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CRISPR editing-mediated antiviral immunity: a versatile source of resistance to combat plant virus infections

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Virus infection poses a constant threat on global crop productivity. It is estimated that virus diseases account for at least 10% of crop losses worldwide. Development of resistant crops using naturally evolved resistance genes has been a main measure for controlling plant virus epidemics. However, isolation of plant resistance genes is costly and time-consuming. Furthermore, most plant resistance genes are pathogen race-specific, with very few of them being broadly specific (Langner et al., 2018). Consequently, artificial immunity with engineered resistance to virus infections has received substantial research, which includes pathogen-derived resistance, introducing single or stacked transgene into crops, and RNA interference (Shepherd et al., 2009). However, these strategies have so far achieved limited success due to narrow availability and/or fitness costs on host plants. Therefore, it is urgent to develop novel and more efficient strategies to combat plant virus infections. Recent breakthrough of CRISPR/Cas-based DNA and RNA editing tools provides a promising direction for engineering artificial immunity to plant viruses. There are now increasing reports demonstrating that CRISPR/Cas systems can be harnessed to develop antiviral immunity in plants with high efficiency and broad specificity (Table S1 in Supporting Information).

In this insight, we first outline the DNA and RNA editing tools available for developing CRISPR-mediated antiviral immunity, followed by a comparison of the two main strategies in this area of research. Lastly, current concerns and future perspectives on CRISPR-mediated antiviral immunity are discussed.

CRISPR/Cas-based DNA and RNA editing tools

CRISPR/Cas systems, originating from bacteria and archaea, can eliminate the mobile genetic elements such as bacteriophages and plasmids in an "immunity-memory-immunity" manner. Plenty of CRISPR/Cas systems with different structures exist in the nature, which differ from each other in the adaptive immune process. CRISPR/Cas systems have been divided into class I (type I, type III and type IV) and class II (type II, type V and type VI) (Makarova et al., 2018). Currently, most DNA and all of the RNA editing tools come from class II systems due to their simplicity.

For DNA editing tools, the most widely used system is the CRISPR/SpCas9 system from *Streptococcus pyogenes*. Naturally, it consists of tracrRNA, crRNA and Cas9, which was engineered as a two-component system with a single-guide RNA (sgRNA) that directs Cas9 to recognize and cleave the target sequence with a NGG PAM (Garcia-Doval and Jinek, 2017). Current reports on CRISPR-mediated antiviral immunity have mainly used CRISPR/SpCas9 system. However, limited PAM range and off-target effect have re-

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stricted the application of CRISPR/SpCas9 (Garcia-Doval and Jinek, 2017).

The CRISPR/Cas12a (CRISPR/Cpf1) and CRISPR/Cas12b (CRISPR/C2c1) are derived from type V systems. Cas12a and Cas12b have a conserved RuvC domain with Cas9 but a distinct Nuc domain, and they prefer T-rich PAMs. The guide segments of both systems are longer than that of CRISPR/SpCas9, which thus lead to higher specificity (Makarova et al., 2018). Although both systems have been successfully applied to modify nuclear genes with expanded target range and higher specificity, there are still no reports on employing them to achieve antiviral immunity.

For RNA editing tools, the Cas9 from *Francisella novicida* (FnCas9) was firstly reprogrammed to target RNA substrate. Although it cannot cleave RNA, the CRISPR/FnCas9 system has been applied to inhibit RNA viruses in both human and plant cells by blocking viral translation and replication processes (Zhang et al., 2018).

The type VI CRISPR/Cas13 systems may provide another family of RNA editing candidates (Makarova et al., 2018). Unlike Cas9 and Cas12, the Cas13 protein is guided by a 28-nt crRNA and finds its target by recognizing a non-G protospacer flanking site. However, upon activation, some Cas13 proteins can degrade not only the target RNA but also collateral RNAs, which may limit their application in RNA editing. Recent studies reported LwaCas13a (*Leptotrichia wadei* Cas13a) and engineered CasRx (*Ruminococcus flavefaciens*) can specifically cleave target RNA transcripts without collateral activity (Garcia-Doval and Jinek, 2017). The lower off-target property of these two systems makes them potentially useful for RNA editing and antiviral immunity.

Two strategies for engineering CRISPR-mediated antiviral immunity

Strategy A achieves antiviral immunity by cleaving and/or interfering viral nucleic acids directly to inhibit viral gene expression and genome replication (Figure 1A).

Geminiviruses are circular single-stranded DNA (ssDNA) viruses causing billions of dollars of economic losses in global crop production. After transmitted to host cells by insect vector, the ssDNA of geminivirus is converted to a double-stranded DNA (dsDNA) form, from which new ssDNA is accumulated by rolling-circle replication. We and Balts et al. are the first to employ CRISPR/Cas9 to confer geminivirus resistance by cleaving the viral dsDNA in plant cells (Baltes et al., 2015; Ji et al., 2015). By introducing highly efficient sgRNA-Cas9 expression vectors into *Nicotiana benthamiana* or *Arabidopsis* plants, transgenic plants showed strong resistance to *bean yellow dwarf virus* or *beet severe curly top virus* (BSCTV) with significantly reduced viral accumulation and symptom production. A following research proved that CRISPR/Cas9 could be engineered to target and inhibit the accumulation of begomoviruses efficiently in *Nicotiana benthamiana* plants (Ali et al., 2015). Subsequent studies have vindicated the simplicity and effectiveness of this antiviral strategy to diverse geminiviruses (Ali et al., 2016; Ji et al., 2018; Kis et al., 2019; Liu et al., 2018; Tashkandi et al., 2018). Significantly, it has been found that a single sgRNA targeting the conserved viral region can confer resistance to multiple viruses (Ali et al., 2015). Thus, the antiviral immunity based Strategy A represents an efficient and broad-spectrum resistance for combating geminivirus infections.

RNA viruses, being the majority of plant viral pathogens, account for 90% crop losses elicited by virus diseases. Initial research showed that CRISPR/FnCas9 could be engineered to inhibit plant RNA viruses with some success (Zhang et al., 2018). But the main advance comes from the use of CRISPR/LshCas13a. Aman and coauthors showed that CRISPR/LshCas13a could be used to engineer resistance to *Turnip mosaic virus* (TuMV) in *Nicotiana benthamiana* with high specificity (Aman et al., 2018). Subsequently, this RNA editing system was successfully used to achieve resistance to *Tobacco mosaic virus* in *Nicotiana benthamiana* and *Southern rice black-streaked dwarf virus* in rice (Zhang et al., 2019). Besides, Zhan and colleagues generated *Potato virus Y*-resistant potatoes with CRISPR/LshCas13a, and noted a correlation between the level of resistance and the degree of Cas13a/sgRNA expression level (Zhan et al., 2019). These reports illustrate the potential of CRISPR/Cas13a in developing antiviral immunity for controlling plant RNA viruses.

Strategy B achieves CRISPR-mediated antiviral immunity by knocking out plant susceptibility genes, which are host factors hijacked by viruses to facilitate their gene expression and genome replication (Figure 1B), through CRISPR editing.

Many single-stranded positive sense RNA viruses require host plant eIF4 complex to express their proteins. In eIF4F complex, eIF4E with their isoforms, eIF(iso)4E and novel cap-binding protein (nCBP), and eIF4G are essential for binding capped mRNAs and recruiting the other translation initiation factors (Chandrasekaran et al., 2016; Pyott et al., 2016). Chandrasekaran et al. mutated *eIF4E* in cucumber using CRISPR/Cas9, and found that the resultant lines exhibited strong and broad-spectrum resistance to *Cucumber vein yellowing virus*, *Zucchini yellow mosaic virus* and *Papaya ring spot mosaic virus-W*, with no apparent fitness cost on plant growth and development observed (Chandrasekaran et al., 2016). A similar finding was made when *Arabidopsis eIF(iso)4E* was knocked out, which yielded high resistance to TuMV (Pyott et al., 2016). *Rice tungro spherical virus* resistant rice was generated by targeting *eIF4G* (Macovei et

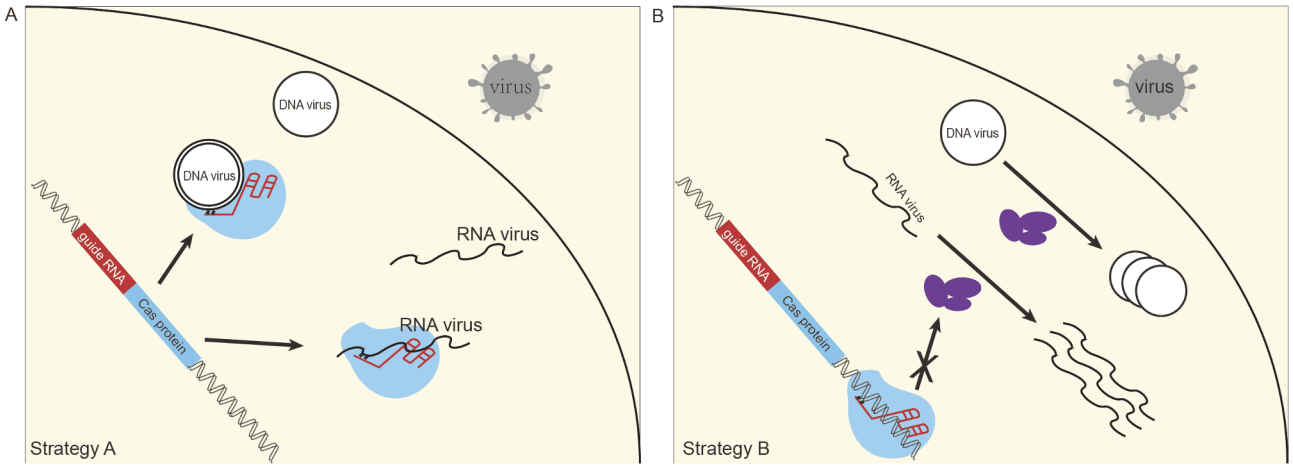

Figure 1 The strategies for developing CRISPR-mediated antiviral immunity to combat plant viruses. Strategy A (A) is achieved by cleaving and/or interfering viral nucleic acids directly, while Strategy B (B) knocks out susceptibility genes to repel virus infection. The red boxes represent guide RNA. The blue boxes represent Cas protein. For Strategy A, the Cas protein can be Cas9, Cas12a or Cas12b for cleaving DNA viruses and Cas13 for cleaving RNA viruses. For Strategy B, the Cas protein can be Cas9, Cas12a or Cas12b for modifying susceptibility genes. The purple complex represents host factors required for virus gene expression and genome replication.

al., 2018). Simultaneous mutation of *nCBP-1* and *nCBP-2* genes in cassava led to tolerance to *Cassava brown streak virus* (Gomez et al., 2019). These examples demonstrate clearly that disabling susceptibility genes by CRISPR editing is a highly efficient approach to engineer potent and broad-spectrum antiviral immunity to plant RNA viruses.

Concerns and future perspectives

Although CRISPR-mediated antiviral immunity is promising and powerful, several concerns need to be addressed in order to realize its full potential. One major concern for both strategies is off-target effects. Since both strategies need to generate transgenic plants, overexpression of CRISPR/Cas systems in transgenic cells can provoke off-target effects at DNA or RNA levels. Recent studies on CRISPR-mediated resistance to geminiviruses have highlighted that evolving viral escapees can pose a biosafe risk. Ali et al. showed that cleaving the coding region of different DNA viruses could induce new mutations that enabled the viruses to overcome CRISPR-mediated antiviral immunity (Ali et al., 2016). Metha et al. identified a mutant of *African cassava mosaic virus* in the plants expressing CRISPR/Cas9, which could replicate depending on wild-type virus (Mehta et al., 2019). Finally, the regulation of CRISPR/Cas-modified plants is another concern. This is particularly relevant to the development of Strategy A since insertion of exogenous DNA to host plant genome cannot be avoided.

Several options can be considered to address the above concerns. For solving the problem of off-targeting, development of virus-inducible genome editing system is a top choice. Using this approach, we have succeeded in developing strong antiviral immunity to the geminivirus BSCTV with no off-target mutations (Ji et al., 2018). This approach may also be useful to avoid off-target editing in engineering antiviral immunity to plant RNA viruses. For Strategy B, careful design of target sgRNAs by software with low off-target scores can decrease unwanted side effects. Delivery of CRISPR/Cas9 reagents in the form of ribonucleoproteins (RNPs) can be adopted to eliminate host susceptibility genes with low off-target effects (Ran et al., 2017). Furthermore, engineered high-fidelity Cas9 and alternative nucleases such as CRISPR/Cas12a and CRISPR/Cas12b, which combine high editing efficiency and enhanced target specificity, may be used to minimize undesired mutations. To avoid the generation of escapees, cleaving the viral genome by large fragment deletion or using several sgRNAs from different locations of the viral genome are worthy approaches. Multiplex CRISPR editing methods, well established in plants (Shen et al., 2017), can be employed to simultaneously target several different sites of a plant viral genome. In addition, novel DNA editing tools such as CRISPR/Cpf1, which can cleave outside the target site at a limited range, may be used to produce CRISPR-mediated antiviral immunity while delaying the generation of viral escapees. As for the concern on transgene insertion in crop genome, this may be lessened for non-food species such as cotton. For eliminating susceptibility genes, CRISPR/Cas RNPs can be utilized to achieve the goal without transgene insertion in plant genome.

In conclusion, CRISPR-mediated antiviral immunity is emerging as an effective, broad-spectrum and versatile source of resistance for combating plant virus infections. As genome editing science is advancing rapidly and more and more CRISPR/Cas systems with novel attributes are characterized (Jiao and Gao, 2017), the prospects of refining CRISPR-mediated antiviral immunity to specific viruses, or to all of the viruses attacking a given crop, are high. Con-

sequently, CRISPR-mediated antiviral immunity will play an important role in the control of plant virus diseases in the future.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Table S1 The reports on generating virus resistant plants by different CRISPR/Cas systems

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