of the PCR products obtained from 9 plants, after amplification of a region of LbCas12a nuclease of 600 bp (D3604-D3605 primer pairs). Right, electrophoresis of the PCR products obtained from 6 plants, after amplification of the complete CDS of *hpt* gene of 1023 bp (D344-D345 primer pairs). Sample 1 was transformed with pLBHig-Cas9 and samples 2-6 were transformed with pLBHig-Cas12att. M1Kb, molecular weight size marker GeneRuler 1 Kb Plus DNA Ladder; C+, positive control, DNA from a known plant presenting the desired T-DNA; C-, negative control, DNA from a wild type melon plant.

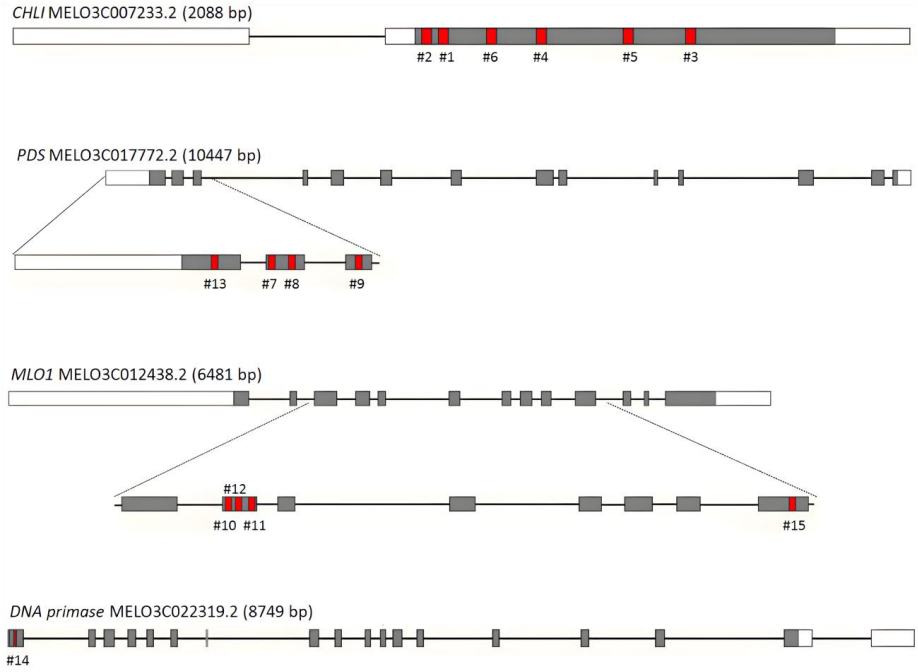


Figure S3. Schematic representation of the gene structure of the four genes targeted in this study, at scale. The orientation represented is 5´->3´. 5´ UTRs and 3´ UTRs are represented as white rectangles. Grey rectangles represent exons, and black horizontal lines represent introns. The location of the target site of each construct (named from #1 to #15 based on **Table S2.**) is represented by a red rectangle. Note that constructs #13 and #13b; and #14 and #14b share the same target site. Nb, *DNA primase* gene presents a small 5´ UTR (17 bp), that is not visible at this scale.

Figure S4. WMV-Vera and its derivatives developed to express gRNAs in transgenic melon plants.

>pGWMV-Vera

TGGCAGGATATATTGTGGTGTAAACGTTCCTGCGGCGGTCGAGATGGATCT**TGG**
CAGGATATATTGTGGTGTAAACGTTCCTGCGGCCGC**GATTCCATTGCCCAGCTA**
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35S Promoter and **terminator**, controlling the expression of the WMV-Vera (GenBank accession number MH469650). Double **LB** and **RB** of the T-DNA. **MluI** site in the T-DNA of pGWMV-Vera.

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>pGWMV-CHLI.2 (#2) (insert in MLUI cloning site of WMV-Vera)
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>pGWMV-CHLI.3 (#3) (insert in MLUI cloning site of pGWMV-Vera)
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>pGWMV-CHLI.4 (#4) (insert in MLUI cloning site of pGWMV-Vera)
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>pGWMV-CHLI.5 (#5) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATCCGAACCAATCAAAATGAATCGA

>pGWMV-CHLI.6 (#6) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATCCTCACTTCAATACCCATTGATT

>pGWMV-PDS.1 (#7) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATTGTGGATTACCCTAGACCACAGA

>pGWMV-PDS.2 (#8) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATGTGCTTCTGCACGTCCCAGGAAG

>pGWMV-PDS.3 (#9) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATGCAGATGCTGGCCACAAACCTGT

>pGWMV-MLO1.1 (#10) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATATCTTCGTCTTGGCTGTCTTCCA

>pGWMV-MLO1.2 (#11) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATTTCTGCCGAAAGCCAAAGTGATG

>pGWMV-MLO1.3 (#12) (insert in MLUI cloning site of pGWMV-Vera)
GTAATTTCTACTAAGTGTAGATGTGTCGGCATATGGAATCCATCA

>pGWMV-PDS.4 (#13 (insert in MLUI cloning site of pGWMV-Vera)
AGCAATGGTTTAGCGTTTTGGTTTCAGAGACAAGTTGAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

>pGWMV-PDS.5 (#13b) (insert in MLUI cloning site of pGWMV-Vera)
AGCAATGGTTTAGCGTTTTGGTTTCAGAGACAAGTTGAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGCATGTCTATAAATATAAGAGAC CCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATCCGTTTAATAGATCA ATCACTCTAAAGGTTACTTATGGCCAA

>pGWMV-ND1.1 (#14) (insert in MLUI cloning site of pGWMV-Vera)
AGAGCGGTAGAGTGGGAGAGGTTTCAGAGACAAGTTGAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

>pGWMV-ND1.2 (#14b) (insert in MLUI cloning site of pGWMV-Vera)

AGAGCGGTAGAGTGGGAGAGGTTTCAGAGACAAGTTGAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCATGTCTATAAATATAAGAGACCCTCTTATAGTAAGCAGAGTTGTTGGAGACGTTCTTGATCCGTTTAATAGATCAATCACTCTAAAGGTTACTTATGGCCAA

>pGWMV-MLO1.4 (#15) (insert in MLUI cloning site of pGWMV-Vera)

AACCAAAACAGGTCATCCCGGTTTCAGAGACAAGTTGAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

Constructs #1 - #12 were designed for LbCas12a and ttLbCas12a gRNA delivery. Constructs #13 - #15 were designed for SpCas9 sgRNA delivery. **G** extra nucleotide added to maintain the ORF. **Spacers** are highlighted in green. **Cas12 direct repeats** are highlighted in yellow. **Compact scaffolds** required for Cas9 editing are highlighted in fuchsia. ***Flowering Locus T* of *A. thaliana*** highlighted in grey.

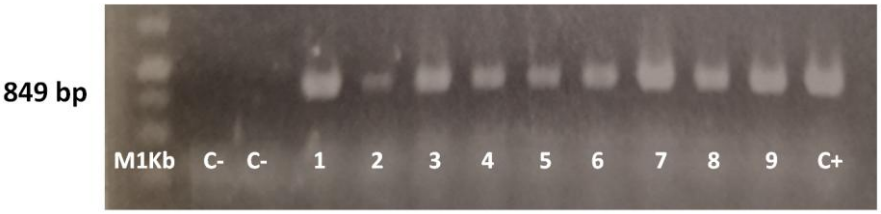


Figure S5. Evaluation of the presence of the WMV in inoculated plants. Gel electrophoresis of the PCR products obtained from 9 MGO melon plants (lanes 1 to 9) inoculated with construct #3, after amplification of a region of the coat protein of WMV of 849 bp (D2380-D3286 primer pairs). RNA samples were first subjected to a retrotranscription reaction, using D1553 primer. M1Kb, molecular weight size marker GeneRuler 1Kb Plus DNA Ladder; C+, positive control, cDNA from a known plant infected with WMV; C-, negative controls, cDNA from a non inoculated *N. benthamiana* plant (left) and a non inoculated MGO melon plant (right).

Figure S6. Full sequence of pBSΔE used for cloning purposes, to sequence the complete T-DNA of 2.17 MGO and 2.1 CH5 plants.

>pBSΔE

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAAT
CAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAA
AGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACT
ATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGA
TGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCG
TAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGG
AAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGC
TAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGC
GCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTG
TTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGG
GGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG
ACGT**TGTAAAACGACGGCCAGT**GAGCGCGCG**TAATACGACTCACTATAGG**GCGA
ATTG**GAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCT**
GCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGG
TACCCAGCTTTTGTTC**CCTTTAGTGAGGGTTAATT**GCGCGCTTGGCGTAATCAT
GGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAC
TCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGT
GCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG
GGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGC
GGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAG
GGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACC
GTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGC
ATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA
GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCC
TGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTT
CTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGC
TGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTA
ACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAG
CCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT
TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCG
CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCA
AACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGC
GCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACG
CTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAA
GGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAA
GTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCAC
CTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCG
TGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGA
TACCGCGAGA**G**CCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAG

CCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGT
CTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGC
GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTA
TGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCA
TGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTA
AGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTA
CTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGT
CATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATAC
GGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAAC
GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA
TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCG
TTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGG
CGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCA
TTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAA
ATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

T7 Promoter in light blue, highlighting **+1** and **+2** nucleotides of transcription in yellow. **T3 Promoter** in red highlighting **+1** and **+2** nucleotides of transcription in yellow. **Polylinker** in bold. **Silencing mutation** (β-lactamase) in red, highlighted in yellow.

Figure S7. Sequence amplified from sgCHLI1-5'FT Cas9 M3 plasmid to develop constructs expressing sgRNAs for SpCas9 genome editing.

> sgCHLI1-5'FT Cas9 M3 insert

GTTTCAGAGACAAGTTGAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGC ACCGAGTCGGTGCATGTCTATAAATATAAGAGACCCTCTTATAGTAAGCAGAGT TGTTGGAGACGTTCTTGATCCGTTTAATAGATCAATCACTCTAAAGGTTACTTA TGGCCAA

Compact scaffold lacking stop codons required for SpCas9 genome editing is highlighted in green. ***Flowering Locus T* of *A. thaliana*** is highlighted in grey. Amplification from the donor vector was done by means of PCR using D6299 (in the case of sgRNA scaffold) or D6300 (in the case of sgRNA scaffold and *AtFT*) as a reverse primer. Forward primers were specific for each construct.

Table S1. Primers used for T-DNA detection and T-DNA sequencing.

Primer*	Primer sequence (5'→3')	Amplification	Purpose
D3602 (F)	CCTGGGAACGTATCACGATCTC	SpCas9 fragment (600 pb)	T-DNA detection
D3603 (R)	TCTTTTATACCCTCTTCAATCC		
D3604 (F)	ATAAGGAGACAGACTATCGGGC	LbCas12a and ttLbCas12a fragment (600 pb)	
D3605 (R)	TGATCTGTCCGTGATTGTTCTC		
D344 (F)	ATGAAAAAGCCTGAACTCACCGCG	Hygromycin fragment (1023 pb)	
D345 (R)	TTTCTTTGCCCTCGGACGAGTGCTG		
D3663 (F)	ATGATTGAACAAGATGGATTGC	Kanamycin fragment (792 pb)	
D3664 (R)	GAAGAACTCGTCAAGAAGGCGA		
D5474 (F)	GCTTCGAAAAAAACCCCATCGAC	T-DNA fragment of pLBHig-Cas9 (2716 pb)	T-DNA sequencing
D5338 (R)	TGGAGAAAAAGATTAATTAAGTACT		
D5476 (F)	ATCTCGACAATCTGCTGGCCCA	T-DNA fragment of pLBHig-Cas9 (2780 pb)	
D5473 (R)	CTTGGGAAGCTTAATGATGAGG		
D5341 (F)	TCAGTCATCACAAAATCAACAATTC	T-DNA fragment of pLBHig-Cas9 (2002 pb)	
D5475 (R)	GTCTGACAGGTTCTTTGCCGCC		
D5478 (F)	CGACGTGGATTTTCTGATCAGC	T-DNA fragment of pLBHig-Cas12 (2373 pb, in combination with D5338)	
D5480 (F)	AGTACGAGGACGATCGGAGAAA	T-DNA fragment of pLBHig-Cas12 (2378 pb)	
D5477 (R)	ATTCTCCTGGGCCTCATAGTTC		
D5479 (R)	TCCTGCAGCTGCTCCAGAGAAA	T-DNA fragment of pLBHig-Cas12 (2313 pb, in combination with D5341)	

*F, forward primer; R, reverse primer.

Table S2. Guide RNAs selected and cloned in the pGWMV vector.

Suitable nuclease	Target gene	Spacer sequence	Tested	Construct code
LbCas12a ttLbCas12a	*CHLI* MELO3C007233.2	CTGTTCCTCTATCACCCATGATC	Yes	#1
		GGTCAATAACGTTCAGTAGAAGA	Yes	#2
		ACCAGAATCCTAAGGAATTCCGT	Yes	#3
		TGGGACTATTGACATTGAAAAAG	No	#4
		CCGAACCAATCAAAATGAATCGA	No	#5
		CCTCACTTCAATACCCATTGATT	No	#6
	PDS MELO3C017772.2	TGTGGATTACCCTAGACCACAGA	No	#7
		GTGCTTCTGCACGTCCCAGGAAG	No	#8
		GCAGATGCTGGCCACAAACCTGT	No	#9
	MLO1 MELO3C012438.2	ATCTTCGTCTTGGCTGTCTTCCA	No	#10
		TTCTGCCGAAAGCCAAAGTGATG	No	#11
		GTGTCGGCATATGGAATCCATCA	No	#12
SpCas9	*PDS* MELO3C017772.2	AGCAATGGTTTAGCGTTTTG	No	#13
		AGCAATGGTTTAGCGTTTTG	Yes	#13b *
	DNA Primase MELO3C022319.2	AGAGCGGTAGAGTGGGAGAG	No	#14
		AGAGCGGTAGAGTGGGAGAG	Yes	#14b *
	MLO1 MELO3C012438.2	AACCAAAACAGGTCATCCCG	No	#15

*Constructs named as #13b and #14b present the *A. thaliana* FT sequence fused to the 3´end of the Cas9 RNA scaffold.

Table S3. Primers used for transformation vector development.

Primer*	Primer sequence (5'→3')	Amplification	Purpose
D4690 (F)	gatcatcgagcaaataagcgaattcTCCAAAAGAGTGATCCTCG	p235SpCas9-fragment (341 bp)	Addition of Nucleoplasmin NLS to p235SpCas9, to generate p235SpCas9-2xNLS
D4691 (R)	Cctggccggccttttttcgtggccgccggcc ttttttgaaccGTCTCCACCGAGCTGAGAG		
D4692 (F)	Cacgaaaaaggccggccaggcaaaaaagaa aaagggatccCCCAAGAAGAAGAGGAAGG	p235SpCas9 fragment (470 bp)	
D4693 (R)	Gtcaaggaacgtcgagggccc CTGGATTTTGGTTTTAGGAATTAG		
D4702 (F)	TAAAGAAGGAGTGCGTCG	pLBHig3 backbone (3553 bp)	Development of pLBKan vector
D4703 (R)	TGCAGATTATTTGGATTGAGAG		
D4704 (F)	ctcaatccaaataatctgcaATGATTGAACAAGATGGATTGC	NptII CDS from pGKan (832 bp)	
D4705 (R)	ttcgacgcactccttctttaGAAGAACTCGTCAAGAAGGC		
D4983 (F)	CGGCTTCTTTCGTAACAGAGAGAATATGTTTTCCGAGGAG	p235kLbCas12a vector (9270 bp)	Development of p235KLbCas12att vector
D4982 (R)	CTCTCTGTTACGAAAGAAGCCGGTGAAGGCTGT		

*F, forward primer; R, reverse primer.

Table S4. Primers used for pGWMV derivatives construction.

Primer*	Primer sequence (5'→3')	Amplification	Developed construct
D4998 (F)	Tgtccctgcaatctggtaaa GTAATTTCTACTAAGTGTAGAT	No template amplification. PCR product of 85 bp using D4998 as forward primer	
D5310 (R)	agggaaactgactcgcaacaGATCATGGGT GATAGAGGAACAGATCTACACTTAGTAG		pGWMV-CHLI.1 (#1)
D5311 (R)	agggaaactgactcgcaacaTCTTCTACTG AACGTTATTGACCATCTACACTTAGTAG		pGWMV-CHLI.2 (#2)
D5312 (R)	agggaaactgactcgcaacaACGGAATTCC TTAGGATTCTGGTATCTACACTTAGTAG		pGWMV-CHLI.3 (#3)
D6371 (R)	agggaaactgactcgcaacaCTTTTTCAAT GTCAATAGTCCCAATCTACACTTAGTAG		pGWMV-CHLI.4 (#4)
D6372 (R)	agggaaactgactcgcaacaTCGATTCATT TTGATTGGTTCGGATCTACACTTAGTAG		pGWMV-CHLI.5 (#5)
D6373 (R)	agggaaactgactcgcaacaAATCAATGGG TATTGAAGTGAGGATCTACACTTAGTAG		pGWMV-CHLI.6 (#6)
D6374 (R)	agggaaactgactcgcaacaTCTGTGGTCT AGGGTAATCCACAATCTACACTTAGTAG		pGWMV-PDS.1 (#7)
D6375 (R)	agggaaactgactcgcaacaCTTCCTGGGA CGTGCAGAAGCACATCTACACTTAGTAG		pGWMV-PDS.2 (#8)
D6376 (R)	agggaaactgactcgcaacaACAGGTTTGT GGCCAGCATCTGCATCTACACTTAGTAG		pGWMV-PDS.3 (#9)
D6377 (R)	agggaaactgactcgcaacaTGGAAGACAG CCAAGACGAAGATATCTACACTTAGTAG		pGWMV-MLO1.1 (#10)
D6378 (R)	agggaaactgactcgcaacaCATCACTTTG GCTTTCGGCAGAAATCTACACTTAGTAG		pGWMV-MLO1.2 (#11)
D6379 (R)	agggaaactgactcgcaacaTGATGGATTC CATATGCCGACACATCTACACTTAGTAG		pGWMV-MLO1.3 (#12)
D6298 (F)	tgtccctgcaatctggtaaaAGCAATGGTTTAG CGTTTTGGTTTCAGAGACAAGTTGAAATA	127 bp product using D6299 as reverse primer; 229 bp product using D6300 as reverse primer. pGTEVdEE sgCHLI1-5'FT Cas9 M3 used as template	pGWMV-PDS.4 (#13) pGWMV-PDS.5 (#13b)
D6297 (F)	tgtccctgcaatctggtaaaAGAGCGGTAGAGT GGGAGAGGTTTCAGAGACAAGTTGAAATA		pGWMV-ND1.1 (#14) pGWMV-ND1.2 (#14b)
D6296 (F)	tgtccctgcaatctggtaaaAACCAAAACAGGT CATCCCGGTTTCAGAGACAAGTTGAAATA		pGWMV-MLO1.4 (#15)
D6299(R)	agggaaactgactcgcaacaGCACCGACTCGGT GCCACTTTT		
D6300 (R)	agggaaactgactcgcaacaTTGGCCATAAGTA ACCTTTAGA		

*F, forward primer; R, reverse primer. Constructs developed to express LbCas12a or ttLbCas12a guides are denoted in light red. Constructs developed to express SpCas9 guides are denoted in green.

Table S5. Primers used for WMV detection and plant genotyping.

Primer*	Primer sequence (5'→3')	Amplification	Purpose
D1553 (R)	AGGACAACAAACATTACCGTACCTC		WMV cDNA synthesis
D2380 (F)	TCAGGAAAAGAAAAAGAAACGGTG	WMV coat protein fragment (849 bp)	WMV detection
D3256 (R)	CTGCGGTGGACCCATACCCAAC		
D5343 (F)	GATTTGTTTCAAGGTCATACGAG	*CHLI* fragment (540 bp)	Genotyping the target site of constructs #1 and #2
D5344 (R)	AGTAGGACATCAACTAAATGATC		
D6113 (F)	GGATTCGGCTGCCTCTGGTT	*CHLI* fragment (340 bp)	Genotyping the target site of construct #3
D6114 (R)	CCGGGAGATTTTCACCTTCAG		
D3483 (F)	GGTAAATGAGAAATTTTCGTG	*PDS* fragment (941 bp)	Genotyping the target site of construct #13b
D3484 (R)	ATAACACATTTTGTCATCGGC		
D4644 (F)	TTACATTGTCGTTTCTTTTCC	*DNA primase* fragment (805 bp)	Genotyping the target site of construct #14b
D4645 (R)	ATAAACACTATAACTCTAGAGG		

*F, forward primer; R, reverse primer.

Chapter IV

Comparative genomics-driven design of virus-delivered short RNA inserts triggering robust gene silencing

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F.P. conceived the study. J.-A.D. and F.P. obtained funding and computational resources. A.G. and F.P. designed experiments with input from the other authors. A.G., V.A., P.O.-G. and F.P. performed the experiments. F.J.H. and J.P. provided materials. A.G., S.G. and F.P. analysed and managed data. F.P. wrote the manuscript with input from A.G. and the other authors. All authors discussed and revised the manuscript.

Abstract

Engineered RNA viruses can downregulate target genes through virus-induced gene silencing (VIGS). Largely optimized using the model plant *Nicotiana benthamiana*, current VIGS protocols rely on viral vectors engineered to deliver 200-400-nt inserts with homology to a target gene, often located in less conserved regions to ensure specificity. Given the *N. benthamiana* allopolyploidy and functional redundancy, we leveraged enhanced genomics and transcriptomics resources to obtain curated locus annotations, which guided the design of virus-delivered short RNA inserts (vsRNAi) for simultaneous targeting of homeologous gene pairs. Our results showed that quantitative silencing phenotypes could be obtained using 24, 28, and 32-nt vsRNAi that targeted conserved regions of functionally redundant gene pairs. vsRNAi triggered informative transcriptome-wide changes, and target transcript downregulation was linked to gene-pair-specific production of 21 and 22-nt sRNAs. The portability of vsRNAi to crops was confirmed in tomato (*Solanum lycopersicum*) and scarlet eggplant (*Solanum aethiopicum*). Overall, our results support the usefulness of vsRNAi-based approaches in functional genomics and trait reprogramming of the model species *N. benthamiana*, as well as of crops that contribute to global and local food provisions.

Keywords: virus-induced gene silencing, tobacco rattle virus, functional genomics, *Nicotiana benthamiana*, *Solanum aethiopicum*.

Introduction

RNA products and RNA virus-based technologies have the potential to transform agriculture by enabling on-demand crop trait reprogramming and effective pest and disease management (Pasin et al., 2024; Rössner et al., 2022). In virus-induced gene silencing (VIGS), engineered RNA viruses can redirect the host RNA interference machineries to target gene silencing through the production of gene-specific small RNAs (sRNAs) (Rössner et al., 2022). Although endogenous sRNAs and those resulting from VIGS are in the 20–30-nt range, VIGS vectors are engineered to deliver larger inserts of 200–400-nt with homology to a target gene, often located in less conserved regions to ensure specificity (Ahmed et al., 2020). Reducing insert sizes, may enhance the VIGS scalability and applicability to non-model species.

Nicotiana benthamiana is the most widely used model for optimizing VIGS protocols. However, this host has a complex, allotetraploid genome with functionally redundant homeologous gene pairs, and for which no high-quality assemblies were available until very recently (Ranawaka et al., 2023). Multi-gene CRISPR-Cas9 mutagenesis was applied to tackle functional redundancy in plants (Berman et al., 2025; Ellison et al., 2020). Here, we hypothesized that VIGS insert sizes could be lowered to match those of endogenous sRNAs by combining enhanced genomics and transcriptomics resources to guide the design of virus-delivered short RNA inserts (vsRNAi) for simultaneous targeting of homeologous gene pairs.

Results and discussion

Genomic and transcriptomic analyses guided the design of vsRNAi

We focused on the *Magnesium chelatase subunit I* (*CHLI*) gene, whose downregulation results in leaf yellowing due to a reduction of chlorophyll biosynthesis and levels. Tomato (*Solanum lycopersicum*) is a diploid relative of *N. benthamiana* with high-quality genome assembly and annotation. The tomato *CHLI* coding sequence (CDS) is distributed across three exons with boundaries supported by transcriptomic expression analysis (materials and

methods). Analysis of reported *N. benthamiana* Niben101 and Niben261 annotations, however, revealed *CHLI* variation including large sequence deletion and insertion, not supported by results obtained by *de novo* transcriptome assembly (**Figure S1**). A high-quality chromosome-level genome assembly was recently reported for *N. benthamiana* (Ranawaka et al., 2023). Sequence searches using the tomato CHLI protein identified homologue loci on chromosomes 5 (NbLab360C05) and 10 (NbLab360C10), whose expression and gene structures were validated by mapping sequencing reads from RNA samples. Leveraging this curated annotation, vsRNAi were designed to target CDS regions conserved in *N. benthamiana* and tomato *CHLI* (**Figure S1**).

vsRNAi can induce transcript level reduction of targeted genes

Custom-synthesized DNA oligonucleotide pairs spanning vsRNAi sequences were inserted into pLX-TRV2 of the JoinTRV vector system, which is based on tobacco rattle virus (TRV) (Aragonés et al., 2022), by one-step digestion-ligation reactions (**Figure 1a**). JoinTRV derivatives with 32, 28, 24 and 20-nt inserts targeting *CHLI* (vCHLI, vCHLI-28, vCHLI-24 and vCHLI-20, respectively) were inoculated to *N. benthamiana*. After 10 days, upper uninoculated leaves of plants treated with vCHLI, vCHLI-28 and vCHLI-24 showed a yellowing phenotype. Fluorometry revealed a significant reduction of chlorophyll levels in vCHLI ($\bar{x}$ = 0.11), vCHLI-28 ($\bar{x}$ = 0.23) and vCHLI-24 ($\bar{x}$ = 0.39) compared with controls ($\bar{x}$ = 1.00; **Figure 1a**) and a significant positive correlation with *CHLI* transcript levels measured by RT-qPCR (**Figure S2**).

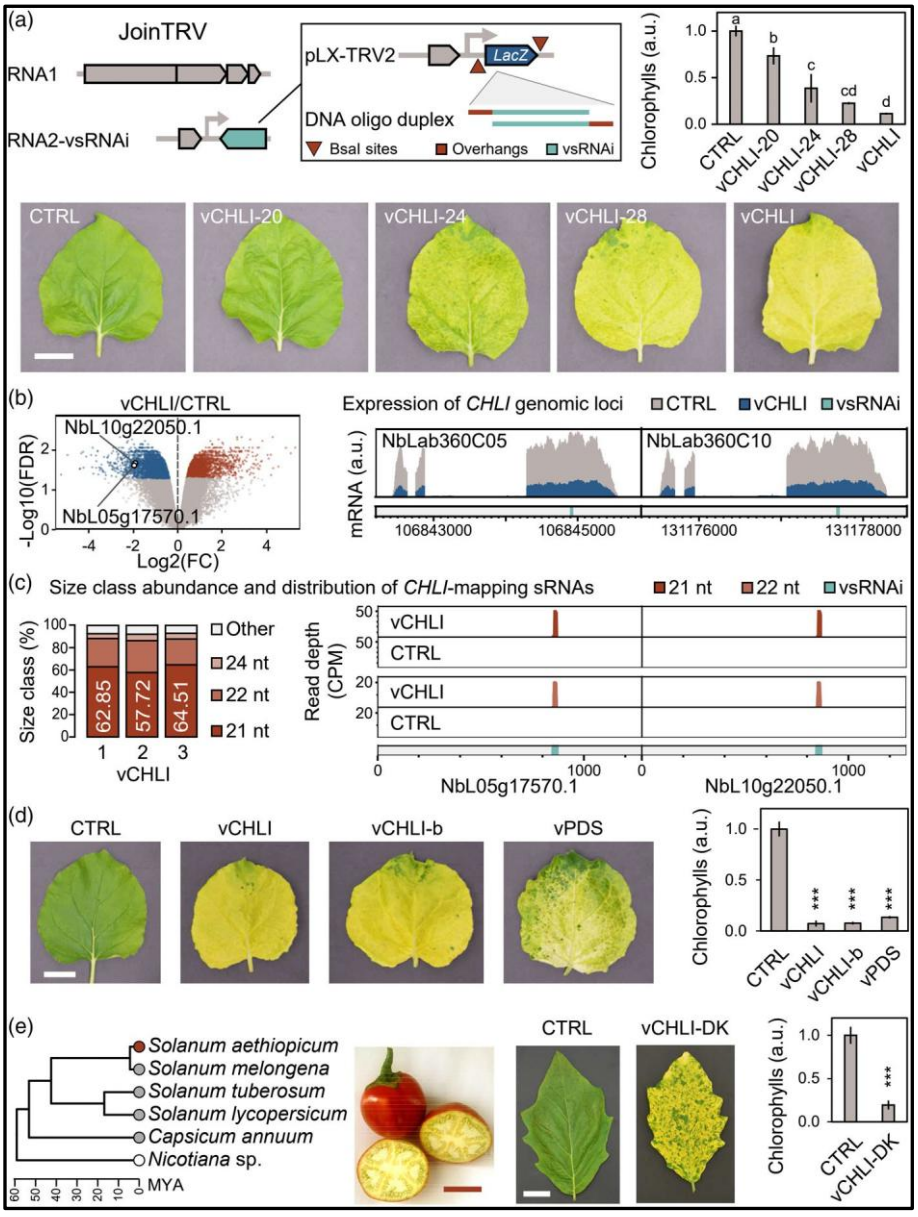


Figure 1. Virus-delivered short RNA inserts (vsRNAi) trigger gene silencing. (**a**) JoinTRV vector assembly and delivery of 32, 28, 24 and 20-nt vsRNAi targeting the two *N. benthamiana CHLI* homeologs (vCHLI, vCHLI-28, vCHLI-24 and vCHLI-20). Leaf phenotypes and chlorophyll levels (mean SD, $n = 3$) are shown; CTRL-control. Different letters indicate significant differences ($P < 0.05$), by one-way ANOVA and Tukey's honestly significant difference (HSD) test. (**b**)

Transcriptomics of vCHLI and CTRL samples (*n* = 3) detects a significant downregulation of *CHLI* expression (FDR <0.05). (**c**) In vCHLI samples, small RNA sequencing shows predominance of 21 and 22-nt sRNAs mapping to *CHLI*, and with a read depth peak (mean, *n* = 3) localized to the vsRNAi-targeted region. (**d**) Leaf phenotypes and chlorophyll levels (mean SD, *n* = 3) are shown for vCHLI-b and vPDS, targeting *CHLI* and *PDS* homeologs, respectively; ***, *P* < 0.001, Student's *t*-test. (**e**) vsRNAi portability to scarlet eggplant (*Solanum aethiopicum*). Phylogeny and fruits are shown, alongside leaf phenotypes and chlorophyll levels (mean SD, *n* = 3) of 'Rossa di Rotonda' plants treated with vCHLI-DK, a pTRV1 + pTRV2 derivative targeting *CHLI*; ***, *P* < 0.001, Student's *t*-test.

vsRNAi mediated *NbCHLI* downregulation leads to robust transcriptional changes

Silencing phenotypes of vCHLI-treated plants were robust and equivalent to those obtained by using a VIGS vector including a 300-nt *CHLI* cDNA fragment (**Figure S3**). Transcriptomes of plants inoculated with the unmodified JoinTRV (CTRL) or its vCHLI derivative were analysed. Abundance of over 4000 transcripts was significantly altered in the vCHLI/CTRL comparison (FDR <0.05; **Figure 1b** and **Figure S4**; Data S1 and S2). Transcriptome-wide functional analysis revealed an enrichment of gene ontology terms associated with light responses, and biological processes involved in carbohydrate metabolism, cellulose biosynthesis and cell wall biogenesis (**Figure S4c**; Data S3-S5), consistent with the anticipated reduction of cell photosynthetic capacity caused by the *CHLI* downregulation.

Among downregulated transcripts, we identified the *CHLI* homeologs (NbL05g17570.1, and NbL10g22050.1; log2(FC) ≤ —1.92, FDR <0.05) and confirmed a global expression reduction of their genomic loci beyond the 32-nt region targeted by vCHLI (**Figure 1b**; **Data S6**). Viral amplification of large VIGS inserts can lead to overestimation of expression levels of homologous host genes (**Figures S5, S6**); by contrast, vsRNAi enable transcriptome-wide quantification of target gene silencing.

vsRNAi trigger gene downregulation by host-derived sRNAs production

We next sequenced sRNAs to assess if their production is involved in the vCHLI-induced phenotypic and transcriptomic changes. In vCHLI samples, vsRNAi

triggered host-derived production of sRNAs (**Figure S7**) and a marked enrichment of sRNAs mapping to *CHLI* transcripts (**Figure S8**; Data S7 and S8). 21-nt sRNAs were predominant, followed by 22-nt sRNAs (**Figure 1c**; Data S9), and their levels showed a significant negative correlation with those of *CHLI* transcripts (**Figure S9**). The vsRNAi-targeted region of *CHLI* transcript sequences exhibited a localized accumulation of 21 and 22-nt sRNAs in vCHLI samples, which was absent in control samples (**Figure 1c**; Data S10). We concluded that vsRNAi trigger target gene downregulation through region-specific enrichment of 21 and 22-nt sRNAs, known end products of Dicer-like 4 (DCL4) and DCL2, respectively (Rössner et al., 2022).

vsRNAi system is portable to different target genes and different crops of agronomical interest

We used vCHLI-b and vPDS to target a second 32-nt region conserved in *CHLI* homeologs or 32-nt of the *PHYTOENE DESATURASE* (*PDS*) gene pair, respectively. In the upper uninoculated leaves of treated plants, we observed a reduction in green pigmentation and chlorophyll levels (**Figure 1d**), confirming the broad applicability of vsRNAi for *N. benthamiana* gene functional characterization.

We next assessed vsRNAi portability to crops. The vCHLI insert is conserved in tomato *CHLI* (**Figure S1**); 'Moneymaker' seedlings inoculated with vCHLI showed leaf yellowing and a significant reduction of chlorophyll levels compared with control plants (**Figure S10a**). Scarlet eggplant (*Solanum aethiopicum*; **Figure 1e**) is an underutilized solanaceous crop (Gramazio et al., 2016). Searches of *de novo* assembled transcriptomes and of a genomic assembly (Benoit et al., 2025) confirmed that the 32-nt insert of vCHLI is conserved in scarlet eggplant *CHLI* (100% identity) (**Figure S10b**). Seedlings of the ecotype 'Rossa di Rotonda' inoculated with vCHLI showed leaf yellowing and a significant reduction of chlorophyll levels compared with control plants (**Figure S10b**). Using vCHLI-DK, a derivative of the pTRV1 plus pTRV2 vector system used for VIGS and genome editing (Ellison et al., 2020), we delivered a *CHLI*-targeting 32-nt vsRNAi and obtained equivalent results (**Figure 1e** and **Figure S10c**), highlighting the versatility of our approach.

Future perspectives on the use of vsRNAi

Overall, our results demonstrate that vsRNAi as short as 24-nt can effectively produce phenotypic alterations. Use of 32-nt vsRNAi results in robust gene silencing phenotypes, informative transcriptome-wide changes and target transcript downregulation linked to gene-specific production of 21 and 22-nt sRNAs. Viral delivery of artificial micro-RNAs or trans-acting small interfering RNAs reduces VIGS insert sizes and off-targets (Cisneros et al., 2025). vsRNAi offer equivalent specificity and greatly simplifies viral vector engineering by eliminating intermediate cloning steps and precursor elements required to activate sRNA production. Simplified cloning of vsRNAi fragments, which are nearly 10-fold smaller than those of conventional VIGS and can be synthesized at low cost, may enable high-throughput functional genomics in model plants and crops that contribute food security and on-demand alteration of crop traits.

Materials and methods

Plants

Plants of *Nicotiana benthamiana*, *Solanum lycopersicum* cv. 'Moneymaker' and *Solanum aethiopicum* ecotype 'Rossa di Rotonda' were grown in soil mixture (3 parts of potting substrate and 1 part of vermiculite) and maintained in a greenhouse chamber at ≈24°C under a 16-h-day/8-h-night photoperiod.

Computational resources

Bioinformatics analyses were done in high performance computing systems through a command-line interface (Castrignanò et al., 2020). Transcriptomics and genomics datasets, and software/algorithms used in the study are listed in **Tables S1**, **S2**.

Comparative genomics and gene annotation

Genome sequences of *N. benthamiana* (NbLab360.genome.fasta.gz), *S. lycopersicum* (S_lycopersicum_chromosomes.4.00.fa) and *S. aethiopicum* (Saethiopicum.fasta) were retrieved from public databases. Available gene annotations of *N. benthamiana* (NbLab360v103, Niben261 and Niben101), and

the tomato (ITAG4.1) were used. To confirm gene expression, NCBI SRA transcriptomic datasets of *N. benthamiana* (SRR11282461, SRR11282462, SRR11282463 SRR24114147),*S.lycopersicum*(SRR28956637)and *S. aethiopicum* (SRR2229192, SRR6431645) were obtained from the Amazon Web Services (AWS) cloud using kingfisher. Adapter sequences and low-quality sequences were filtered using fastp to yield clean reads, which were mapped to the reference genome using the spliced aligner HISAT2 with the --dta option; obtained alignment .bam files were visualized using IGV and per base coverage of genomic loci were obtained using samtools depth. *De novo* transcriptome assembly was done using rnaSPAdes, a pipeline implemented within the SPAdes package. Transcripts of interest from assembled transcriptomes and available gene annotations were identified by DIAMOND blastx searches against a custom protein database.

Multi-species transcripts and genomic loci were aligned using MAFFT to identify conserved regions for vsRNAi design; transcript sequences used for vsRNAi design are shown in Data S11. Dated molecular phylogeny for the Solanaceae was reported (Messeder et al., 2024).

Viral vector systems

The tobacco rattle virus (TRV) vector system JoinTRV was used, which relies on pLX-TRV1 (Addgene:180515) and pLX-TRV2 (Addgene:180516), two T-DNA vectors of the pLX series (Pasin et al., 2017) for simultaneous *Agrobacterium*-mediated inoculation of TRV genomic components (Aragonés et al., 2022). A pLX-TRV2 derivative including an *N. benthamiana CHLI* cDNA fragment of 300-nt was described before (Aragonés et al., 2022). An alternative TRV system consisting of pTRV1 (Addgene:148968) and pTRV2 (Addgene:148969) was also used (Liu et al., 2002).

One-step assembly of vsRNAi vectors

For one-step assembly of vsRNAi into pLX-TRV2, oligonucleotide pairs were synthesized as follows: (i) a first oligonucleotide consisting, from the 5’ end, of the TGAA sequence followed by the target gene sequence in the antisense orientation, and (ii) a second oligonucleotide consisting, from the 5’ end, of AGAG followed by the target gene sequence in the sense orientation. Custom

DNA oligonucleotides were purchased desalted after synthesis as 100 mM solutions (Integrated DNA Technologies; Table S3). To recover oligonucleotide duplexes with overhangs compatible with BsaI-digested pLX-TRV2 (Table S4), pairs were mixed (10 µM each) in 1× T4 DNA ligase buffer (New England Biolabs) in a final volume of 10 µL, then incubated at 95°C (5 min), and cooled to 25°C (−0.1°C/s) and diluted by adding 190 µL of ultrapure water.

Recovered duplexes were assembled into pLX-TRV2 by one-step digestion-ligation reactions (Marillonnet and Grützner, 2020) including 1 µL duplex, ≈50 ng of pLX-TRV2, 0.5 µL T4 DNA ligase (M0202S, New England Biolabs), 0.5 µL BsaI-HFv2 (R3733S, New England Biolabs), 1× T4 DNA ligase buffer (New England Biolabs) in a final volume of 10 µL. Reaction mixtures were subjected to 30 cycles (5 min at 37°C and 5 min at 16°C), followed by 5 min at 60°C. Reactions were then taken to ice and transformed in *E. coli* DH5α competent cells, which were spread onto medium plates supplemented with 50 µg/ml kanamycin and 40 µl 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; 40 mg/ml stock), and incubated (37°C, ≈24 h). White colonies were selected and recovered DNA plasmids were verified by Sanger sequencing.

For one-step assembly of vsRNAi into pTRV2, the duplex obtained using the oligonucleotide pair vCHLI-DK_F/vCHLI-DK_R (**Tables S3, S4**) was assembled into pTRV2 by a one-step digestion-ligation reaction in which BsaI-HFv2 was replaced with MluI (ER0561, Thermo Fisher Scientific); the resulting vector pTRV2-vCHLI with the *CHLI* sequence in the antisense orientation (vCHLI-DK) was identified by Sanger sequencing of recovered DNA plasmids. The assembled vsRNAi vectors are summarised in **Table S4**, and their sequences are shown in Data S12.

Viral vector delivery

Agrobacterium AGL1 was used for viral vector delivery to plants (Pasin, 2022). In the JoinTRV system, AGL1 cells were electroporated with pLX-TRV1; colonies were selected on plates supplemented with rifampicin and gentamicin. Competent cells of the recovered strain AGL1 (pLX-TRV1) were then electroporated with pLX-TRV2 and its derivatives. AGL1 transformant simultaneously hosting the two vectors of TRV genomic components were selected on plates supplemented with rifampicin, gentamicin, and kanamycin.

In the pTRV1 + pTRV2 system, rifampicin and kanamycin were used to select AGL1 transformants hosting the vectors and their derivatives. Antibiotics were used at final concentrations of 30 µg/ml gentamicin, 50 µg/ml kanamycin, and 50 µg/ml rifampicin.

Suspensions of the transformed bacteria were prepared in 10 mM 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES; M8250, Sigma-Aldrich), 10 mM MgCl2, 150 µM 3´,5´-dimethoxy-4-hydroxyacetophenon (acetosyringone; D134406, Sigma-Aldrich), pH 5.6, as described (Senthil-Kumar and Mysore, 2014; Uranga et al., 2023). Bacterial suspensions were adjusted to an optical density at 600 nm (OD600) of 0.5 and infiltrated in the abaxial side of one leaf of 3-week-old *N. benthamiana* plants using 1-ml needle-less syringes. For *S. lycopersicum* and *S. aethiopicum*, bacterial suspensions were adjusted to OD=2 and applied to fully expanded cotyledons of 10-day-old seedlings.

Chlorophyll quantification

Chlorophyll fluorometric quantification was done using the DUALEX® optical leaf clip meter (FORCE-A) (Cerovic et al., 2012); data were obtained from upper uninoculated leaves and measurement was done with the adaxial leaf side facing the light sources, as previously reported (Villanueva et al., 2023).

Total RNA purification

Samples from upper non-inoculated leaves were ground in liquid nitrogen in a mortar, and total RNA was extracted from ≈100 mg of plant material powder with the FavorPrep™ plant total RNA mini kit (FAPRK 001, Favorgen); during the preparation a mix of 5 U RNase-free DNase I (EN0521, Thermo Fisher Scientific), 100 U RiboLock RNase inhibitor (EO0381, Thermo Fisher Scientific), 1× DNase I reaction buffer with MgCl2 in a final volume of 50 µL was added on column and incubated 10 min at 25°C to remove genomic DNA. RNA was eluted in RNase-free ultrapure water, quantified using a NanoDrop 1000 (Thermo Fisher Scientific), and stored at −80°C until further use.

RT-qPCR analysis

For RT-qPCR analysis, cDNA was synthesized using 1.2 µg of purified RNA and the iScript™ gDNA Clear cDNA Synthesis Kit (1725035, Bio-Rad

Laboratories), following manufacturer instructions. The cDNA samples were used in qPCR reactions that included gene-pair-specific primers (**Table S3**) and 1× SsoAdvanced™ Universal SYBR® Green SuperMix (1725271, Bio- Rad Laboratories) and were run on a QuantStudio™ 3 Real-Time PCR system (Thermo Fisher Scientific). Expression was normalized using *PP2A* as a reference gene (Liu et al., 2012), and fold changes relative to the control condition were calculated by the $\Delta\Delta^{CT}$ method (Pasin et al., 2020).

Transcriptome and small RNA sequencing and data analysis

For each experimental condition, three biological replicates were used for transcriptomic analysis (RNA-seq; **Table S5**) and small RNA sequencing (sRNA-seq; **Table S6**); total RNA samples were processed at BGI (Hong Kong, China).

For RNA-seq, polyA-tailed RNA molecules were enriched and cDNA sequencing libraries were prepared following the DNBSEQ Eukaryotic mRNA library protocol, and sequenced (2 x 100-nt paired-end reads) on a DNBSEQ platform. Adapter sequences and low-quality sequences were filtered using fastp to yield clean mRNA reads. Transcripts were quantified by Salmon using the options–`keepDuplicates-k31`, and a decoy-aware transcriptome index built using *N. benthamiana* annotated CDS (NbLab360.v103.gff3.CDS.fasta.gz) and, as a decoy, the complete genomic sequence (NbLab360.genome.fasta.gz). The obtained transcript-per-million matrix was used to assess transcriptome variation of the samples analysed by principal component analysis. Differential expression analysis was calculated by edgeR using Salmon transcript read counts and trimmed mean of M values (TMM) normalization; quasi-likelihood F-test was applied to identify genes that are differentially expressed between sample groups, and adjusted *p* values (FDR) were computed by the Benjamini–Hochberg method. To inspect sequencing depth of *CHLI* genomic loci, clean reads were mapped using the spliced aligner HISAT2 with the --dta option against the *N. benthamiana* genome (NbLab360.genome.fasta.gz); obtained alignment .bam files were visualized using IGV and per base coverage were obtained using samtools depth. For each RNA-seq sample, per base coverage of *CHLI* genomic loci were normalized against the total number of mapped reads obtained by samtools `idxstats`.

For sRNA-seq, small RNA molecules were size enriched and cDNA sequencing libraries were prepared following the UMI small RNA library protocol, and sequenced (1 x 50-nt single-end read) on a DNBSEQ platform. Adapter sequences and low-quality sequences were filtered to yield clean sRNA reads. To analyze sRNA size classes, for each sample individual datasets with read length of 20, 21, 22, 23, 24, and 25 were obtained using fastp. Reads were mapped *to N. benthamiana* transcripts (NbLab360.v103.gff3.CDS.fasta.gz) using STAR, requiring at least an 18-nt perfect match (`--outFilterMatchNmin 18`) and 1 mismatch maximum (`--outFilterMismatchNmax 1`). Obtained alignment .bam files were visualized using IGV and per base coverage of transcripts were obtained using igvtools count. For each sRNA-seq sample, number of mapped reads per transcript and per base coverage of *CHLI* transcripts were normalized against the total number of mapped reads obtained by samtools `idxstats`, and reported as counts per million (CPM).

In plots showing the normalized sequencing depth per nucleotide, CPM values were obtained using the equation:

$$\frac{counts\ of\ reads\ mapped\ per\ nucleotide}{number\ of\ reads\ mapped\ to\ the\ reference\ dataset}\ \text{x}\ 1\text{M}$$

wherein, the reference dataset consisted in the complete plant genome, transcriptome or the TRV RNA1 and RNA2 sequences from pLX-TRV1 and pLX-TRV2-vCHLI.

Gene ontology term enrichment

Gene ontology (GO) terms associated to *N. benthamiana* transcripts were retrieved by searchingan *A.thaliana*proteindatabase (Araport11_pep_20240409 _ representative_gene_model.gz) with DIAMOND `blastx` and retaining one target sequences per query (`-k 1`); GO annotation was then obtained from the Bioconductor database org.At.tair.db via topGO. Enrichment analysis of GO terms of biological processes in a transcriptome-wide analysis (global) was done by Kolmogorov-Smirnov testing considering edgeR computed FDR values; enrichment significance of GO terms of differentially expressed gene lists was determined by Fisher's exact test.

Statistics

Student's *t*-test was used for pairwise comparisons; one-way ANOVA and Tukey's honestly significant difference (HSD) test were used for multiple comparisons. Pearson correlation coefficient was calculated to estimate the linear relationship between the two variables. In differential expression analysis, the Benjamini–Hochberg multiple testing control method was used to compute adjusted *p* values (FDR); enrichment significance of GO terms was determined by Kolmogorov-Smirnov or Fisher's exact testing. Significance levels of *p* values are indicated in the figures and supporting information.

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Data availability

Supplemental data accompanies this work. The transcriptomic and small RNA sequencing datasets generated are deposited under NCBI BioProject PRJNA1217923. These datasets are also available at Wiley.com (https://onlinelibrary.wiley.com/doi/10.1111/pbi.70254). pLX-TRV2- vCHLI is available at Addgene (239842, https://www.addgene. org/239842/).

Supplementary Data

Supplementary data is available at the end of this chapter.

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Supporting information

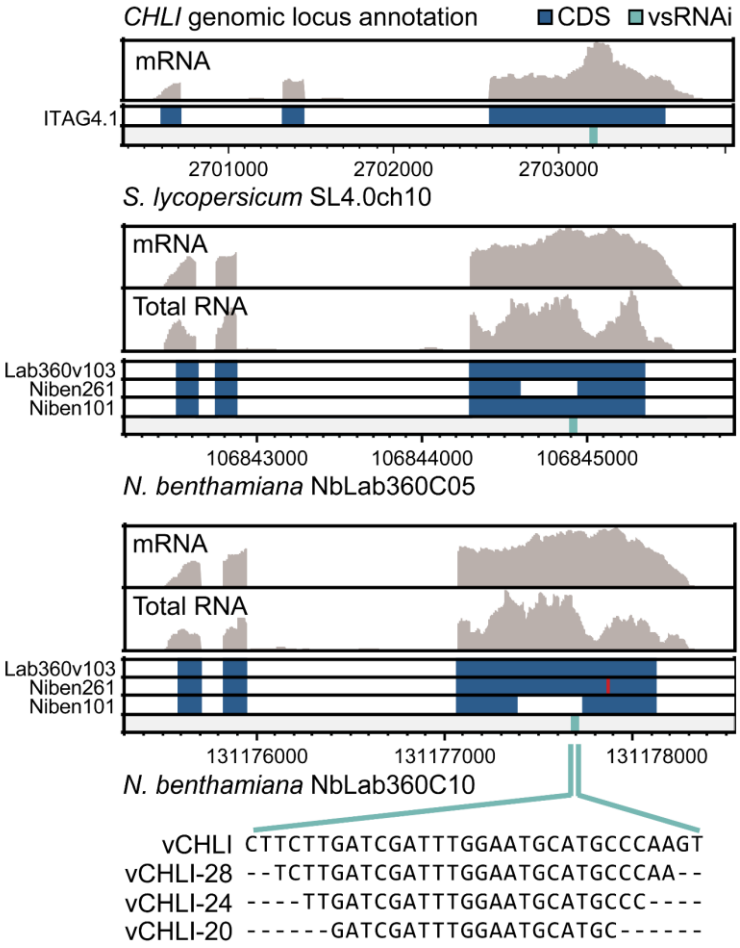


Figure S1. Annotation of *Nicotiana benthamiana CHLI* genomic loci and design of vsRNAi. Comparative genomics and transcriptomics guided the design of vsRNAi of 32, 28, 24 and 20-nt (vCHLI, vCHLI-28, vCHLI-24, and vCHLI-20) with sequences conserved in tomato (*S. lycopersicum*) and *N. benthamiana CHLI* genomic loci. Chromosomal regions were identified by DIAMOND blastx searches; for each genomic locus, expression and gene structure were confirmed by mapping sequencing reads obtained by either rRNA-depleted total RNA (Total RNA) or polyA-tailed RNA (mRNA) samples. Coding sequences (CDS) from previously reported annotations are shown as blue boxes; the red line indicates a sequence insertion unsupported by transcriptomic analysis.

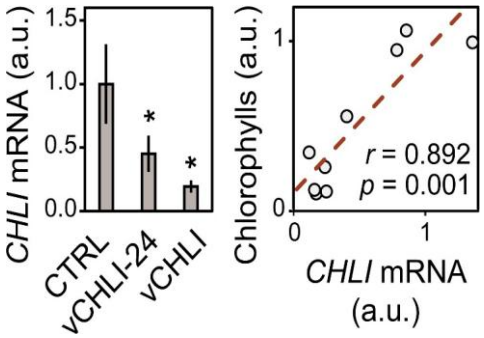


Figure S2. RT-qPCR of *N. benthamiana CHLI* transcripts. Left, *N. benthamiana CHLI* transcript quantification in vCHLI, vCHLI-24 and control (CTRL) samples (mean ± SD, n = 3); significance levels versus the control condition as per Student's t- test are shown; $*$, $p < 0.05$. Right, linear relationship between *CHLI* transcript and chlorophyll amounts (n = 9); Person's r and p value are indicated.

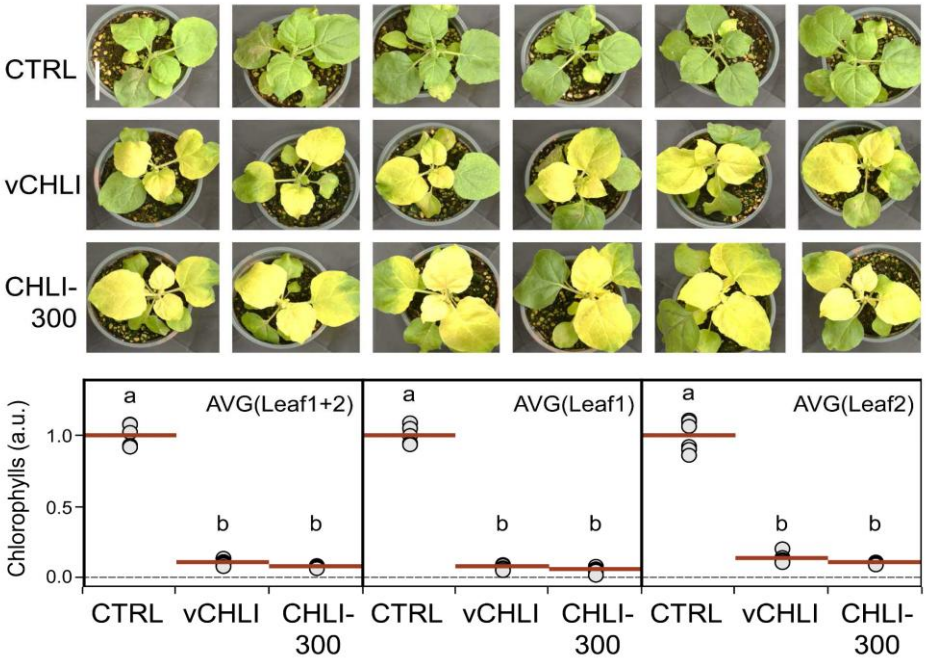


Figure S3. Delivery of a 32-nt vsRNAi triggers robust silencing phenotypes. Plants were treated with the unmodified JoinTRV (CTRL), or derivatives including a 32-nt vsRNAi (vCHLI) targeting the two *N. benthamiana CHLI* homeologs, and a 300-nt *CHLI* cDNA fragment previously reported (Aragonés et al., 2022). Top, plant phenotypes (scale = 5 cm). Bottom, chlorophyll levels, with each condition represented by a red bar (average of 6 plants). Individual dots show the average chlorophyll content of a single plant, measured at the most and second-most symptomatic leaf, and both leaves combined (AVG(Leaf1), AVG(Leaf2), and AVG(Leaf1+2), respectively). For control plants, equivalent leaves were measured. Five measurements were taken per leaf and averaged to calculate the values shown. Different letters indicate significant differences ($p < 0.05$), by one-way ANOVA and Tukey's HSD test.

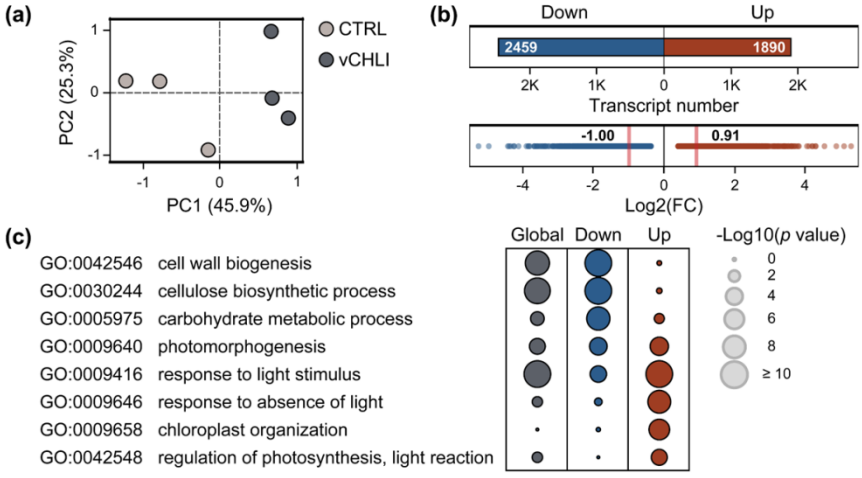


Figure S4. Functional analysis of transcriptome-wide changes triggered by vsRNAi. (**a**) Principal component analysis of samples analysed by transcriptomics; CTRL, control condition samples; vCHLI, samples from *N. benthamiana* plants inoculated with TRV vector comprising a 32-nt vsRNAi targeting *CHLI* homeologs (n = 3). (**b**) Total number (top) and fold change (bottom; median values are shown) of differentially expressed transcripts (FDR < 0.05; Data S1, Data S2). (**c**) Enrichment of gene ontology (GO) terms of biological processes in a transcriptome-wide analysis (global; Data S3), and in subset lists including down- or up-regulated transcripts (Data S4, S5); representative terms are shown with their significance levels.

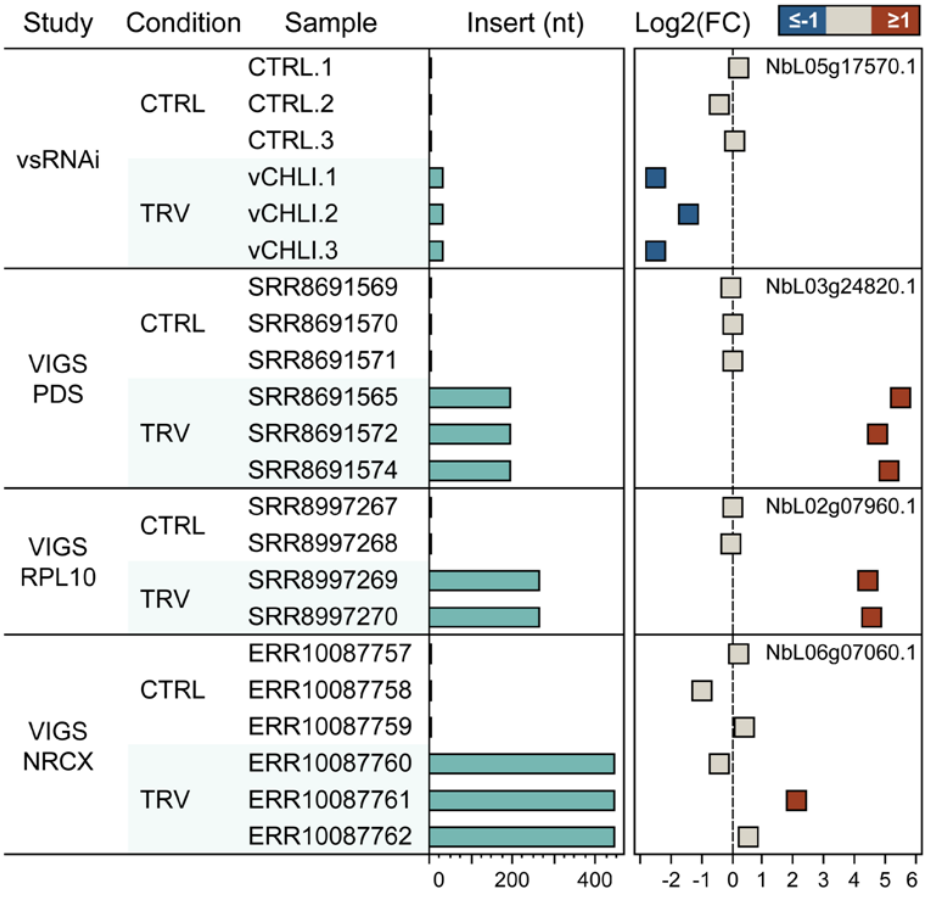


Figure S5. vsRNAi enable transcriptome-wide quantification of target gene silencing. Transcriptomes of *N. benthamiana* plants treated with TRV vectors containing vsRNAi or larger VIGS inserts were analysed using the alignment-free method Salmon (Patro et al., 2017). The obtained transcript-per-million (TPM) values were used to calculate the abundance of target genes compared to control samples, and expressed as log2 fold change (FC). Target gene downregulation was confirmed in vsRNAi samples from this study, whereas no downregulation was detected in the VIGS studies analysed (PDS, RLP10, and NRCX). VIGS transcriptomes and the insert sizes were reported (Adachi et al., 2023; Ahmed et al., 2020); NCBI SRA identifiers of the RNA-seq samples analysed are shown.

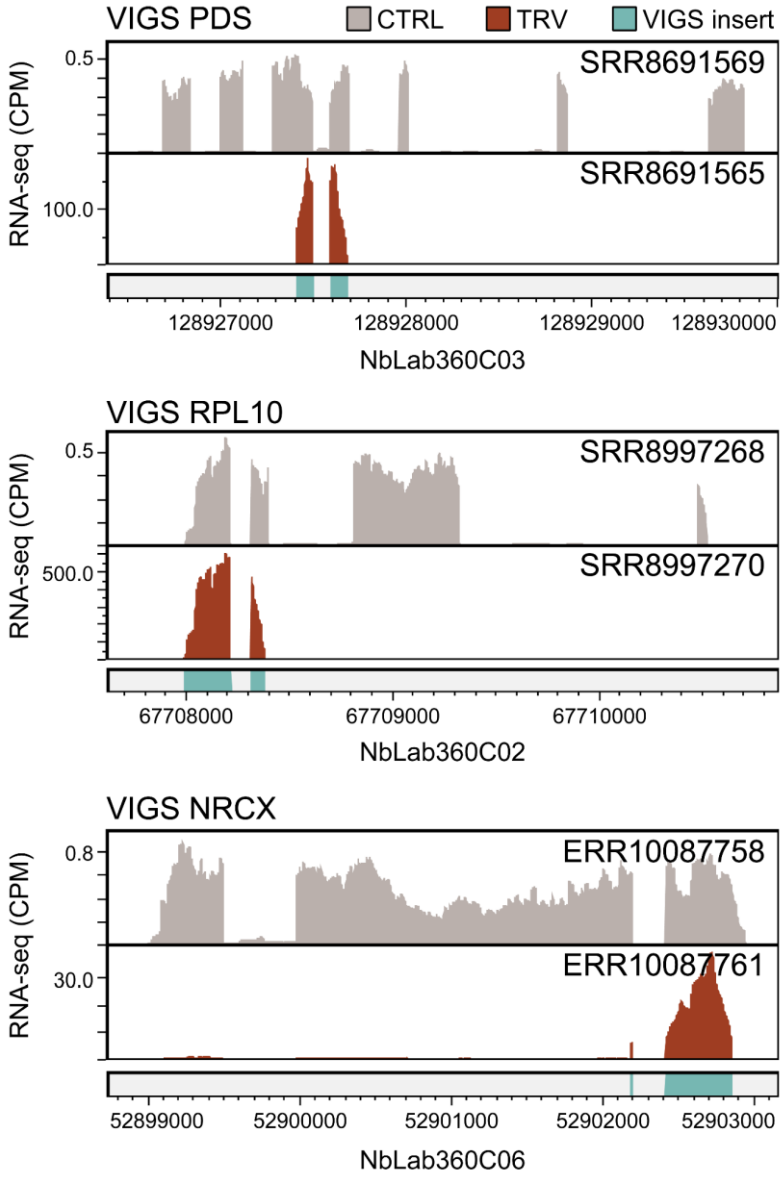


Figure S6. Viral amplification of large VIGS inserts interferes with quantification of target genes. RNA-seq reads from TRV VIGS (TRV) and control (CTRL) samples were mapped to the *N. benthamiana* genome using the HISAT2 aligner (Kim et al., 2019). Plots show the number of reads, expressed as counts per million (CPM), mapped to the genomic loci of target genes. TRV samples showed high CPM values within the genomic regions that share homology with the VIGS inserts, which greatly exceeded those of control

samples. VIGS transcriptomes were reported (Adachi et al., 2023; Ahmed et al., 2020), and NCBI SRA identifiers of the datasets analysed are shown within each plot.

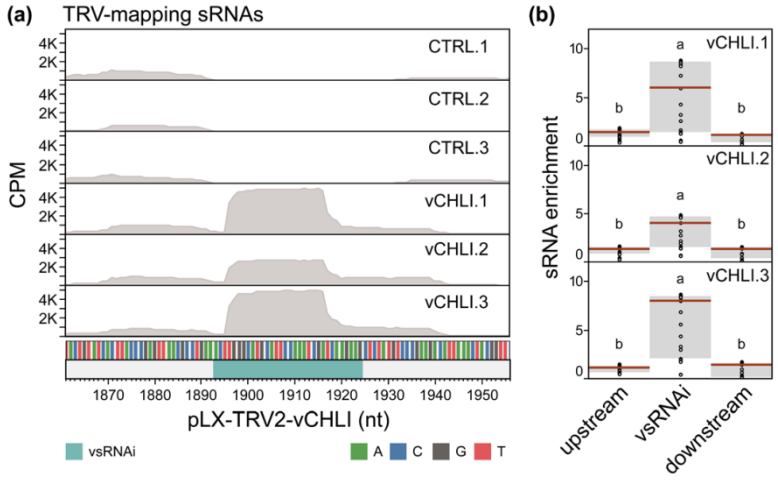


Figure S7. vsRNAi trigger host-derived production of sRNAs. (**a**) Small RNA sequencing (sRNA-seq) reads of vCHLI and CTRL samples were mapped to the TRV RNA2 sequence of pLX-TRV2-vCHLI, including a 32-nt vsRNAi targeting *CHLI* homeologs. Plots show the number of mapped reads. vCHLI samples showed high CPM values spanning the vsRNAi position, which greatly exceeded those of control samples. (**b**) Enrichment of sRNA mapping to vsRNAi compared to flanking viral sequences. The 32-nt vsRNAi sequence had significantly higher per nucleotide CPM values than the 32-nt viral sequences immediately up- and downstream, indicating viral-independent and host-derived sRNA production. Letters indicate $p < 0.05$, one-way ANOVA and Tukey's HSD test.

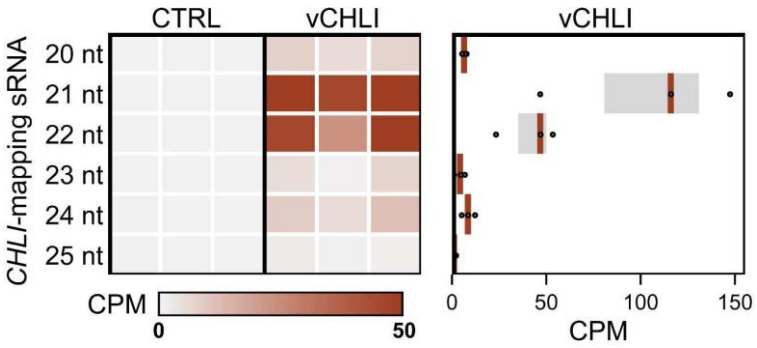


Figure S8. Production of gene-specific sRNAs triggered by vsRNAi. Small RNA sequencing (sRNA-seq) reads of vCHLI and CTRL samples (*n* = 3) were mapped to *N. benthamiana CHLI* transcripts (Data S7, S8). Left, heatmap of counts per million (CPM) per sample and size class; right, plot of CPM values of vCHLI samples (median, upper and lower quartiles are marked; *n* = 3).

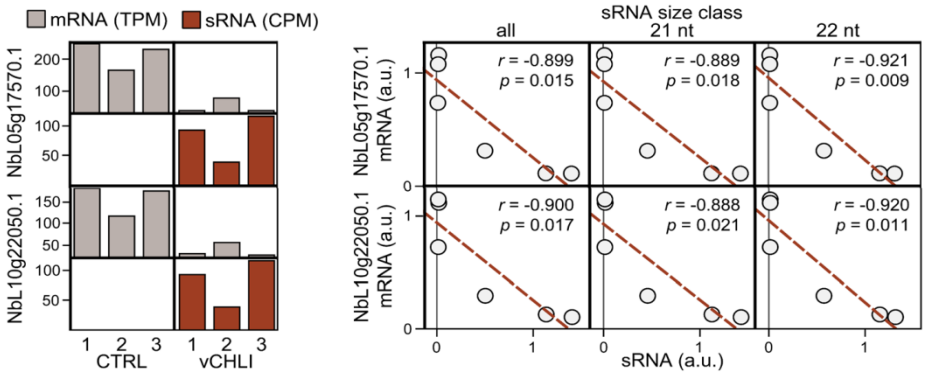


Figure S9. Abundance of CHLI-mapping sRNAs negatively correlated with *CHLI* expression. Left, abundance of *CHLI* mRNA (transcript per million, TPM) and *CHLI*-mapping sRNAs (counts per million, CPM) in CTRL and vCHLI samples are shown for the two *CHLI* homeologs (NbL05g17570.1, NbL10g22050.1). Right, scatter plots show correlation (n = 6) among the abundance of CHLI-mapping sRNAs and *CHLI* transcripts; Pearson correlation coefficients (r) and significance levels (p) are shown.

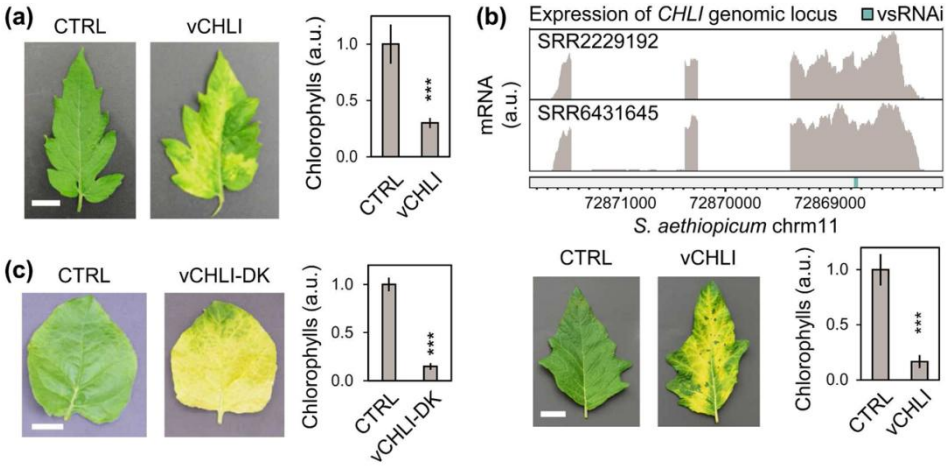


Figure S10. Portability of vsRNAi approaches to crops and viral vector systems. (**a**) The JoinTRV derivative vCHLI causes a chlorophyll reduction in tomato (*S. lycopersicum*). Phenotypes of upper uninoculated leaflets of 'Moneymaker' plants and chlorophyll fluorometric quantification results are shown (mean ± SD, $n = 4$; ***, Student's *t*-test $p < 0.001$); CTRL, control; scale = 1 cm. (**b**) The JoinTRV derivative vCHLI causes a chlorophyll reduction in scarlet eggplant (*Solanum aethiopicum*). Top, identification and annotation of the *S. aethiopicum CHLI* genomic locus; results of transcriptomic analysis and the vsRNAi targeted sequence of the identified *CHLI* genomic locus are shown. Bottom, phenotypes of upper uninoculated leaves of 'Rossa di Rotonda' plants, and chlorophyll fluorometric quantification results are shown (mean ± SD, $n = 3$; ***, Student's *t*-test $p < 0.001$); scale = 2.5 cm. (**c**) Use of a 32-nt vsRNAi derivative of the pTRV1 + pTRV2 vector system from Liu et al. (2002) to target the *N. benthamiana CHLI* gene pairs (vCHLI-DK) results in a chlorophyll reduction. Phenotypes of *N. benthamiana* upper uninoculated leaves and chlorophyll fluorometric quantification results are shown (mean ± SD, $n = 3$; ***, Student's *t*-test $p < 0.001$); scale = 2.5 cm.

Table S1. Transcriptomics and genomics resources used in the study.

Dataset	Source	Identifier
N. benthamiana transcriptomic and small RNA sequencing reads for vsRNAi and control samples	This study	NCBI BioProject PRJNA1217923
N. benthamiana genome and annotations	(Ranawaka et al., 2023)	Assembly: https://solgenomics.net/ftp/genomes/Nicotiana_benthamiana/LAB360/NbLab360.genome.fasta.gz NbLab360v103: https://solgenomics.net/ftp/genomes/Nicotiana_benthamiana/LAB360/NbLab360.v103.gff3.CDS.fasta.gz Niben261: https://solgenomics.net/ftp/genomes/Nicotiana_benthamianaV261/Nbenthamiana_Annotation/Niben261_genome.annotation.transcripts.fasta.gz Niben101: https://solgenomics.net/ftp/genomes/Nicotiana_benthamiana/annotation/Niben101/Niben101_annotation.transcripts.fas ta.gz
N. benthamiana transcriptomic datasets	(Pasin et al., 2020; Yue et al., 2023)	NCBI SRA: SRR11282461 SRR11282462 SRR11282463 SRR24114145 SRR24114146 SRR24114147
N. benthamiana VIGS *PDS* transcriptomic datasets	(Ahmed et al., 2020)	NCBI SRA: SRR8691569 SRR8691570 SRR8691571 SRR8691565 SRR8691572 SRR8691574

N. benthamiana VIGS *RPL10* transcriptomic datasets	(Ahmed et al., 2020)	NCBI SRA: SRR8997267 SRR8997268 SRR8997269 SRR8997270
N. benthamiana VIGS *NRCX* transcriptomic datasets	(Adachi et al., 2023)	NCBI SRA: ERR10087757 ERR10087758 ERR10087759 ERR10087760 ERR10087761 ERR10087762
S. lycopersicum genome and annotations	(Hosmani et al., 2019)	Assembly: https://solgenomics.net/ftp/genomes/Solanum_lycopersicum/assembly/current_build/S_lycopersicum_chromosomes. 4.00.fa ITAG4.1 https://solgenomics.net/ftp/genomes/Solanum_lycopersicum/Heinz1706/annotation/ITAG4.1_release/ITAG4.1_cDNA. fasta
S. lycopersicum transcriptomic dataset	(Giovannini et al., 2024)	NCBI SRA: SRR28956637
S. aethiopicum genome	(Benoit et al., 2025)	https://cshl-lippmanlab.s3.amazonaws.com/SolPanGenomics/Saethiopicum.genome_annotation.gz
S. aethiopicum transcriptomic datasets	(Gramazio et al., 2016; Wei et al., 2020)	NCBI SRA: SRR2229192 SRR6431645
Arabidopsis thaliana proteome	(Cheng et al., 2017)	Araport11 https://www.arabidopsis.org/download/file?path=Sequences/Araport11_blastsets/Araport11_pep_20240409_representative_gene_model.gz

Table S2. Software and algorithms used in the study.

Software/ Algorithm	Source	Identifier
DIAMOND	(Buchfink et al., 2021)	https://github.com/bbuchfink/diamond
kingfisher	(Woodcroft et al., 2024)	https://github.com/wwood/kingfisher-download
fastp	(Chen et al., 2018)	https://github.com/OpenGene/fastp
HISAT2	(Kim et al., 2019)	http://daehwankimlab.github.io/hisat2/
SPAdes	(Bushmanova et al., 2019	https://github.com/ablab/spades
MAFFT	(Katoh et al., 2019)	https://mafft.cbrc.jp/
salmon	(Patro et al., 2017)	https://github.com/COMBINE-lab/salmon
STAR	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
samtools	(Li et al., 2009)	http://www.htslib.org/
edgeR	(Robinson et al., 2010)	https://www.bioconductor.org/packages/edgeR
topGO	N/A	https://doi.org/doi:10.18129/B9.bioc.topGO
Integrative Genomics Viewer (IGV)	(Thorvaldsdóttir et al., 2013)	https://igv.org/

Table S3. Primers used in the study.

Primer[a]	Primer sequence (5'→3')[b]	Purpose
vCHLI_F	TGAAACTTGGGCATGCATTCCAAATCGATCAAGAAG	Cloning
vCHLI_R	AGAGCTTCTTGATCGATTTGGAATGCATGCCCAAGT	
vCHLI_20_F	TGAAGCATGCATTCCAAATCGATC	
vCHLI_20_R	AGAGGATCGATTTGGAATGCATGC	
vCHLI_24_F	TGAAGGGCATGCATTCCAAATCGATCAA	
vCHLI_24_R	AGAGTTGATCGATTTGGAATGCATGCCC	
vCHLI_28_F	TGAATTGGGCATGCATTCCAAATCGATCAAGA	
vCHLI_28_R	AGAGTCTTGATCGATTTGGAATGCATGCCCAA	
vCHLI-b_F	TGAACTCATTACTTCTTGGTCATCTGGATCTGAATT	
vCHLI-b_R	AGAGAATTCAGATCCAGATGACCAAGAAGTAATGAG	
vPDS_F	TGAACAACATAGACTGATTGGGGTTGTAATATTCCT	
vPDS_R	AGAGAGGAATATTACAACCCCAATCAGTCTATGTTG	
vCHLI-DK_F	CGCGATTGAAACTTGGGCATGCATTCCAAATCGATCAAGAAGCTCT	
vCHLI-DK_R	CGCGAGAGCTTCTTGATCGATTTGGAATGCATGCCCAAGTTTCAAT	
CHLI_F	GGAGGAAGTTTTATGGAGGGATTAG	RT-qPCR
CHLI_R	GGATCAATTACATTCAGCAAAAGACA	
PP2A_F	TGGGGATGGCTCCTGTTTTG	
PP2A_R	CTCGCCAATGCCTGTCCTCT	

[a]F, forward primer; R, reverse primer. [b]Overhangs compatible with BsaI-digested pLX-TRV2 are underlined.

Table S4. Oligonucleotide duplexes used for vsRNAi vector assembly.

Oligonucleotide pair	Sequence	Assembled vector	Vector code
vCHLI_F vCHLI_R	5' TGAAACTTGGGCATGCATTCCAAATCGATCAAGAAG 3' 3' TGAACCCGTACGTAAGGTTTAGCTAGTTCTTCGAGA 5'	pLX-TRV2-vCHLI	vCHLI
vCHLI_20_F vCHLI_20_R	5' TGAAGCATGCATTCCAAATCGATC 3' 3' CGTACGTAAGGTTTAGCTAGGAGA 5'	pLX-TRV2-vCHLI-20	vCHLI-20
vCHLI_24_F vCHLI_24_R	5' TGAAGGGCATGCATTCCAAATCGATCAA 3' 3' CCCGTACGTAAGGTTTAGCTAGTTGAGA 5'	pLX-TRV2-vCHLI-24	vCHLI-24
vCHLI_28_F vCHLI_28_R	5' TGAATTGGGCATGCATTCCAAATCGATCAAGA 3' 3' AACCCGTACGTAAGGTTTAGCTAGTTCTGAGA 5'	pLX-TRV2-vCHLI-28	vCHLI-28
vCHLI-b_F vCHLI-b_R	5' TGAACTCATTACTTCTTGGTCATCTGGATCTGAATT 3' 3' GAGTAATGAAGAACCAGTAGACCTAGACTTAAGAGA 5'	pLX-TRV2-vCHLI-b	vCHLI-b
vPDS_F vPDS_R	5' TGAACAACATAGACTGATTGGGGTTGTAATATTCCT 3' 3' GTTGTATCTGACTAACCCCAACATTATAAGGAGAGA 5'	pLX-TRV2-vPDS	vPDS
vCHLI-DK_F vCHLI-DK_R	5' CGCGATTGAAACTTGGGCATGCATTCCAAATCGATCAAGAAGCTCT 3' 3' TAACTTTGAACCCGTACGTAAGGTTTAGCTAGTTCTTCGAGAGCGC 5'	pTRV2-vCHLI	vCHLI-DK

Table S6. Summary of the small RNA sequencing (sRNA-seq) datasets generated.

Condition	Biological replicate	Clean read count	Average read length (nt)	Sample description
CTRL	1	12153131	19.67	Total RNA sample from upper uninoculated leaves of *Nicotiana benthamiana* plants inoculated with pLX-TRV1 and the pLX-TRV2 empty vector, and collected after 13 days; small RNA molecules were size enriched and sequenced (SE50).
	2	9775190	19.85	
	3	11982178	19.21	
vCHLI	1	10562799	19.44	Total RNA sample from upper uninoculated leaves of *Nicotiana benthamiana* plants inoculated with pLX-TRV1 and the pLX-TRV2-vCHLI, and collected after 13 days; small RNA molecules were size enriched and sequenced (SE50).
	2	10891736	19.88	
	3	11735408	20.17	

General discussion

Plants have been used to satisfy human consumption needs since immemorial time. The domestication of species over decades and centuries has led to the development of cultivable varieties that, to some extent, meet current nutritional and consumption requirements. However, the notable increase in the world's population, combined with other factors such as global warming (which brings about the spread and thus the increase of pests and pathogens), highlights the urgent need to expedite plant breeding processes. To achieve this, the most convenient and effective way is to implement newer biotechnological tools in the world of agriculture.

There is no doubt that one of the most valuable technologies for plant breeding is CRISPR/Cas-mediated gene editing. The most appealing features of this system are its ease of design, low production cost, high specificity, versatility, rapidity and reproducibility. The application of CRISPR/Cas technology in plants has been reported for over a decade (Feng et al., 2013), and it is now widely used. A critical step for successful plant editing is finding an efficient way for the nuclease and gRNA complex to reach the nucleus of the target cell. While stable transformation mediated by *Agrobacterium tumefaciens* is frequently used, the use of viral vectors to express the editing reaction components has generated significant interest in recent years.

Plant virus-derived vectors have been a topic of interest for virologists for at least thirty years, who have studied them as means to overexpress heterologous proteins (Siegel, 1985). One of the most important applications is their use as silencing vectors (virus-induced gene silencing, VIGS). This technique, used for decades, has been of great importance in gene functional studies (Senthil-Kumar and Mysore, 2011). With the application of CRISPR/Cas editing systems to plants, the use of viral vectors as a tool to express editing components was proposed due to their ability to invade systemic tissue. There are numerous examples where viral vectors are used in different species to successfully express gRNAs (Ali et al., 2015; Cody et al., 2017; Ellison et al., 2020; Kang et al., 2025; Wang et al., 2022). Despite cargo limitations, some viral vectors can also be used to express nucleases in plants (Liu et al., 2023; Ma et al., 2020).

In the first three chapters of this thesis, we have tried to advance and deepen the use of viral vectors as tools applicable to CRISPR/Cas-mediated gene editing. In **Chapter I**, we describe the development of a VIGE system in tomato plants that express the Cas9 nuclease, using a viral vector derived from the potato virus X (PVX). In **Chapter II**, we managed to express gRNAs for Cas12a nuclease-mediated editing for the first time in plants using potyviral vectors. In **Chapter III**, we worked on implementing a VIGE system in a species of agronomical interest, melon, which presents serious difficulties, particularly some agronomically relevant varieties, when it comes to being transformed by *A. tumefaciens*. Even so, significant advances were made that we hope will help in the development of this VIGE system in this species. Finally, in **Chapter IV**, we developed a VIGS strategy using a tobacco rattle virus (TRV) vector, in which we minimized homologous insert size, and maximized the simplification of viral vector engineering.

Advancing the VIGE technology in tomato

In the first chapter of this thesis, we describe the development of a viral induced gene editing (VIGE) system. This system uses a vector derived from PVX as a tool to express sgRNAs in tomato plants that constitutively express the SpCas9 nuclease. The use of PVX as an expression vector has been validated since more than three decades ago (Chapman et al., 1992). Its application as a biotechnological tool is highly significant due to its ability to infect more than 60 different plant species, including agronomically important crops such as those in the Solanaceae family (Zhang et al., 2022). As far as we know, VIGE has been successfully applied to model plants, but it had not yet been optimized for the horticultural trait breeding of tomatoes until the publication of the article corresponding to **Chapter I**. The use of a PVX vector as a guide RNA delivery tool had already been reported by our group; we obtained multiplexed and heritable gene editing in *Nicotiana benthamiana* Cas9 expressing plants (Uranga, Aragonés, et al., 2021). After this achievement, we investigated whether this method could be transferred to a species of agronomical interest, such as tomato.

To validate the portability of our previously developed VIGE system to other crops, we first proved that detectable CRISPR edits could be found when a reporter gene was targeted (*SlPDS*) in upper non-inoculated leaves of a Micro-Tom SpCas9 tomato line (MT-Cas9). To obtain solid mutants, plants were regenerated from infected tissue, and latterly selfed. Albino plants presenting the homozygous +1 allele (*pds* +1/+1) were obtained after selfing a bi-allelic mother plant (*pds* +1/-3) (**Figure 1F**). With these results, we found that our VIGE method, combined with tissue culture, produces inheritable genetic edits and can be used to obtain progeny with a loss-of-function mutation.

Following the same approach, we were able to obtain Micro-Tom *sgr1* mutants, which displayed fruits with the typical mutant *green-flesh* phenotype (**Figure 1G**). Aiming to transfer the obtained outcomes to traditional tomato varieties, we replicated our experiments in a SpCas9 expressing 'Pera' tomato line (Pera-Cas9). Again, we were able to find CRISPR edits in *SlPDS* and *SlSGR1* genes in systemic non-inoculated tissue (**Figure S6**). Altogether, these results validate the efficacy of the designed and employed VIGE system to edit genes of agronomical interest not only in research-focused lines, such as the Micro-Tom tomato, but also in commercial and traditional varieties.

The editing strategy described in **Chapter I** validates for the first time the use of PVX-derived vectors for sgRNA expression and the generation of CRISPR edits in tomatoes. Very recently, another article was published supporting the use of a PVX vector to edit the same reporter gene (*SlPDS*) in tomato plants, in this case from the cultivar 'Moneymaker' (Kang et al., 2025). Although the results were similarly successful, the authors were unable to obtain edited progeny after self-pollinating the edited plants. Therefore, they had to resort to a similar *in vitro* culture strategy to obtain stable mutants.

One of the most common and effective approaches to increase mutation heritability is to fuse tRNA-like sequences to gRNA transcripts to enhance RNA mobility within the plant (Yang et al., 2023). The ability to edit the germline depends on various factors, including the viral vector itself. *FLOWERING LOCUS T* (FT) from *Arabidopsis thaliana* encodes a mobile hormone (florigen) that regulates flowering time. An essential requirement for this regulation is its prior

transportation to the shoot apical meristem (Shibuta and Abe, 2017). However, not only the FT protein has been proved to be mobile, but also the FT mRNA, which migrates from shoots to apical meristems (Jackson and Hong, 2012; Yu et al., 2022). By fusing an FT mRNA fragment to the 3' end of the sgRNA, Ellison et al. (2020) achieved a more than threefold increase in heritable edits when targeting the *NbPDS* gene with a TRV-derived vector. In the constructs, we used for our VIGE approach with both tomato cultivars, the sgRNAs were also fused to an *AtFT* sequence, although no heritable edits were found after self-pollination.

We hypothesize that, for future improvements to our VIGE system, a promising strategy to achieve heritable gene editing and thus avoid the tissue culture regeneration step is to analyze the literature to identify the appropriate tomato FT ortholog, being *SINGLE-FLOWER TRUSS* (*SFT*) a suitable candidate (Lifschitz et al., 2006) that could direct our sgRNAs to the germline. Very recently, following this approach with a TRV vector, Lee et al. (2025) successfully obtained heritable gene editing in tomato by fusing the sgRNA to a *Tomato Flowering Locus T* (*SlFT*) mRNA. As a future approach, this strategy should be implemented in our VIGE platform.

Incorporating potyviruses into the VIGE technology

One of the main requirements when implementing a new technology in the world of plant breeding is the versatility and adaptability of the technique. In the case of CRISPR/Cas technology, the wide range of available nucleases, each with its characteristic properties, provides a variety of possibilities to satisfy the requirements of each particular case. Among these, the Cas12a nuclease has certain characteristics that make it attractive to be used in VIGE strategies. Firstly, this endonuclease requires a T rich PAM (TTTV, being 'V' a A, C, or G) (Zetsche et al., 2015), which opens the possibility of editing certain A and T-rich regions of the genome, such as promoters (Tjaden and Coruzzi, 1994), and thus controlling gene expression. Furthermore, the Cas12a nuclease possesses a self-processing capacity, because it is able to process the pre-CRISPR RNA (pre-crRNA) into mature crRNA, thanks to its endoribonuclease activity (Charpentier

et al., 2015; Fonfara et al., 2016). This particular capacity makes it especially attractive for expressing several crRNAs in plants at the same time (multiplexing). In addition, the capability of Cas12a to generate large indels makes it an ideal nuclease for generating gene knockouts (Bernabé-Orts et al., 2019).

However, there are scarce cases in which the Cas12a nuclease has been applied to VIGE assays. A notable example is the article by Uranga, Vazquez-Vilar, et al. (2021), in which the authors were able to express the LbCas12a nuclease by means of a vector derived from the tobacco etch virus (TEV; genus *Potyvirus*), from which the *NIb* gene had been previously removed to increase its cargo capacity. In this study, wild-type *N. benthamiana* plants were co-inoculated with a PVX-derived vector that expressed the NIb polymerase, as well as the gRNAs, achieving editing in non-inoculated systemic tissue. In addition, Liu et al. (2023) were able to express CRISPR/Cas12a reaction components using a tomato spotted wilt virus (TSWV; genus *Orthotospovirus*) and obtain CRISPR edits in crops of agronomical interest, such as tobacco, tomato, chili pepper, sweet pepper, and peanut.

The versatility and adaptability of the VIGE technique are highly restricted by the viral vector used. Each viral vector has certain characteristics such as its cargo capacity, replication mode, or infectivity. There is no single viral vector capable of infecting all existing species of interest. However, if an 'ultimate or universal vector' were to be developed, one of the desirable characteristics would undoubtedly be a broad host range. The genus *Potyvirus* is considered the largest group of plant RNA viruses. Collectively, potyviruses infect a wide range of host plant species, being able to infect some of them in up to 38 host families, many of which are of high agronomical interest (Gadhave et al., 2020; Wylie et al., 2017). Within this genus, the most commonly used viruses as vectors are the plum pox virus (PPV), the clover yellow vein virus (CYVV), and the TEV (Mikhaylova, 2025). In **Chapter II** of the thesis, we implemented a VIGE system in which potyviral vectors were used to deliver gRNAs in transgenic plants overexpressing LbCas12a nuclease. Our purpose is to expand the scope of VIGE technology to potyviral vectors. To our knowledge, this is the first time that a potyviral vector has been used to deliver gRNAs in a VIGE approach.

The first objective in **Chapter II** was selecting a proper crRNA that could generate CRISPR edits in *N. benthamiana* Cas12a plants. For this purpose, we chose a TRV-derived vector to express the crRNAs *in planta*. We efficiently targeted both homeologs of *NbCHLI* gene and proved the heritability of the mutations (**Figure 3**). We then tried to replicate the obtained outcome using a TEV vector, but we encountered the problem of the development of severe symptoms in infected plants, which impaired seed production. To overcome this situation, we decided to generate four mutant variants of the HC-Pro cistron, which is involved in RNA silencing suppression in the host. These attenuated TEV versions were reported to induce mild symptoms after infection (Torres-Barceló et al., 2008). All four developed mutant TEV variants were able to generate CRISPR edits (**Figure 4C**). This supports the high efficiency of the VIGE system applied to TEV, and its great capacity to express crRNAs even in attenuated versions, in which a lower viral load in the plant is assumed (values not empirically quantified). Plants infected with TEV-CLA2 attenuated variant were self-pollinated, and we were able to find edited progeny without the virus. In addition, we also obtained solid mutants after regeneration of complete plants by means of tissue culture, using infected tissue as explant source (**Figure 5**). These results demonstrate the possibility of using attenuated versions of TEV to express crRNAs in plants. Furthermore, heritable mutations have been obtained using two different approaches, after tissue culture regeneration, as well as after plant selfing.

We then decided to test whether our TEV based VIGE system could be transferrable to other species of agronomical interest. We proved that TEV could efficiently deliver crRNAs in tomato and tobacco Cas12a overexpressing lines, and generate indels in reporter genes (*SlCHLI* and *NbCHLI* homeologs) in systemic non-inoculated tissue (**Figure 6**). By using the same approach followed in *N. benthamiana*, *N. tabacum* edited plants were regenerated after tissue culture, but we failed to obtain edited seedlings after selfing. Surprisingly, one of the regenerated edited plants was free of virus (**Figure 6**). Although special treatments are usually required to eliminate viral presence during in vitro regeneration (thermotherapy or ribavirin addition as an example) (Liu et al., 2023), it has been reported that virus-free tissue sectors may arise from calli of infected plants (Wang and Hu, 1980). We finally were able to prove that our

VIGE system was transferrable to other potyviral vectors, such as lettuce mosaic virus (LMV) and turnip mosaic virus (TuMV). However, we could only test these vectors in *N. benthamiana* plants, because of the lack of Cas12a lines of their natural hosts, lettuce and brassica.

In order to validate the results obtained in other species of agronomical interest, it would be advisable to propose a future genetic transformation program that would allow us to obtain transgenic lines of host crops for the potyviruses used, such as brassica species, lettuce, pepper, or eggplant. The results of **Chapter II** conclusively validate the use of potyviruses as vectors for expressing crRNAs in different plant species, a fact that we believe has been demonstrated for the first time. The next necessary objective in this project would be to successfully express the nuclease by means of a viral vector, thus avoiding the prior transformation process. Despite its large cargo capacity, we have not been able to express the LbCas12a nuclease with our TEV-derived vector in previous experiments performed in our laboratory. However, the adaptation of so-called miniature Cas nucleases to plant breeding can completely overcome the cargo limitations of viral vectors. Thus, examples already exist in the literature that support the expression of versions of Cas12 nucleases, such as SpCas12f1, AsCas12f1, and CasMINIv3.1, using viral vectors in plants, giving rise to CRISPR edits in endogenous genes (Gong et al., 2024; Ishibashi et al., 2024). The expression of these orthologs in our TEV vector would be the next step to advance the topic proposed in **Chapter II**.

Towards a VIGE system in melon and other cucurbits

One of the primary goals of plant biotechnology is to transfer the results obtained from model plants, such as *A. thaliana* and *N. benthamiana*, to cultivated species with agronomical importance that are consumed by humans in one way or another. This is how we will be able to meet the needs demanded by the constant increase in the global population and climate change. The use of VIGE technology has been widely demonstrated in agronomically important species, such as tomato (Kang et al., 2025), wheat (Wang et al., 2022), barley (Tamilselvan-Nattar-Amutha et al., 2023), chili

pepper, sweet pepper, or peanut (Liu et al., 2023). However, there is a certain group of plants, such as those in the Cucurbitaceae family, and more specifically melon, where CRISPR/Cas editing technologies have not been as widely implemented as in other crops of equal agronomical importance. This is mainly due to the existing difficulty in stably transforming (recalcitrance) and regenerating melon plants (Chovelon et al., 2011). Although significant progress has been made in this field over time, and gene editing in melon has been obtained (Hooghvorst et al., 2019; Nonaka et al., 2023; Pechar et al., 2022; Sasaki et al., 2025), to our knowledge, there is no report supporting VIGE in cucurbits. Because of this, we aimed to develop the first reported VIGE system in melon plants (**Chapter III**). To achieve this, we combined the *A*. *tumefaciens*-mediated stable transformation of five different melon genotypes to obtain lines that expressed SpCas9, LbCas12a, and ttLbCas12a (a thermotolerant variant of LbCas12a) nucleases. Different gRNAs were expressed in transgenic plants by means of a vector derived from watermelon mosaic virus (WMV; genus *Potyvirus*). Despite our efforts, we have not been able to achieve gene editing in any of the targeted genes so far.

Nevertheless, the considerable difficulty to stably transform and regenerate melon plants should be mentioned (Chovelon et al., 2011). Although this characteristic is influenced by genotype, it affects every melon variety to a greater or lesser extent (Wan et al., 2023). In the experiments conducted in **Chapter III**, we were able to obtain a total of 43 transformed melon plants, the combined transformation efficiency being 0.95%. In order to evaluate the results obtained, we can compare the efficiency of the transformations with the 'Védrantais' type, which is below the generic values (0.63% versus 3%), and with the 'Piel de Sapo' type, which is above the generic values (1.25% in the case of CH5 and 0.78% in the case of PO, versus 0.1%, as a generic value) (Castelblanque et al., 2008) (**Table 1**). Certain studies report obtaining transformation efficiencies up to 3.3% in T111 'Piel de Sapo' line (the same one used in Castelblanque et al., 2008) and up to 23.3% in Near Isogenic Lines (NILs) with T111 background (Menéndez et al., 2012). However, the transformation efficiency in this last study is calculated taking into account the total amount of transgenic plants, and not the amount of organogenic callus obtained (i.e. the amount of callus that gave rise to a transgenic shoot), as it was done in **Chapter**

III and also in Castelblanque et al., 2008. This could overestimate the transformation efficiency of the study.

It should be taken into account that the fundamental objective of this chapter was not to improve the efficiency of genetic transformation, but to obtain melon lines that express the nucleases to be tested. We therefore obtained a satisfactory transformation efficiency in our experiments. However, if we wanted to obtain better results, one of the possible strategies to increase transformation efficiency is to co-express growth-regulating factors in the T-DNA. The expression of a fusion protein combining GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) has been shown to substantially increase the efficiency and speed of regeneration in wheat, which may be due to the complex's ability to promote cell proliferation in a wide range of organs (Debernardi et al., 2020). A similar strategy has been tested in cucurbits such as watermelon (Feng et al., 2021) and melon (Wan et al., 2023) with outcomes that validate the use of this approach to increase transformation efficiency. With a view to future possible transformations, the orthologs of melon *GRF* genes could be expressed in our transformation constructs.

Unfortunately, we were not able to find any gene editing event after expressing the gRNAs in our transgenic plants. The reasons that could explain this issue are diverse. Lack of Cas gene expression or Cas nuclease translation; inefficient Cas nuclease performance due to environmental factors such as temperature; a sample taken where the viral vector has not replicated or low efficiency of the selected guides are simply some of the hypotheses discussed in **Chapter III**. Despite all these theories, the necessary tests to clarify the situation have not yet been performed. If this research were to continue, the next steps would be to test the remaining vectors (and gRNAs) already constructed, in an attempt to detect some editing event. At the same time, RT-qPCR analysis should be performed to verify the correct expression of the Cas nucleases, as well as a western blot to validate their translation.

Facilitating VIGS for plant research and biotechnology

VIGS is a tool that has been used for decades, predating CRISPR/Cas technology. Its application in gene functional studies has been widely validated, and it is a powerful tool for performing forward and reverse genetics to decipher the involvement of plant genes in various processes (Senthil-Kumar and Mysore, 2011). Various viral vectors have been employed in plant gene silencing studies. Among these, the use of TRV stands out. TRV is a virus with a broad host range that induces relatively mild symptoms, which are desirable characteristics for VIGS studies. The TRV vector has been engineered and used in silencing assays in *N. benthamiana* and tomato plants for more than two decades (Y. Liu, Schiff, and Dinesh-Kumar, 2002; Y. Liu, Schiff, Marathe, et al., 2002). During a set of experiments in which we tried to generate CRISPR edits in *CHLI* gene in *N. benthamiana* and tomato Cas12a plants, expressing the crRNAs by means of a TRV vector, an unexpected outcome arose. We realised that plants presented whitish dots, a typical phenotype related to *CHLI* inactivation. However, after plant genotyping, we could not find any CRISPR edit in the targeted gene. Surprisingly, the same phenotype appeared in control wild-type plants, inoculated with the same TRV vector. We hypothesized that this phenotype was then caused by *CHLI* silencing.

The induction of gene silencing caused by CRISPR guides has already been reported, and termed as guide-induced gene silencing (GIGS) (Sharma et al., 2022). In this study, authors reported to obtain a reduction in *PDS* transcripts when expressing Cas13 guides (28 nucleotides), by means of a TRV vector in *N. benthamiana* plants, although no photobleaching phenotype was observed. There are also reports supporting the possibility of silencing heterologous genes in transgenic plants, expressing short homologous fragments (23-nt) by means of a PVX vector (Thomas et al., 2001). Nevertheless, when trying to silence endogenous genes, a homologous fragment of at least 33-nt was required. However, the ability to generate silencing with small cloned inserts in viral vectors is not fully understood. Liu and Page (2008) were not able to induce *NbPDS* silencing using TRV constructs with 103-nt and 54-nt inserts. In **Chapter IV**, we aimed to determine which was the smaller insert size required to efficiently generate endogenous gene silencing, accompanied to the

expected phenotypic alterations. For this, we generate a VIGS system based on a TRV vector, whose main characteristics were its specificity and its great simplification of viral vector engineering.

Four different TRV derivatives with 32, 28, 24 and 20-nt inserts targeting *NbCHLI* were tested. We proved that 24-nt inserts where the shortest in size, that lead to a distinguishable phenotype change (photobleaching), supported by a reduction in *CHLI* mRNA transcripts (**Figure 1A**, **Figure S2**). By small RNA sequencing, we also proved that the targeted regions presented an accumulation of 21 and 22-nt small RNAs (sRNAs), which are end products of Dicer-like 4 (DCL4) and DCL2 (Rössner et al., 2022). Finally, we proved the adaptability of the developed system by targeting another conserved region in *CHLI* and *NbPDS* homeologs, achieving similar results (**Figure 1D**). We also proved the portability of the system to other crops, by achieving gene silencing with the corresponding phenotypic alteration in *S. lycopersicum* and scarlet eggplant (*Solanum aethiopicum*) (**Figure S10**).

Taken together, obtained results demonstrate that small inserts of 24-nt size can produce phenotypic alterations due to silencing of endogenous genes. Our findings open the door to a new cloning strategy in which homologous fragments are nearly 10-fold smaller than those used in conventional VIGS assays. This allows a low cost fragment synthesis eliminating any error prone step (like a polymerase chain reaction). This strategy allows to easily perform functional genomics studies both in model plants and crops of agronomical interest.

What is next?

The effects of climate change, as well as the massive increase in the world's population, compel us to imminently seek alternatives that allow to increase crop production globally. To complete this race against time, researchers have developed efficient techniques based on plant biotechnology, such as the use of viral vectors applied to plant genetic improvement.

Diseases caused by plant viruses have a tremendous negative impact on agriculture worldwide. In 2014, virus disease pandemics and epidemics were estimated to have a global economic impact higher than 30 billion of US dollars annually, an amount that has considerably increased among recent years. It is calculated that 47% of the pathogens causing emerging and re-emerging plant disease epidemics around the world are viruses (Jones, 2021).

Fortunately, over the years, molecular biology techniques have emerged and have allowed us to modify plant viruses at will and use them as biotechnological tools, taking advantage of features that are a priori negative, such as a broad host range or a high infectivity. For more than three decades, plant viral vectors have been used as expression vectors for reverse genetics studies, later applied to silencing studies, and in the current era, to CRISPR/Cas gene-editing experiments (Cody and Scholthof, 2019). In this work, we aimed to demonstrate the high applicability of plant viruses in the world of plant biotechnology. To achieve this, we developed biotechnological strategies based on RNA silencing and CRISPR/Cas-mediated gene editing, using plant viruses from three different genera (*Potexvirus*, *Potyvirus*, and *Tobravirus*).

While promising, it is important to note that the strategies developed here are not without a certain degree of controversy. For now, the VIGE systems used in this thesis require the prior generation of transgenic organisms that express the corresponding nucleases. Although these can be removed from the plant's genome through conventional crossing and selection techniques, the modified organisms obtained via CRISPR/Cas technology are still subjected to stringent regulations by the European Union. This makes their cultivation and market entry exceedingly difficult. Although there is a proposal for them to be considered organisms obtained through new genomic techniques (NGTs) and therefore regulated more permissively, there has been no practical change in European legislation to date, something that has occurred in certain countries like the United Kingdom (EUR-Lex, 2025; RSC, 2025).

Additionally, the use of viral vectors in healthy plants can generate some societal apprehension. It is essential to educate the public about the real risks associated with using viral vectors in controlled experiments, where their

dispersion into nature is highly improbable if proper safety measures are followed. Furthermore, we must clarify that, as plant pathogens, these viruses cannot infect mammals, including humans. In addition, the three genus of viruses used in our studies (*Potexvirus*, *Potyvirus*, and *Tobravirus*) are positive single-stranded RNA virus, which replicate in the cytoplasm, not entering the nucleus and not undergoing any DNA-based replication phase. This makes the probability of integrating viral sequences into the plant genome practically null (da Fonseca et al., 2016).

The tools we humans have at our disposal are really powerful. Proof of this is our ability to precisely modify an organism's DNA. The ethical and responsible use of these tools can allow us to address the current and future problems we will face due to global food shortages. In the huge available toolbox, viral vectors must never be absent. Their application in plant biotechnology has been implemented for several decades, and their potential has not yet been fully exploited. It is up to us, the researchers, to harness the advantages they provide for the common good of humanity.

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Conclusions

1. Potato virus X (PVX) can be engineered for efficient sgRNA delivery into Micro-Tom SpCas9 expressing plants, leading to high indel percentages (up to 46%) in different genomic loci. Solid mutant plants can be obtained after tissue culture regeneration. Mutation heritability was proven after analyzing the progeny of regenerated plants. In addition, the system was shown to be transferrable to traditional tomato cultivars with similar results.

2. Tobacco rattle virus can induce heritable (seed transmissible) gene edits by delivering crRNAs into the model plant *N. benthamiana* expressing LbCas12a. Different vector architectures can lead to this outcome, showing the best heritable editing when using a single direct repeat and a 5FT fragment flanking the spacer (DR1-CHLI2-5FT).

3. Tobacco etch virus (TEV), a potyviral vector, can be engineered to deliver crRNAs into the model plant *N. benthamiana* expressing LbCas12a, obtaining indel percentages up to 75%. Heritable mutations (both by tissue culture regeneration, and by seed transmission from inoculated plants) can be achieved when using attenuated versions of the virus, such as TEV-CLA2. This viral vector is also able to induce somatic mutations in crops of agronomical interest, such as tobacco and tomato.

4. The ability to induce somatic mutations by expressing crRNAs in transgenic plants is not restricted to TEV in the *Potyviridae* family. Lettuce mosaic virus (LMV) and turnip mosaic virus (TuMV) can also be engineered to induce gene edits in *N. benthamiana* LbCas12a expressing plants.

5. The developed VIGE strategy in melon plants, was not able to induce gene edits when expressing the gRNAs by means of a watermelon mosaic virus (WMV) vector. However transgenic melon plants were obtained with a relatively high transformation efficiency for this species (0.95%).

6. It is possible to effectively induce gene silencing in *N. benthamiana* plants using a TRV vector, by expressing 10-fold smaller fragments than the ones used in conventional VIGS assays (vsRNAi). Inserts as short as 24-nt can

effectively produce phenotypic alterations. This silencing effect is mediated by the endogenous production of gene-specific 21 and 22-nt small RNAs.

7. The vsRNAi system is not only efficient in model plants, but is also portable to crops of agronomical interest, such as tomato and scarlet eggplant (*S. aethiopicum*), proving its applicability to functional genomics assays.

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