# Potential for gene editing in antiviral resistance

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Declarations of interest: none

Key points:

- Virus diseases significantly threaten agriculture
- Genetic resistance is the most effective strategy to combat virus diseases in crops
- CRISPR/Cas technology has revolutionised the field of genome editing and has already been successfully employed to generate the first wave of virus resistant plants
- Continued research into both the basic science of plant-virus interactions and also technological advances is needed to expedite the next generation of resistance genotypes
- Engagement between scientists and the public/governing bodies will be essential to ensure the successful, safe and ethical deployment of genome editing technologies in agriculture

**Abstract**

The discovery of CRISPR/Cas systems and their subsequent application in genome modifications and in gene expression control have fundamentally changed both basic and applied research. They have already been employed to generate novel virus resistance traits either by modifying host factors in the plant genome or by directly inducing targeted virus degradation. Here we summarise the latest developments in this field and discuss the potential applications and concerns around this technology.

## Introduction

The global population is predicted to reach 9 billion by the middle of this century [1] which will undoubtedly result in a variety of challenges that must be faced at the global scale. Food security is one such challenge that will become increasingly pertinent in years to come. Estimates suggest that over the period of 2005 to 2050 annual crop production worldwide will need to at least double to support the growing global population [2]. Reducing the crop losses incurred by plant diseases is therefore an important area of focus to ensure that crop production continues to meet the rising demand. Of the various causal agents of plant disease, viruses can cause considerable damage resulting in an estimated 10-15% reduction of global crop yields [3]. Moreover, the fast evolution rates of many viruses render them formidable pathogens that can be difficult to control by breeding.

Within the plant virology field, it is widely accepted that the deployment of genetic resistance is one of the most effective strategies to control virus diseases. This relies on identifying genetic loci associated with resistance to a given virus disease and introducing these alleles that confer resistance into the crop of interest. While many of these resistance alleles operate in a monogenic dominant manner it is noteworthy that, compared to the those identified for other plant pathogens, a disproportionally large number of loci associated with viral resistance behave in a recessive manner [4]. The majority of these recessive resistance loci have been characterised as genes encoding susceptibility factors (S-genes/S-factors) which are host factors required by the virus to complete its lifecycle within the host. Hence homozygous mutations at S-gene loci can result in very effective resistance if they prevent the usurpation of their gene products by the virus. A detailed discussion of natural resistance alleles and their modes of action

is beyond the scope of this review and hence we refer the reader to the following excellent reviews for further detail on this topic [4-6].

Traditionally the introduction of resistance alleles into crop varieties has been achieved through classical breeding, whereby the resistance loci are introgressed by crossing susceptible varieties with other cultivars or wild relatives bearing the resistance alleles. While such methods have been, and continue to be, widely used for crop improvement they suffer from several limitations. They are expensive in both time and money as multiple backcrosses to the parental crop variety are required to ensure that desirable crop traits are not lost through genetic segregation. Furthermore, linkage drag (genetic linkage between the resistance allele and undesirable loci) or breeding incompatibility (sexual barriers between the crop variety and the genotype containing the resistance allele) can significantly hinder the success of breeding programmes. The advent of technologies that allowed a more direct introduction of resistance alleles into crop varieties via genetic transformation offered practical solutions to some of limitations of classical breeding. Furthermore, in addition to introducing natural resistance alleles to crops via cis-genesis (introduction of foreign DNA that originates from the same or a closely related species) these technologies offer the possibility to create artificial resistance alleles that can be introduced to crops via trans-genesis (introduction of foreign DNA that originates from a distantly related species). This method of creating transgenic resistance alleles has often involved overexpressing genes from the viral pathogen itself to achieve a type of resistance referred to as pathogen derived resistance (PDR). In some cases, PDR is reached by the constitutive overexpression of fully functional viral proteins (for specific examples see [7]). However, in many cases PDR is attained by the transgenic expression of truncated, hence non-functional, viral genes that trigger an innate immune response to viral infection known as RNA silencing or RNA interference (RNAi). Consequently, it results in the production of short interfering RNAs (siRNAs) which then direct the plant's RNA-induced silencing complex (RISC) to silence or destroy the viral genome in a nucleotide sequence-specific manner [8]. Furthermore, artificial resistance alleles can be created by expressing a variety of non-viral proteins that disrupt viral function [9], or by triggering RNAi of virus genomes using transgenic expression of artificial short silencing RNAs known as microRNAs [10]. While the use of transgenesis has been demonstrated as an effective method for introducing virus resistance in crops (a very notable example is the production of

transgenic papaya that are resistant to *Papaya ringspot virus* [11]), strict regulations and lack of public acceptance prevents the deployment of this approach to create virus resistant crops in many parts of the world.

The last decade has seen a rapid expansion of the field of site-specific genome editing, largely resulting from the development of a biotechnological technique referred to as CRISPR/Cas technology [12]. This technology is based on an adaptive immune response of bacteria and archaea whereby sequences from invading phage or plasmids are integrated into Clustered Regularly Interspaced Short Palindromic Repeat (CRIPSR) loci. Transcription of these CRISPR loci, along with processing of the nascent CRISPR transcript, results in the formation of mature CRISPR RNAs (crRNAs) which can associate with Cas nuclease proteins and direct them to the invading nucleic acid through sequence complementarity and trigger cleavage. A biotechnological breakthrough came from the discovery that transgenic expression of Cas nucleases and modified versions of crRNAs could be used to direct site-specific cleavage of DNA in eukaryotic cells, triggering the error-prone non-homologous end joining (NHEJ) DNA repair pathway, hence offering a mechanism for inducing mutations at specific genomic loci [13]. The key advantage of this technology is that the targeting of the nuclease relies on an RNA molecule and hence the system is much cheaper and more versatile than previous genome editing technologies such as Zinc-Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALEN) that require nucleases to be custom-designed for each DNA target. In the first generation of CRISPR/Cas technology the Cas9 DNA endonuclease from *Streptococcus pyogenes* was used in conjunction with a synthetic crRNA to direct knock-out mutations at target loci. In recent years the 'CRISPR toolbox' has greatly expanded from the exploitation of the natural diversity of Cas proteins including Cpf1 (Cas12a) and the RNA targeting Cas13a, and also from bioengineering natural Cas proteins to confer new functions. This has expanded the utility of CRISPR/Cas technology for a variety of purposes including: non-random mutations through the development of base-editing [14,15], knock-in mutations (introduction of foreign DNA at specific sites) [16] and CRISPR/Cas-directed RNA cleavage [17]. CRISPR/Cas technology hence offers tantalising new prospects for engineering viral resistance in crop plants (Figure 1). Recently, several research groups have begun to demonstrate the power of this new technology for this purpose using a variety of different strategies.

**Direct virus targeting using CRISPR/Cas**

CRISPR/Cas9 evolved as an RNA-programmable DNAse to cleave and subsequently destroy bacteriophage DNA. Thus not surprisingly, the first antiviral application of the CRISPR/Cas technology in plants was to control DNA viruses. Around the same time, three independent research groups reported similar strategies to transgenically introduce Geminivirus resistance in *Nicotiana benthamiana* using CRISPR/Cas9 [18-20]. In each of these reports, transgenic expression of Cas9 was used along with the delivery of a crRNA directed against the viral DNA genome to create resistant plants. Recently, similar approaches have been adopted to create transgenic plants with resistance to RNA viruses by targeting the viral RNA genomes using Cas ribonucleases such as Cas13a [21,22] and FnCas9 [23]. While these reports demonstrate that CRISPR/Cas systems can be reprogrammed to create novel, transgenic resistance alleles there remain a number of concerns around the utility of these methods in an agricultural context. First, there is the concern that the programmed nucleases may target and cleave host DNA or RNA bearing a close enough homology to the crRNA designed against the virus. While this issue of 'off target' effects is of a lower concern in plants compared to other eukaryotic kingdoms [24] it remains a significant concern. To address this issue, Ji and colleagues recently developed a virus-inducible CRISPR system to ensure that the Cas nuclease is only expressed upon virus infection [25]. A second concern is that these resistance methods that rely on cleavage of the viral genome are inherently mutagenic and thus may serve to accelerate the evolution of new viral strains which could then overcome the resistance. More worryingly still, CRISPR-directed cleavage of the virus genomes during mixed infections could stimulate genome recombination resulting in the emergence of new viral strains that could potentially become even more pathogenic. However, while these concerns have been previously raised [26] no research has yet been conducted to rigorously test these potential risks. Finally, the examples listed in this section all rely on the persistent expression of transgenes and as such will be limited by the same, if not more, scrutinous legislation that prevents the use of these technologies for agriculture throughout large parts of the world.

**Using CRISPR technology to create resistance alleles by mutation of host susceptibility factors**

An alternative approach to generate novel resistance alleles using CRISPR technologies is to mutate host S-factors such that they can no longer be co-opted by the virus. Contrary to the examples in the previous section, this approach does not require the continuous expression of a CRISPR/Cas transgene for the resistance to be maintained and hence the transgene can be removed by genetic segregation once the desired mutation at the S-gene locus has been created. The efficacy of this strategy was demonstrated by two proof-of-concept studies whereby CRISPR/Cas9-induced knock-out mutations in S-factors were used to engineer resistance to RNA viruses in *Arabidopsis thaliana* [27] and *Cucumis sativus L.* [28]. Both of these studies targeted members of the eukaryotic initiation factor (eIF) gene family which are required for the translation of polypeptides from virus RNA molecules. Knock-out mutations for single genes within this family can be tolerated in some cases due to functional redundancy between eIF paralogues in plants. However, certain RNA viruses have evolved to use multiple eIF paralogues to translate their genomes and hence knock-out mutations at more than one locus would be required to achieve a virus resistance phenotype in these cases. While the aforementioned studies provided a proof-of-concept for delivering this strategy using CRISPR technology, there remain several drawbacks that will need to be addressed before this technique can be applied confidently in an agricultural context. First, while both studies [27,28] reported no growth defects of the eIF mutant plants, which were grown under laboratory conditions, it is likely that under certain natural growth conditions the knock-out mutations could result in a decrease in plant fitness. Secondly, it has been previously demonstrated that viral strains can evolve to overcome the resistance (a phenomenon known as 'resistance breaking') associated with eIF loss-of-function mutations by co-opting alternative, functional eIF paralogues [29]. Using knock-out mutations restricts the spectrum of resistance (the range of viruses the resistance is effective against) that can be achieved in one plant because knock-out mutations for more than one paralogue of eIFs can be lethal to the plant. Potential solutions for all three of these limitations were proposed recently in an excellent review [30]. The authors posited that instead of creating complete knock-out mutations for eIF, subtle mutations that disrupt the interaction of the viral genome with the eIF should be employed to engineer resistance alleles that closely resemble natural resistance alleles. As such alleles have been naturally selected multiple times, it is unlikely that the introduction of analogous mutations by artificial means would result in a significant fitness cost to the plant. Furthermore, it was recently demonstrated (albeit using

classical transgenic technology rather than CRISPR/Cas gene editing) that such subtle mutations that copy natural S-factor mutations can be stacked in one plant to achieve resistance to multiple viruses that have evolved specificity for alternative eIF alleles [31]. Such stacking would not be possible using knock-out mutations due to plant lethality. Furthermore, resistance gene stacking is likely to significantly reduce the likelihood of resistance breaking. Encouragingly, two recent studies reported the use of CRISPR/Cas9 and CRISPR-nCas9-cytidine deaminase base editor to engineer resistance to an RNA virus in rice and in *Arabidopsis* respectively, by creating a subtle eIF mutation that disrupts the viral usurpation of this host factor without completely ablating its function [32**,33**]. In order to generate the most effective resistance alleles, basic research is still required to better understand the molecular interactions that exist between specific virus species/strains and their hosts. This was recently exemplified by a study showing that long deletion mutations in the coding sequence of tobacco *eIF4E-1* conferred a more effective and durable viral resistance than frameshift mutations, early stop-codon mutations, or small exonic deletions. Moreover, a dominant mutation at a paralogous locus, *eIF4E-2*, was found to enhance the durability of the virus resistance when combined with the large deletion mutation at *eIF4E-1* indicating that effective strategies to engineer virus resistance may require modifications at multiple loci [34]. Hence, a two-pronged approach to gain deeper insights into the molecular interactions between viral proteins and plant S-factors, alongside technological research to improve methods for generating precise mutations *in planta* will hopefully pave the way for a new generation of durable and broad-spectrum resistance alleles that can be generated *de novo* in crops.

**Challenges and outlook**

Our knowledge is still limited about plant-virus interactions including viral-host factors. Identifying and characterising resistance genes in varieties and wild species can increase the plethora of single nucleotide polymorphisms (SNP) known to be associated with resistance or reduced virus titre and subsequently inform gene editing efforts to attain virus resistance in staple and emerging crops.

One of the major limitations to the use of CRISPR technology in agriculture is inefficient plant transformation and regeneration technologies. *Agrobacterium*- and particle bombardment-mediated plant transformation methods have been established

for more than three decades, yet they remain suboptimal due to the low frequency of stably transformed cells, low delivered DNA titres for promoting efficient homologous recombination, and inefficient tissue culture for recovering transgenic plants from engineered cells and tissues [35]. Beside further improving, optimising and automating the plant transformation and regeneration protocols, novel approaches are required to enable the direct modification of germline or meristematic tissue *in planta*. This would overcome the species- and variety-dependent constraints that are currently associated with tissue culturing and regeneration processes, and at the same time could simplify and accelerate the generation of gene-edited lines.

Another limitation to CRISPR-mediated crop improvement is the low efficacy of precision gene engineering. CRISPR/Cas has been extensively used to produce loss-of-function alleles by introducing random frame-shift mutations and subsequently early stop codons at the targeted loci. However, gene knockouts can be associated with trade-offs including developmental and yield penalties. To overcome this issue, gene-editing needs to become predictable and efficient without affecting the original function of targeted genes. This approach requires a template for the DNA repair machinery that is either present in the cell or co-delivered with the CRISPR reagents. The current technologies are ineffective due to low level of homologous recombination and inefficient directed DNA repair. Increasing the simultaneous delivery of CRISPR nuclease and DNA templates, suppressing NHEJ, as well as improving the efficacy of DNA-base editors and DNA-templated repair mechanisms, are likely to contribute to the development of designer virus resistant plants with nucleotide precision edits. However, even with predictable and efficient gene-editing platforms it will be necessary to stringently test novel genotypes as fitness penalties could potentially occur for any type of genomic modification.

Gene edited plants harbour a genetic modification. If this modification involves the introduction of large exogenous DNA to confer a new trait, such as resistance to a particular virus, it is evident that both the process and the product fall under GMO regulations [36]. However, if the precise modification is restricted to a single nucleotide or to short stretches of nucleotides that mimic natural resistant alleles in other species, especially when DNA-free genome editing methods are used (including, but not restricted to the direct delivery of RNPs and base editors), then it is likely to trigger fewer regulatory and safety concerns [37*]. In addition, these technologies do not leave

traces behind. Therefore it is impossible to determine whether a point mutation occurred naturally or was generated by CRISPR or chemical/radiation mutagenesis. Thus, gene-edited products will pose a significant challenge to international trade, market surveillance and regulation. Consequently, the global harmonisation of genetic modification regulations is required to ensure the safe release and trade of gene-edited crops.

The gene editing technology is simple, can massively accelerate crop research and breeding, and in most applications it is non-traceable: nearly ready to take over the world by storm. Are we prepared?

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**Figure legend**

Schematic overview of the currently available gene-editing tools, the predicted gene-editing outcomes and the engineered virus resistance traits. The solid and dashed arrows indicate published and potential applications, respectively. ssODN, single-stranded oligodeoxynucleotides.

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