CellPress
REVIEWS

programmable endonucleases, CRISPR/Cas9. In this system, a catalytically inactive form of Cas9 is fused to a cytidine deaminase enzyme and directed to its genomic target with a guide RNA (gRNA), where it mediates the direct conversion of cytidine to uridine, thereby effecting a C→T (or G→A) substitution without introducing dsDNA breaks [6]. The introduction of the base editing tool caused a revolution in the genome engineering field and allowed researchers to introduce precise modifications in virtually any part of the DNA with ease – with one notable exception: the mitochondrial genome. Although mitochondria allow the import of proteins tagged with a specific peptide known as a mitochondrial targeting sequence (MTS), they cannot import DNA or RNA due to the strong electrochemical potential across the inner mitochondrial membrane. This feature essentially precludes the use of base editing in mitochondria because these tools require a gRNA to locate the DNA target. One way to circumvent this issue would be to develop a gene editing tool comprising solely protein components; for example, by combining the DNA recognition capability of ZFNs or TALENs and the base editing function of deaminases. The main bottleneck, however, is that deaminases typically act on single-stranded DNA (ssDNA), but not dsDNA. Unlike Cas9, which possesses helicase activity, ZFNs and TALENs are incapable of unwinding dsDNA to provide a suitable ssDNA substrate for deaminases.

A recent publication by Mok *et al.* [7] describes the identification and characterization of a novel bacterial cytidine deaminase that the authors named 'dsDNA deaminase toxin A', or DddA, isolated from the Gram-negative bacterium *Burkholderia cenocepacia*. As the name implies, this DddA enzyme specifically converts cytosines to thymines (C→T) present in dsDNA, unlike other reported DNA cytidine deaminases which operate predominantly on ssDNA [8]. To prevent DddA from converting DNA bases at random and thereby causing unwanted edits potentially leading to considerable toxicity, the authors first had to engineer a split version of the protein comprising two parts that would be functional only when the two DddA halves assembled adjacently on target DNA, similar to the assembly of FokI endonuclease monomers reconstituting dsDNA cleavage activity in ZFNs and TALENs. On optimizing the split DddA architecture, the two halves were subsequently fused to MTS-linked TALE array proteins that bind neighbouring DNA sites in the target gene *MT-ND6* located in the mitochondrial genome. When these constructs were transfected into different human cell lines (HEK293T and U2OS), specific C→T conversions were observed almost exclusively in the predicted *MT-ND6* target with efficiencies of up to 27% without perturbing mtDNA copy numbers. This observation suggests that they edit bases without generating dsDNA breaks, which could result in subsequent mtDNA degradation. On further tweaking of the MTS-TALEN-DddA fusion proteins, the optimized mitochondrial base editing tool was named the 'DddA-derived cytosine base editor' (DdCBE). To show that these DdCBEs were no one-trick pony, the team managed to precisely edit five different mitochondrial genes using seven pairs of TALE arrays, with efficiencies ranging from 4.6% to 49%. Collectively, these results show for the first time that installing defined modifications in the mitochondrial genome is possible in mammalian cells. This breakthrough is likely to pave the way for the generation of new genetically engineered cell lines and animal model systems to gain a better understanding of the cellular mechanisms involved in mitochondrial diseases caused by mutations in mtDNA.

Although the newly described mitochondrial gene editors are a huge leap forward, there remain some limitations to this new technology, the main one of course being the number of editable sites; in its current form, only Cs in the context 5′-T**C**-3′ can be converted into Ts, generating 5′-T**T**-3′. Of the 83 pathogenic mtDNA point mutations currently listed in the MITOMAP database, only 41 could in theory be corrected by the current generation of DdCBEs. Therefore, it would be interesting to see whether these editors can be engineered in a way that will alter their substrate preference and further expand the scope of mtDNA editing. One thing is for sure, however: this is going to be an exciting year for scientists studying mitochondrial biology.

[1]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

*Correspondence:
joey.riepsaame@path.ox.ac.uk (J. Riepsaame).

https://doi.org/10.1016/j.tig.2020.08.001

References

1. Gorman, G.S. *et al.* (2015) Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Ann. Neurol.* 77, 753–759
2. Gammage, P.A. *et al.* (2014) Mitochondrially targeted ZFNs for selective degradation of pathogenic mitochondrial genomes bearing large-scale deletions or point mutations. *EMBO Mol. Med.* 6, 458–466
3. Bacman, S.R. *et al.* (2013) Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. *Nat. Med.* 19, 1111–1113
4. Moretton, A. *et al.* (2017) Selective mitochondrial DNA degradation following double-strand breaks. *PLoS One* 12, e0176795
5. Hagström, E. *et al.* (2014) No recombination of mtDNA after heteroplasmy for 50 generations in the mouse maternal germline. *Nucleic Acids Res.* 42, 1111–1116
6. Komor, A.C. *et al.* (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424
7. Mok, B.Y. *et al.* (2020) A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. *Nature* 583, 631–637
8. Iyer, L.M. *et al.* (2011) Evolution of the deaminase fold and multiple origins of eukaryotic editing and mutagenic nucleic acid deaminases from bacterial toxin systems. *Nucleic Acids Res.* 39, 9473–9497

Spotlight

Virus-Based CRISPR/Cas9 Genome Editing in Plants

Huawei Liu[1,*] and Baohong Zhang[2,*]

CRISPR/Cas9 is a versatile tool for plant gene function studies and crop improvement. However, traditional CRISPR/Cas9-mediated genome editing requires plant tissue culture that is both time-consuming and genotype-dependent. Ma *et al.* recently reported a novel virus-based method for delivering CRISPR/Cas9 into plant cells, and this should further expand the application of CRISPR/Cas9-mediated genome editing.

Traditional CRISPR/Cas9 Genome Editing Holds Promise but Has Shortcomings

Since it was first recognized as a genome editing tool in 2012, CRISPR/Cas9 has revolutionized biological and biomedical research for investigating gene function, gene therapy, and crop improvement [1]. CRISPR/Cas9 can be used not only to knock out or knock in individual genes but also to repair individual nucleotides and regulate gene expression at the epigenomic and genomic levels [2]. However, these applications are hampered in plants because the current CRISPR/Cas9 delivery method generally requires plant tissue culture and plant regeneration, both of which are species- and genotype-dependent, and are not achievable in many plant species. Although it is feasible to generate transgenic genome-edited plants, there are several problems related to government regulations and genetic mutagenesis. Thus, there is an urgent need to develop a genotype-independent CRISPR/Cas9 delivery method that does not require transgene insertion. In a recent study, Ma *et al.* employed a virus for the first time to successfully deliver the entire CRISPR/Cas9 system *in vivo* and achieve genome editing in plants [3]. This could potentially revolutionize the use of CRISPR/Cas9 genome editing for plant gene function studies and crop improvement.

Viruses as Vectors for Delivering CRISPR/Cas9 Components

Viruses have been widely used for delivering the CRISPR/Cas9 system into animal cells *in vivo* and *in vitro* for fundamental studies and gene therapy [4]. Viruses have also been used in plants to deliver small molecules such as small interfering RNAs for virus-induced gene silencing (VIGS); viruses have been successfully employed to knock out an individual gene by delivering meganucleases and zinc-finger nuclease [5]. Recently, viruses have also been employed to deliver CRISPR guide RNA (gRNA) for targeting specific genes in plants [5]. However, to date there have been no reports describing delivery of the entire CRISPR/Cas9 system into plants for genome editing. Two factors limit virus delivery of the CRISPR/Cas9 system into plant cells. The viruses currently used for plant gene function studies are all DNA viruses or positive-strand RNA viruses: these two virus types have limited capacity for inserting DNA/RNA fragments. Moreover, the

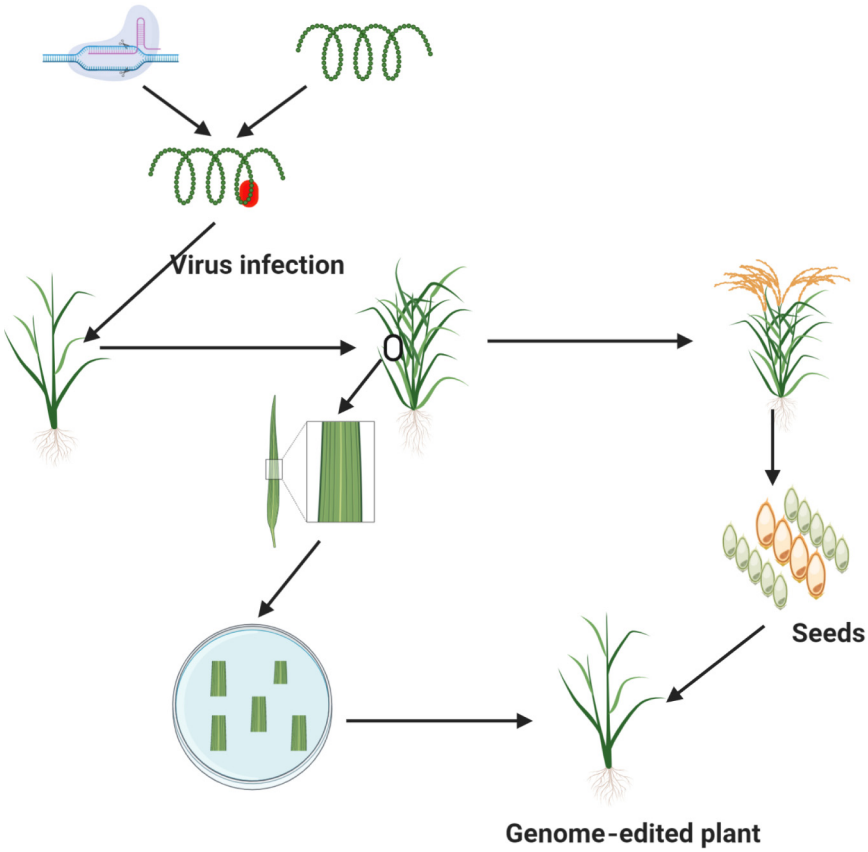

Figure 1. A New Strategy for Genome Editing in Plants by Viral Delivery of CRISPR/Cas9 Independently of Tissue Culture. In this strategy, both Cas nuclease (gray) and the guide (g)RNA (pink) are engineered into the virus genome (green helix), and the plants are then infected *in vivo* with the engineered virus. Virus infection leads to the synthesis of Cas nuclease and gRNAs with the help of the endogenous plant RNA-processing machinery, leading to editing of the plant genome. Because viruses can invade different cells, and also move between cells alone or with the help of a mobile RNA molecule, the CRISPR/Cas regents may also enter meristem and/or germline cells for genome editing. Once editing is complete in the germline cells, the modification is transmitted to seeds, and screening of progeny plants can then identify genome-edited plants. This method allows the generation of transgene-free genome-edited plants without recourse to tissue culture.

CRISPR/Cas9 system is large (usually >4.5 kb) and therefore it is difficult to use DNA viruses or positive-strand RNA viruses to deliver the large CRISPR/Cas9 system into plant cells by virus infection.

Recently, Ma *et al.* successfully delivered the entire CRISPR/Cas9 system into plant cells and obtained CRISPR/Cas9 genome-edited plants with high efficiency [3]. This success was achieved by using a negative-strand RNA virus instead of a DNA or positive-strand RNA virus. Although many negative-strand RNA viruses are known to infect plants, few have been exploited as vectors for delivering nucleotide fragments into plant cells, principally because it is difficult to engineer this class of viruses. This technical barrier was recently solved by using sonchus yellow net rhabdovirus (SYNV) as a model, in which Wang *et al.* successfully rescued infectious SYNV from cloned cDNA [6]. Because negative-strand RNA viruses such as SYNV can be engineered with a large sequence capacity, the success of engineered SYNV opens the possibility of delivering the CRISPR/Cas9 system into plant cells. Using the above finding as a starting point, Ma *et al.* successfully inserted *Streptococcus pyogenes* Cas9 (SpCas9) and gRNAs into the SYNV genome and used this engineered virus to knock out different genes in plants [3].

To achieve CRISPR/Cas9 genome editing with high efficiency, Ma *et al.* designed a SYNV tgtRNA (tRNA–gRNA–tRNA)–Cas9 cassette to ensure that both Cas9 and gRNA activities are preserved, and that endogenous tRNA processing enzymes precisely cleave the quadruple-fusion SYNV viral transcript to release authentic gRNA. They first tested this system by targeting the *GFP* gene in transgenic tobacco plants, and obtained 77 and 91% genome-editing efficiency, respectively, for two different targeted sites. They went on to target three endogenous tobacco genes, *PHYTOENE DESATURASE* (*PDS*), *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*), and *SUPPRESSOR of GENE SILENCING 3* (*SGS3*) by targeting conserved sites in each gene. PCR–restriction digestion (PCR–RE) assays showed that plant genome editing was achieved for all three genes with a mutagenesis frequency of 40–90%. They also tested the multiplex editing ability of virus-delivered CRISPR/Cas9 by designing multiple gRNAs for a single gene or for different genes in a tRNA–gRNA1–tRNA–gRNA2–tRNA configuration; their results show that multiplexed gRNA expression did not affect the efficacy of a single gRNA. Their study also confirmed that virus-delivered CRISPR/Cas9 generated transgene-free and virus-free genome-edited plants that stably pass the genome alteration on to subsequent generations [3]. This work confirms that SYNV-delivered CRISPR/Cas9 system can be used to edit individual genes in plants with high efficiency.

Concluding Remarks

The research performed by Ma and colleagues opens up a new strategy for genome editing in plants, and this will boost the rapid development and application of CRISPR/Cas9 genome-editing technology in both fundamental and applied fields – including gene function studies and crop biotechnological improvement. SYNV-mediated delivery of CRISPR/Cas9 is an easy way to deliver Cas9 nuclease and gRNAs into plants, and this can be performed in any laboratory, greenhouse, or even in the field with no additional requirement for instruments. However, the method developed by Ma *et al.* still requires plant tissue culture to finally obtain genome-edited plants because plant rhabdoviruses only rarely invade plant germline or meristem cells. Thus, SYNV-mediated genome editing only works efficiently in somatic cells, and these need to be isolated and cultured to regenerate an individual genome-edited plant. This limits the application of this newly developed technology because regeneration of whole plants is limited to a small range of genotypes in certain plant species. This technical barrier can be overcome through further research. First, it may be possible to identify a new negative-strand RNA virus that can host and deliver the CRISPR/Cas9 system while displaying good ability to enter plant germline or meristem cells. Many negative-strand RNA viruses are known to affect plants [7]; like SYNV, it is likely that some of these can be engineered to host the CRISPR/Cas9 system for delivery into plant cells, while displaying good capacity to infect germline or meristem cells. Once the CRISPR/Cas9 reagents are delivered into germline, meristem, or stem cells, CRISPR/Cas9 can target a specific DNA location to cut, edit, and regulate those DNA sequences for different purposes. Although it is currently not possible to deliver the CRISPR/Cas9 system into plant cells by positive-strand RNA or DNA viruses, in the future new ways might be discovered that permit these viruses to be engineered to host large DNA/RNA sequences without affecting their infectivity. Currently, the VIGS system has been used to deliver gRNA for genome editing *in planta*; it may be possible to modify the current VIGS system to host both Cas nuclease and gRNA for genome editing.

Although positive-strand RNA viruses and DNA viruses cannot deliver the entire CRISPR/Cas9 system owing to cargo size limitations, both have been used to deliver small gRNAs into transgenic plants carrying Cas9 nuclease and thereby achieve genome editing (e.g., [8]). However, all the genome-edited plants are transgenic because they carry the gene encoding Cas9. In addition, as part of these studies, it was discovered that gRNAs are unable to enter germline cells for genome editing, and the only successful study reported a very low genome-editing efficiency of 1 in 438 seedlings [9]. However, a recent study by Ellison *et al.* shows that genome-

editing efficiency can be significantly increased to 65–100% if the gRNAs are fused to a mobile RNA sequence, such as truncated *FLOWERING LOCUS T* (*FT*) [10]. This suggests that a mobile RNA sequence allows gRNAs to move between cells, thus enabling targeting of genomic sequences in meristem and germline cells. It may therefore be possible to fuse the Cas9/gRNA system with a mobile RNA sequence, and then use the corresponding engineered SYNV virus to infect plants, thus achieving genome editing completely independently of tissue culture (Figure 1). With these improvements, one can envisage editing a trait of choice in virtually any plant, be it in the field or elsewhere, to obtain transgene-free genome editing for biotechnological improvement of crops.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31772403) and the International Cooperation and Exchanges Project of Shaanxi Province (2018KW-047) to H.L. This work is also partially supported by NSF (#1658707) and the Cotton Incorporated (#15-770) to B.Z.

[1]College of Life Science, Northwest A&F University, Yangling, Shaanxi 712100, PR China
[2]Department of Biology, East Carolina University, Greenville, NC 27858, USA

*Correspondence:
bioche@nwafu.edu.cn (H. Liu) and zhangb@ecu.edu (B. Zhang).

https://doi.org/10.1016/j.tig.2020.08.002

References

1. Adli, M. (2018) The CRISPR tool kit for genome editing and beyond. *Nat. Commun.* 9, 1911
2. Bak, R.O. *et al.* (2018) Gene editing on center stage. *Trends Genet.* 34, 600–611
3. Ma, X. *et al.* (2020) Highly efficient DNA-free plant genome editing using virally delivered CRISPR–Cas9. *Nat. Plants* 6, 773–779
4. Yip, B.H. (2020) Recent advances in CRISPR/Cas9 delivery strategies. *Biomolecules* 10, 839
5. Cody, W.B. and Scholthof, H.B. (2019) Plant virus vectors 3.0: transitioning into synthetic genomics. *Annu. Rev. Phytopathol.* 57, 211–230
6. Wang, Q. *et al.* (2015) Rescue of a plant negative-strand RNA virus from cloned cDNA: insights into enveloped plant virus movement and morphogenesis. *PLoS Pathog.* 11, e1005223
7. German, T.L. *et al.* (2020) New technologies for studying negative-strand RNA viruses in plant and arthropod hosts. *Mol. Plant-Microbe Interact.* 33, 382–393
8. Hu, J. *et al.* (2019) A barley stripe mosaic virus-based guide RNA delivery system for targeted mutagenesis in wheat and maize. *Mol. Plant Pathol.* 20, 1463–1474
9. Ali, Z. *et al.* (2015) Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. *Mol. Plant* 8, 1288–1291
10. Ellison, E.E. *et al.* (2020) Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nat. Plants* 6, 620–624

Glossary

Expression quantitative trait locus (eQTL): a genomic locus that explains the variation in gene expression of nearby genes.
Insertion/deletion (Indel): an insertion or deletion of bases in the genome.
Minor allele frequency (MAF): frequency at which the second most common allele occurs in a given population.

Forum

Host Polymorphisms May Impact SARS-CoV-2 Infectivity

Patrick Brest,[1] Sadal Refae,[2,3,5] Baharia Mograbi,[1] Paul Hofman,[1,4] and Gerard Milano[2,*]

Based on a broad public database compilation, we support the hypothesis that germinal polymorphisms may regulate the expression of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cellular target itself and proteases controlling the process of its shedding or, conversely, its internalization. Consequently, a genetic influence on individual susceptibility to coronavirus disease 2019 (COVID-19) infection is strongly suspected.

General Background

In addition to the need for virus detection, evaluation of individual serological response [1], and biological analytical tools to manage COVID-19 on a population level, there is an urgent need to obtain objective information to identify at-risk individuals and to understand the marked variability in the severity of the disease in general, as well as in given populations. A current hypothesis is that SARS-CoV-2 clinical manifestations are governed by human genetics [2]. Thus, in this context, here we develop two complementary themes: (i) a more thorough examination of the membrane shedding of angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 cellular target, and its potential repercussion on virus propagation; and (ii) a description of the interindividual variability of the genes (SNPs) involved in ACE2 processing and their potential impact on the risk of contracting COVID-19.

ACE2 Expression and COVID-19

Chen *et al*. recently examined a large Genotype–Tissue Expression (GTEx) database and investigated the expression of *ACE2* in different human tissues [3]. The authors stressed that, counterintuitively, expression of the SARS-CoV-2 target was inversely related to certain risk factors, showing higher levels in Asian females compared with Asian males and a significant decrease in patients with type 2 diabetes mellitus. Globally, at a population level, there was a negative correlation between ACE2 expression and COVID-19 severity. Recent data provide evidence that ACE2 is effectively shed from membranes, a process that is fine-tuned at different levels [4] involving two cell membrane proteases: disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) and transmembrane protease serine 2 (TMPRSS2) [4]. More precisely, ADAM17 acts directly on ACE2