Development of CRISPR/Cas9 mediated virus resistance in agriculturally important crops

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ABSTRACT
Clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) system of targeted genome editing has already revolutionized the plant science research. This is a RNA guided programmable endonuclease based system composed of 2 components, the Cas9 nuclease and an engineered guide RNA targeting any DNA sequence of the form N20-NGG for novel genome editing applications. The CRISPR/Cas9 technology of targeted genome editing has been recently applied for imparting virus resistance in plants. The robustness, wide adaptability, and easy engineering of this system has proved its potential as an antiviral tool for plants. Novel DNA free genome editing by using the preassembled Cas9/gRNA ribonucleoprotein complex for development of virus resistance in any plant species have been prospected for the future. Also, in this review we have discussed the reports of CRISPR/Cas9 mediated virus resistance strategy against geminiviruses by targeting the viral genome and transgene free strategy against RNA viruses by targeting the host plant factors. In conclusion, CRISPR/Cas9 technology will provide a more durable and broad spectrum viral resistance in agriculturally important crops which will eventually lead to public acceptance and commercialization in the near future.

INTRODUCTION

Plant viruses infect many agriculturally important crops, from cereals to vegetables limiting the crop yield and posing a serious threat to the food security for feeding the increasing world population.1,2 The best possible solution is to improve the resistance of host plants against the plant viruses which would protect the food crops from the damage. Next Generation Sequencing technology has made easy discovery and detection of variability of viruses and computational biology also helped in understanding plant-virus interactions to find potential targets for development of virus resistant plants.3 But still the virus resistance durability in improved plants is the major drawback because of the diversity and rapid evolution in viruses. The development of plants having resistance against viruses is considered to be the most in exhaustive approach to check the swift evolution of plant viruses for the prosperity of economy and environment. The CRISPR (clustered regularly interspaced palindromic repeats)/CRISPR-associated 9 (CRISPR/Cas9) system has emerged as a promising tools for plant genome engineering.4,5 It has become a simple, most user friendly and efficient, precise genome editing tool for development of genetically edited crops.1,6,7 It has become most promising and extremely versatile tool for crop improvement for providing sustainable productive agriculture for better feeding of rapidly growing population in a changing climate.1,6,7 The CRISPR/Cas9 system is a RNA guided programmable endonuclease based technology composed of 2 components, the Cas9 nuclease and an engineered guide RNA targeting any DNA sequence of the form N20-NGG used for novel genome editing applications in many organisms including plants.4,6,7 Recently many studies reported CRISPR/Cas9 mediated virus resistance development in the plants with promising resistance durability.1,8,9 In this review we have summarized the current status and future prospects of CRISPR/Cas9 mediated virus resistance development.
in plants by direct cleaving of the viral genes and/or by deleting the host plant factors required for virus cycle to generate transgene free virus resistant plants. Delivery of the preassembled Cas9/gRNA ribonucleoprotein complex for development of virus resistance in any plant species have been prospected as Novel DNA free genome editing technique.

The Cas9/gRNA system enables precise genomic modifications in many different organisms and this has been recently used extensively in plants for genome editing.6,10 The CRISPR/Cas9 technology offers additional advantage over RNA interference (RNAi) or artificial microRNAs (amiRNAs) for engineering virus resistance in plants by disruption of essential viral genes instead of silencing those genes at RNA level.10,11 In addition, the potential off-target effects of RNAi and viral resistance durability of amiRNA-mediated silencing could also be prevented by sequence-specific antiviral strategies of the CRISPR/Cas9 technology.10,11 The RNA guided endonuclease (Cas9) creates targeted double-stranded breaks within a 20nt short sequence provided by the complementary guide RNA which leads to disruption of the target by indels in the host DNA repair. This CRISPR/Cas9 targeted genome editing system is used for novel applications in plants to impart resistance to Geminiviruses, a damaging family of DNA viruses by desired gene disruption.9,10

The CRISPR approaches to make virus resistant plants

The CRISPR/Cas9 technology has recently become a novel antiviral tool for plants.10 The novel application of CRISPR/Cas9 technology for genome editing used to combat viral infection in plants to destruct invading viral DNA for virus resistance development in plants was first reported in 2015 by 3 independent communications; 2 in Nature Plants by Ji et al.12,13 & Baltes et al.13 followed by one in Genome Biology by Ali et al.14 against geminiviruses. Then after many research groups started testing this and have reported the feasibility of viable virus resistance development in different plants using CRISPR/Cas9 technology.1

The reports of CRISPR/Cas9 mediated virus resistance development in plants can be basically divided considering 2 broad strategies used. The first approach targets the viral factors for viral genome editing in viruses and the second approach targets the host plant factors responsible for the viral cycle for plant genome editing. The applications of CRISPR/Cas9 mediated virus resistance in plants have been so far limited mainly to model species demonstration like Tobacco and Arabidopsis targeting the viral genes responsible for replication (Table 1).

Targeting viral genes: A highly efficient GM approach for DNA viruses

Four recent reports of CRISPR/Cas9 mediated virus resistance demonstrated the applicability of this technology as antiviral weapon for plants against geminiviruses.12-15 These are summarized in Table 1 and schematic representation is presented in Fig. 1. CRISPR/Cas9 system have been used to specifically target the dsDNA of a geminivirus by gRNA to inhibit virus replication by disruption of the essential replication genes. Ji et al.12 demonstrated the CRISPR/Cas9 mediated virus resistance in Nicotiana benthamiana against the geminivirus, beet severe curly top virus (BSCTV).

Table 1. Applications of CRISPR/Cas9 mediated virus resistance in plants.

| Virus/viruses | Plant | Target (Viral/host) | GM/Transgene free | References |
|---------------|-------|---------------------|-------------------|------------|
| BSCTV         | N. benthamiana and A. thaliana | IR, CP, and Rep | GM | Ji et al., 201512 |
| BeYDV         | N. benthamiana | LIR and Rep/RepA | GM | Baltes et al., 201513 |
| TYLCV, BCTV, and MeMV | N. benthamiana | IR, CP, and Rep | GM | Ali et al., 201514 |
| CLCuKoV, TYLCV 23, TYLCsv, MeMV, BCTV-Logan, BCTV-Worland | N. benthamiana | IR, CP, and Rep | GM | Ali et al., 201615 |
| TuMV          | A. thaliana | Host factor eIF(iso)4E | Transgene free | Pyott et al., 201616 |
| CVYV, ZYMV, and PRSMV | Cucumis sativus | Host factor eIF4E | Transgene free | Chandrasekaran et al., 201617 |

Genetically Modified (GM), Beet severe curly top virus (BSCTV), Bean yellow dwarf virus (BeYDV), Tomato yellow leaf curl virus (TYLCV), Beet curly top virus (BCTV), Merremia mosaic virus (MeMV), Cotton leaf curl Kochrana virus (CLCuKoV), Tomato yellow leaf curl Sardinian virus (TYLCsv), Turnip mosaic virus (TuMV), Cucumber vein yellowing virus (CVYV), Zucchini yellow mosaic virus (ZYMV), Papaya ring spot mosaic virus (PRSMV), intergenic region (IR), coat protein (CP), replication associated protein (Rep), long intergenic region (LIR). (Modified from Zaidi et al., 2016).
severe curly top virus (BSCTV), by introducing mutations at the viral target sequences. They developed stable transgenic Arabidopsis and N. benthamiana plants by overexpressing Cas9 and gRNAs which showed high resistance to virus infection. They also correlated Cas9 and gRNA expression with the levels of virus suppression and reported higher level of Cas9 expression resulted in no obvious viral symptoms. Baltes et al., targeted the replication initiator protein (Rep) gene of bean yellow dwarf virus (BeYDV) using CRISPR/Cas9 system in transgenic N. benthamiana plants and introduced mutations which resulted in virus resistance.

Ali et al. also validate the applicability of the Cas9/gRNA system for virus resistance development in plants by targeting the viral Rep, coat proteins genes and the conserved intergenic region (IR) in N. benthamiana plants against the Tomato yellow leaf curl virus (TYLCV), Beet curly top virus (BCTV) and Merremia mosaic virus (MeMV). The gRNA targeting the IR region having origin of replication could provide broad-spectrum geminivirus resistance against TYLCV, BCTV and MeMV in N. benthamiana plants overexpressing the Cas9 protein. Further, it was investigated that targeting the open reading frames (ORFs) of geminiviruses results in viral variants which could evade the resistance and capable of replication and systemic movement in plants. But targeting the conserved IR region, resulted in the broad spectrum high virus interference against multiple viruses without viral variant escapes, so providing a strategy for broad and durable resistance in N. benthamiana plants. Iqbal et al., proposed the utility of CRISPR/Cas9 system against cotton leaf curl disease (CLCuD) in cotton plants by in silico designing of multiple gRNAs targeting CLCuD-associated begomovirus along with the associated DNA-satellites as a broad spectrum plant virus resistance strategy.

Although, all of these studies proved the applicability of targeting the viral genome with Cas9/gRNA system as a broad spectrum and durable resistance strategy against the plant viruses, but there is a need of generation of stable transgenic plants overexpressing the Cas9 protein to impart durable resistance. That will again raise the never ending ethical issues of genetically modified crops and in addition overexpressing Cas9 could result in off target mutations in the plants also.

**Targeting Plant’s genes: Transgene free approach for RNA viruses**

To create resistance to RNA viruses, instead of targeting the virus genome, the CRISPR/Cas9 system can be used to target the plant genes responsible for viral infection to impart durable virus resistance. Plant RNA viruses require host factors like eukaryotic translation initiation factor (eIF) to maintain their life cycle. Many plant genes, including eIF4E and eIF(iso) 4E, have been identified as recessive resistance alleles for conferring resistance to potyviruses in different plants. As summarized in Table 1 and schematic representation is presented in Fig. 2. The first report of non-transgenic virus resistance plants was published in 2016, where the CRISPR/Cas9 technology have been used for targeted genome editing of the cucumber plants by creating mutations in the eIF4E gene.
gene, which results in development of resistance against 3 economically important cucumber viruses of the Potyviridae family, Cucumber vein yellowing virus (CVYV), Zucchini yellow mosaic virus (ZYMV) and Papaya ring spot mosaic virus-W (PRSV-W). Homozygous non-transgenic cucumber plants with the disruption of the eIF4E gene were screened by backcrossing after segregation in T3 generation showed virus resistance. In another study, knock out mutations created in the eIF(iso)4E gene using CRISPR/ Cas9 technology in Arabidopsis thaliana resulted in transgene free virus resistance to Turnip mosaic virus (TuMV). So, by using this strategy, heritable and homozygous mutations could be segregate out from Cas9 construct to get the transgene-free genetically edited crops in self-pollinating species to obtain Potyvirus resistance without transgenes. This approach will also result in broad spectrum and durable resistance against RNA viruses because knock out of gene will result in complete absence of host protein, instead of silencing at RNA level but further analysis over more generations will require to prove the same.

**DNA free approach of Cas9/gRNA-RiboNucleoProtein mediated targeting**

With the development of novel delivery methods for CRISPR/Cas9 technology Woo et al., developed a DNA-free genome editing method in plants by delivering the preassembled CRISPR-Cas9 ribonucleoproteins. The DNA free genome editing in plants will produce complete non transgenic plants and alleviate the GM crops related regulatory concerns. The purified Cas9 protein and guide RNA were preassembled into the Cas9/gRNA complex and then transfected into plant protoplasts (Arabidopsis thaliana, tobacco, lettuce and rice; bread wheat and (Grapevine & Apple) which resulted in successful targeted mutagenesis as that of naturally occurring genetic variation.

Another study reported biolistic delivery of Cas9/gRNA complex into maize embryos and demonstrated DNA free gene mutagenesis. This DNA free genome editing strategy could be used for development of virus resistance in plants by targeting viral genome and/or the plant’s genes against DNA and RNA viruses in the agriculturally important crops as depicted in the Fig. 3. The complete DNA free genetically edited virus resistant crops will also may not require any regulatory clearance and speed up the process of crop improvement as parental phenotype will be modified only for the targeted gene without changing the other characteristic aspects.

**Future outlook**

Although novel applications of CRISPR/Cas9 mediated virus resistance development in plants have revolutionized the crop improvement in just few years. Future development and commercialization of this technology of plant genome editing for crop improvement will require further analysis over more generations in the changing climate. CRISPR/Cas9 mediated virus resistance can be developed in any plant species with well-established genome sequences against any virus and/or multiple viruses (Gemini-viruses and Potyviruses) by using the CRISPR- P 2.0 online tool of gRNA design and validation. This technology has opened new ways for studying plant-pathogen interaction and designing broad spectrum durable resistance against viruses. Novel host factors can be identified for CRISPR/Cas9 mediated editing which interacts with the virus proteins using molecular dynamic studies of computational biology to provide broad spectrum resistance against rapidly evolving viruses. DNA free editing with avoiding off-target effects by using direct delivery of Cas9/gRNA ribonucleoprotein complex could further simplify and advanced through nanoparticles mediated delivery approach. The DNA and/or transgene free editing for
virus resistance in plants will be less regulated compared with transgenics and have more public acceptance and will be of prime importance to commercialize and large scale cultivation. In conclusion, this approach will result in a more durable and broad spectrum viral resistance in agriculturally important crops which will eventually lead to commercialization. Hence, utilization of CRISPR/Cas9 technology may provide an efficient and publicly acceptable method for crop improvement against viruses in the near future.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by Amity University, India.

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