PROSPECTS OF TOBACCO RATTLE VIRUS (TRV) AS A VECTOR

Abstract

Tobacco Rattle Virus is a plant-borne virus of the genus Tobravirus, which has a wide range of host species. Its unique characteristics and versatility have made it a highly sought-after tool in plant sciences and biotechnology. TRV is characterized by its bipartite genome structure, which is composed of two RNA1s and two RNA2s, allowing for the efficient insertion of foreign genes into RNA2 for gene delivery. Additionally, TRV is associated with the viral gene silencing phenomenon (VIGS), which involves the insertion of a fragment of the desired gene into the virus genome. This has become a useful tool in the study of gene function and understanding of plant-microbe interactions. Finally, TRV is discussed in relation to its RNA interference-mediated resistance (RNAi) against plant pathogens, which is induced by the expression of short interfering RNA molecules (siRNAs).

In this chapter, TRV is presented as a vector with numerous potential benefits, however, it is also discussed that there are challenges and limitations to its use. For example, due to the narrow host range, TRV is only applicable to certain plant species. Therefore, efforts must be made to expand the host range or to develop alternative vectors. Additionally, optimizing viral constructs, delivery techniques, and purification methods is essential for increasing the efficiency and scaling of TRV-induced gene expression and gene suppression. In conclusion, TRV is a valuable tool with the potential to transform plant biotechnology and promote sustainable agriculture practices.

Keywords: Tobacco Rattle Virus, viral vectors; crop improvement; virus-induced gene silencing; future breeding; genome editing

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I. INTRODUCTION

Since the advent of the age of biotechnology and bioinformatics, the field of genetic engineering has gained prominence throughout the world of biological research. Despite strong regulations and sanctions, it is safe to say that genetic engineering alone may be the biggest step in tracing back as well as leading to the next biological revolution. One of the major components of genetic engineering includes gene cloning. It involves the introduction of a desired gene into host cells where they can be expressed on a usually amplified level. In doing so, we need a vector. A vector acts a delivery method through which the desired gene of expression may be transferred. In the plant world, most of the gene cloning activity has been carried out by the process of plasmid agro infection or through the biolistic method. The major constraint in such methods is the partial transformation of some cells only. With the use of virus as a delivery agent, we can ensure that the infection is systemic and the whole groups of cells or tissues are transformed.

Since the t-DNA does not get incorporated in host DNA, these plants technically are not transgenic and exhibit transient characters of genetic modification. It serves as a robust method as some virus families are capable of infecting a large number of crop species- another area where Agrobacterium fails to be applied successfully. Due to these reasons, the application of plant viruses in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR Associated Protein 9 (Cas9) and RNA Induced Silencing Complexes (RISCs) has been increasing. Viral vectors can be studied under the broad categories of DNA and RNA viruses with respect to their mode of infections. This chapter primarily deals with the use of RNA viruses in transgenic crop development and largely focuses on Tobacco Rattle Virus (TRV).

Tobacco Rattle virus (TRV), a plant pathogenic virus which mainly infects dicots and is known for infecting over 400 species of plants from 50 families. Naturally it is transmitted by nematode such as Trichodorus and Paratrichodorus. Viruses are used as vectors because of their small molecular weight and high infection efficiency for carrying host-derived sequence inserts that induces silencing of the corresponding genes in infected plants. Virus-induced gene silencing (VIGS) is a high-throughput reverse genetics technique that exploits an RNA-mediated antiviral defense mechanism [post-transcriptional gene silencing (PTGS)] for functional gene analysis (Ratcliff et al., 1997; Sunilkumar et al., 2006). The viral genome [complementary DNA (cDNA)] is modified to a recombinant virus vector containing sequences homologous to the host genes, which could trigger homologous endogenous gene silencing in plants.

TRV can be modified to facilitate insertion of non-viral sequence and subsequent different plant species due to its ability of transmission in the entire length of plant along with the ability to transmit in meristematic part of plant which contains undifferentiated cells to which most of the virus vector cannot transmit. TRV is composed of two genomic components TRV1 or RNA1 and TRV2 or RNA2. RNA1 encodes for two replicase proteins, a cysteine-enriched protein and a movement protein which is essential for replication and movement within the plants whereas RNA2 encodes for coat protein (CP) and non-structural protein which allows the formation of virions and nematode- mediated transmission across plants.

II. IITRV: A PLANT GENOME EDITING VECTOR

1. **An overview of Tobacco Rattle Virus:** The TRV is a positive single stranded RNA virus belonging to the family Virgaviridae and can cause infections in more than 400 recorded species of plants from 50 families. The natural mode of infection is through nematode vectors of the family Trichodoridae. Other modes of transmission may include seeds and mechanical tools. Symptoms of TRV vary with respect to the host plant with common symptoms including systemic necrosis, mottling, chlorotic or necrotic lesions, etc. With discovery of Zinc Finger Nucleases (ZFNs), Transcription Affector like Effector Nucleases (TALENs) and more recently, the CRISPR/Cas9 systems of gene editing, TRV have been used in studies involving transgenomics as a vector for silencing of specific genes of target.

   Initially applied in to express a ZFN reporter, the TRV RNA has been used extensively since then to express single guide RNAs (sgRNAs) in under the CRISPR/Cas framework. Pioneer work on TRV mediated transformation was carried out on Nicotiana benthamiana. There have been further improvements in applying the RNA virus in several other plant species as well.

   In comparison to DNA viruses, there are a significant number of advantages with RNA viruses. DNA viruses belonging to the family Geminiviridae have been employed in gene editing too. But due to their compact size, rapid systemic infection rates and simplicity in working mechanisms enables TRV to be a versatile vector. The TRV genome is bipartite, which means that there are two RNA strands. The TRV1 and TRV2 strands have a division of functions among themselves. For instance, the TRV1 strand is responsible for production of RNA dependent RNA polymerase (RdRP) as well as the movement protein (MP) that helps it in becoming systemic throughout the host. On the other hand, TRV2 is responsible for the coat protein and this part can be modified to incorporate our gene of interest. Both of these RNA strands are required for systemic infection in the host. Infectious cDNA derived from such strands have been utilized in the host to obtain end-products of agricultural and pharmaceutical significance.

2. **Construction of Tobacco Rattle Virus clone:** cDNA clones of TRV (strain PPK20) RNA1 and modified RNA2 [TRV-Green fluorescent protein (GFP) RNA2] is constructed under the control of cauliflower mosaic virus (CaMV) 35S promoters on the transferred (T) DNA of plant binary transformation vectors. GFP is transcribed from coat protein promoter (P) of the pea early browning virus by replacing the 29.4K (29.4 kDa) and 32.8K (32.8 kDa) open reading frames (ORF) for the expression of GFP in tobacco. The GFP gene is tagged in 3’ terminus of the coat protein of the original TRV2 vector, and the silencing efficiency of the modified TRV–GFP vector was tested in several plants. GFP could be used as an indicator of the degree of silencing of a target gene. By replacing the 29.4k genes and 32.8k genes, multiple cloning sites (MCS) were introduced leaving the viral coat protein and 5’-3’ untranslated region. TRV-GVF vector can cause gene silencing efficiently in solanaceous as well as non-solanaceous crops such as rose. TRV–GFP can be detected by using a hand-held UV lamp. This provides an effective tool for monitoring the efficiency of VIGS in functional genomics of non-solanaceous plants. For construction of vector the cDNA sequence of RNA1 and RNA2 is placed between left and right border and CaMV 35S promoters and the terminators (T). The TRV RNA 1

construct known as pBINTRA6 and TRV RNA 2 construct as pTV00. As the clones were constructed separately, the Agrobacterium cultures carrying the above two constructs i.e., pBINTRA6 and pTV00 were mixed and infiltrated into the leaves of plant for silencing the NbPDS gene.

For silencing of the NbPDS gene of Nicotiana benthamiana, the partial c DNA sequence was inserted to the multiple cloning sites of pTV00 which knocked down the NbPDS gene expression and silenced the gene in the plant producing mild viral symptoms with the efficiency of 47-73%.

In order to increase the silencing efficiency to 90-98%, instead of single CaMV 35S promoter, duplicated CaMV 35S promoter along with self-cleaving ribozyme (Rz) this in turn would increase the ability of virus to transmit infection. It was placed before the nopaline synthase terminator (NOSt) on the transferred Agrobacterium tumefaciens T-DNA of plant vectors. TRV enables non transgenic genome modification as the vectors do no integrate to the genome and can be transferred to the host cell through Agrobacterium mediated gene transfer or mechanical inoculation.

Genomic double strand breaks induced by endonucleases enables genome editing. The endonucleases in the targeted plant cell are introduced by direct gene transfer followed by regeneration of mutated plants. It can be carried out using homing endonuclease or artificial restriction nuclease i.e. ZFN (Zinc Finger Nuclease) which is formed by fusion of an artificial zinc finger DNA-binding domain and a DNA cleavage domain. These endonucleases can also be transferred into the target cells indirectly using vectors which help in production of the mutated plants. The use of tobacco rattle virus for the delivery of ZFNs into the plant would increase the efficiency and lead to permanent modification in the plant cells which can be also heritable to the next mutant generation. The ability of TRV to cause systemic infection in host helps in efficient delivery of ZFNs and as the TRV can cause infection in the meristematic tissues of the host plant, the mutated plants can be generated without relying on the tissue culture. This mutated plant would give rise to mutated inflorescence eventually to mutated seed. The approach was being used in Arabidopsis in which ZFN expression was controlled by inducible promoters to occur at a specific stage of plant development. The cloning of larger genome sequence becomes difficult due to the packaging capacity of vectors. The small size of ZFNs enables efficient cloning and expression of vectors. ZFNs require expression of two different monomers simultaneously. TRV-based vectors enable the co-expression of two ZFN monomers in the target cell. These vectors are also known for mediating the expression of chloroplast targeted proteins in plants and mitochondrial targeted protein in humans.

3. **Mechanism for Gene Editing by TRV:** The most popular application of TRV vector is Viral Induced Gene Silencing (VIGS) which is associated with the Native Plant Post Transcriptional RNA Silencing (PTGS) mechanism. The first virus to be utilized in this model was the Tobacco Mosaic Virus (TMV). RNA viruses are preferred for conducting VIGS since they have a smaller size and desirable infection ratio as compared to DNA viruses. Among RNA viruses, TRV has an added advantage of being systemic to such extents that successful transformations of meristems have been observed across various crop families such as Solanaceae, Cruciferae, Poaceae, etc.

The TRV genome is composed of two RNA strands namely, TRV1 and TRV2. The TRV1 strand is responsible for the movement of the virion particle inside the host cell and cause systemic infection. It also houses the replication proteins essential for viral multiplication. The TR2 strand consists of genes which express the capsid protein of the virus as well as have other structural functions. It is in this strand that we can incorporate our gene of choice by using ZNF dimers, monomers or sgRNA under CRISPR/Cas systems. There have been several experiments using the TRV as a vector for gene editing and it has given good results due the fact that the genome size is small, allowing for rapid multiplication. The infection becomes systemic in plants without any integration with the host genome. There have been instances where infections to the meristem have been found. This indicates that TRV can be sued to infect germ lines of plants as well with unequivocal accuracy. Therefore, TRV can be used a vector in gene editing as explained briefly later in the chapter.

The delivery of TRV vector inside the host can be achieved by first converting the bipartite RNA strands into plasmid strand. It can then be incorporated in the cytoplasm of Agrobacterium. A wide array of options is available to transfer the vector into the host cell- injection of tissues, airbrushing, sap inoculation, agro-drenching, vacuum infiltration, etc. Among these, leaf injection, agro-drenching and vacuum infiltration has very high efficiencies. Once introduced inside the host cell, it is transcribed by RNA polymerase which produces a double stranded RNA (dsRNA). This dsRNA is then recognized by DICER enzymes which convert them into short interfering RNAs (siRNAs). These siRNAs are in turn recognized by the RNA Induced Silencing Complex (RISC) and break down targeted strands.

Leaf injection is a very common and easy way to introduce the vector inside a plant. It is widely practiced in dicots and has good efficiency (97 per cent). The major constraint remains in the fact that it is not suitable for monocot plants and may become labour intensive when conducted on a large scale.

Airbrushing involves the principle of rubbing and contact with host surface. The vectors are sprayed on the host tissue which may take up the vector with an efficiency of more than 90 per cent. It is suitable while working on monocot plants where injections are not that much of a success.

Sap inoculation is carried out in order to achieve high amount of transformation with enhanced concentration on vector suspension. The suspension is rubbed on the host tissue surface along with carborundum dusting and phosphate buffers. It is a somewhat complex process but has high efficiency range.

Vacuum infiltration is another highly efficient method of gene transformation. It can be carried out even when the plant is in the form of a seed which ensures a 100 per cent result. It can be used on a large scale of plant population without the cost of raising them and ensure a 100 per cent transformed plant. It is equally efficient in cases of monocot and dicot plants.

III. CASE STUDY

In an elaborate experiment conducted by scientists to study the function of a specific gene for plant development and resistance against certain strains, Arabidopsis thaliana, a

model organism was chosen. A TRV-based vector for gene silencing was employed and gene expression was analyzed. The vector was designed to carry a fragment of the target gene in an inverted repeat orientation. It also had a reporter gene in the form of Green Fluorescent Protein (GFP) which could indicate the effects of gene silencing and act as a marker for the same.

The vector was introduced into the host plant by mechanical inoculation. The vector replicated in the plant body and enabled the silencing mechanism against the targeted gene. This led to the suppression of the expression of the gene in question.

Modified plants were kept under observation for any sort of visible phenotypic changes which could be associated with the gene silenced. To confirm the specificity of the target gene silenced, the research group performed molecular analysis such as quantitative PCR in order to ascertain the levels of expression in the target gene in comparison with the control plants.

In another case, the researchers conducted a gene expression study in which a second set of transmissible ribosomal vector-based vectors (TRV-based) vectors were designed to express an alien gene of interest. The alien gene was inserted into the TRV by a powerful viral promoter, and was then inoculated into the Arabidopsis plant. To assess the specificity of the gene silencing process, molecular analyses were conducted to measure the reduction in the target gene expression levels in the silenced plants compared to those without the alien gene. Additionally, phenotypic analysis was conducted to assess the effect of expressing the alien gene on the phenotypes of the plants, including growth patterns, resistance to disease, and stress tolerance, and was compared to control plants that did not contain the alien gene. Finally, molecular analysis was conducted using RT-PCR and Western blot analysis, respectively, to identify the presence of either a gene transcript or the corresponding protein product. TRV-based vectors were used to successfully perform gene silencing and expression studies on Arabidopsis. The silenced plants showed altered phenotypes related to the targeted gene, indicating its functional role. The expression of foreign genes also resulted in observable changes in the plant traits, allowing for further investigation of their functions and applications.

This case study demonstrates the utility of TRV-based vectors in genetic engineering for gene silencing and gene expression studies, providing valuable insights into plant biology and potential applications in crop improvement.

Table 1: RTV mediated gene-editing in various crops/plants (Adapted from Zaidi and Mansoor, 2017)

GE Platform	Plant species	Target	Reference
ZFN	*Nicotiana tabacum and Petunia hybrida*	uidA	Marton et al., 2010
Meganuclease	*Nicotiana alata*	DFR	Hoing et al., 2015
CRISPR	*Nicotiana benthamiana*	PDS	Ali et al., 2015b
CRISPR	*Nicotiana benthamiana*	PDS, PCNA	Ali et al., 2015a
CRISPR	*Nicotiana benthamiana*	Plant virus	Ali et al., 2015c
CRISPR	*Nicotiana benthamiana*	Plant virus	Ali et al., 2016

IV. APPLICATION OF TRV AS A VECTOR IN PLANTS

1. **Non Transgenic Genome Modification in Plants:** RTV vectors were initially used in ZFN experiments by incorporating ZFN monomers to the TR2 strand and then introduced into the host along with the TR1 strand. A mutation could then be observed on the basis of a certain marker such as fluorescence or speckling on leaf surface. Once infected individuals were identified, they were furthered on for tissue culture techniques. The regenerated plants also showed significant mutations which were stable and could be passed on. Also, the regenerated plants obtained are virus-free.

2. **Virus Mediated Genome Editing Using CRISPR/Cas:** In case of CRISPR/Cas system of gene editing, a major constraint arises from the fact that the components involved are somewhat problematic to be incorporated into the RTV genome. While the sgRNA can easily be incorporated with the RTV2 strand, the larger Cas9 protein proves to be a hindrance. Even if we somehow managed to incorporate the protein sequence, it would simply get cut out of the picture as the RdRP will not allow its transcription. In order to avoid such complexities, a transgenic plant has to be created first which expresses the Cas9 protein intrinsically. TRV can then be used with the modified sgRNA strand for desired site mutations.

3. **Diversification of RNA Based Editing to Various Virus Vectors:** Apart from the success received in application of TRV as a vector, there are several more viral genomes that can be utilised in the same way and infect even a larger array of hosts. Viruses such a Wheat Dwarf Virus (WDV), Barley Yellow Striate Virus (BYSV), Beet Necrotic Yellow Vein Mosaic Virus (BYVMV), Tobacco Mosaic Virus (TMV), Foxtail Mosaic Virus (FoMV), etc. can be used as substitutes for infecting various crop plants with more specificity. The experiments with TRV have been largely conducted on plants belonging to Solanaceae and Cruciferae families which are dicots. In order to perform more detailed experiments in other plant families, we need to identify suitable infection vectors who already have a well establishes bio-chemical pathway with the host in question and apply the TRV model of gene editing to them.

   There is also a proposition that negative sense RNA viruses such BYSV may act as better vectors since they have a larger information carrying capacity compared to positive sense RNA viruses. Will host-pathogen specificity, we can also ensure that the transformations may become heritable.

4. **Heritable Gene Editing:** A very large challenge in transgenic crop improvement lies in the fact that it requires the establishment of a sophisticated tissue culture laboratory. Once a desirable phenotype is identified and checked for genotypic confirmations, it needs to be cloned in large numbers in order to further the programme. With the use of RTV vectors, genes can be transmitted to progeny lines in a much more efficient way, reducing the need for tissue culture.

   The sgRNA can get a better access to the host and heritable transformed plants are obtained as a result. In experiments conducted upon Nicotiana benthamiana, a Flowering Locus T (FT) motif was added in the sgRNA sequence. This enabled the viral RNA to move and infect even the meristem. The experiment yielded somatic mutagenesis up to 80 per cent and germinal transmissions were estimated to be around 65 per cent.

Multiple sgRNAs can be incorporated in the TR2 strand of the TRV genome to further improve the transformation efficiency. This indicates that there is vast room for improvement in RTV mediated transformation. Also, there is scope to diversify this technology to perennial crop families.

V. FUTURE PROSPECTS AND CONCLUSION

With CRISPR/Cas bringing in a major revolution in gene editing, there is no doubt that the upcoming focus of genetic manipulation will be spearheaded by affordability and efficiency. As more and more organisms are being subjected to CRISPR/Cas systems, it is high time that studies on vectors such as TRV and other ssRNA viruses be done with more depth. Development in the field VIGS has a limitation in the form of non-inclusion of the Cas9 protein sequence which can be researched upon. Seeds or other germinal tissue can be edited to produce heritable modified plants.

As more and more plants species expressing Cas9 proteins are being infected with the desired sgRNAs of TRV genome, the load on tissue culture laboratories will be reduced significantly.Primary research on TRV vectors has been largely confined to plants of the Solanaceae family, a dicot population. There is a need to enlarge the pool of research to other families and preferably to monocots. This technology can enable the production of foreign gene free plants which can be adopted for commercialization relatively easily.

Various combinations of application and inoculation can be used to further improve the infection efficiency of vectors. There is also scope to work on negative strand RNA viruses as potential vectors to be utilized in a procedure similar to the TRV/VIGS pathway. This will help in identifying host specific vectors for rapid development of modified plant varieties. VIGS can be researched further with respect to plant development and environmental interactions. A robust VIGS procedure which can be applied to large number of species-vector dynamics can be developed for crop development programmes. All of these prospects can provide for a bright future for the use of TRV as a large scale, gene editing vector.

VI. CONCLUSION

In this Chapter, we examined the potential of the Ttobacco RrattlesnakeVvirus, TRV, to be used as a vector in a variety of scientific and biotechnological applications. We also looked at the characteristics of TRV that make it an attractive candidate for plant gene engineering. TRV has a bipartite genomic structure, with RNA1 providing foreign genetic material to be inserted into RNA2 while RNA1 is responsible for replication and encapsulation. This system allows for the expression of novel genes of interest into plant cells, allowing for the investigation of gene function, the improvement of crop characteristics, and the formation of novel plant cultivars.

One of TRV's key advantages is its capacity to induce gene suppression through the process of virus-induced-genetic-silencing, also known as VIGS. This technique involves the insertion of a segment of the desired gene into the host plant's viral genome, resulting in a decrease in the expression of the endogenous gene. As a result of this process, researchers have been able to observe phenotypic alterations related to the silenced gene.

In addition, TRV has the potential to be used for plant protection strategies, especially in the area of plant pathogen resistance (RNAi) induced by TRV. TRV expresses short interfering RNAs that target essential genes of pathogens and induce RNAi in infected plants, specifically suppressing pathogen gene expression resulting in disease resistance. This approach has the potential to lead to the development of eco-friendly and sustainable crop protection strategies.

However, TRV has some challenges and limitations as a viral vector. One limitation is that TRV is limited to a specific host range, which limits its use to specific plant species. Therefore, it is important to work on expanding the host range or developing alternative vectors for non-native plants.

The use of Tobacco Rattle Virus (TRV) as a platform for gene expression and functional genomics, as well as for plant protection strategies, is a valuable asset to plant science and biotechnologies. However, further optimization of viral constructs and delivery protocols, as well as purification methods, is necessary to enhance the efficacy and scalability of TRV-mediated viral gene expression and gene silencing in order to effectively infect plants. Furthermore, the ability of TRV to induce gene silencing via VIGS provides new opportunities to investigate gene function and improve crop performance. With ongoing research and development, the development of TRV systems has the potential to transform plant biotechnology and to contribute to sustainable agricultural practices in the future.

Authors' contribution: AAN conceived the idea and collected data; AAN and SK wrote and edited and the manuscript; SK did some addition and correction to the manuscript; SK prepared the table; AAN added the pictorial diagrams; AANSD revised the manuscript. All authors read, finalized and approved the manuscript.

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