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Chapter 5

Tobacco rattle virus-A pathogen Based Vector System as a Tool for Functional Genomics

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Abstract

In this era of genomic research, it has become highly important to understand the mechanisms by which plant genes are influenced and respond to their immediate environment. However, dissecting these crucial mechanisms is a challenge in many non-model and crop plant species due to the unavailability of reliable genetic transformation protocols. Virus-Induced Gene Silencing (VIGS) has emerged as the most suitable alternative approach to bypass these processes as it does not require any stable transgenic lines. Among various VIGS systems developed till date, *Tobacco Rattle Virus* based Induced Gene Silencing (TRV-VIGS) is the most extensively preferred one. It has been frequently used in many members of *Solanaceae, Rosaceae, Brassicaceae, Euphorbiaceae, Apocynaceae, Amaranthaceae* and some other plant families to study the plant–pathogen and plant-herbivore interactions, evolution and various plant developmental processes. The experimental range of plant species amenable to TRV-VIGS is increasing day by day with refinement in inoculation techniques and improvement of new TRV viral vectors and protocols. This book-chapter explores the methodology and recent advancements in currently available widely adopted TRV-VIGS system.

Keywords: Transgenic Plants; Gene silencing; VIGS; TRV; Transformation; Agroinfiltration.

1. Introduction

In past 20 years, scientists have revolutionized the plant science research. The completion of various plant genome and transcriptome sequencing projects, along with the advancements in microarray platforms and Next Generation Sequencing (NGS) technologies, have paved the way for functional annotation of genomic information. To determine the function of any gene, two basic approaches are frequently applied i.e. gain of function and loss of function. Among widely used approaches, Virus-Induced Gene Silencing (VIGS) has emerged as the most convenient and effective method due to its simple methodology, robustness, cost-effective nature and quick results. This is also evident from the fact that the keyword "VIGS" was fetched by 7900 publications in Google Scholar website (https://scholar.google.co.in).

Researchers have exploited the properties of many plant viruses as a gene delivery tool to develop functional genomics platforms. due to their efficient machinery, comprehensive genome structure and ability to replicate autonomously within the plant cells. Initially, these viral vectors were used for gain-of-function assays by overexpressing the full-length cDNAs of endogenous or non-endogenous genes and the effects were studied *in planta* [1,2]. Later, these vectors were also used to perform the loss-of-function assays [3,4].

Kumagai and co-workers reported VIGS for the first time using a *Tobacco Mosaic Virus* (TMV) expression vector in *Nicotiana benthamiana* [3]. They knocked down the *phytoene desaturase* gene by expressing a cDNA fragment of *phytoene desaturase* in antisense orientation and observed a loss-of-function phenotype. Afterwards, other virologists too over-expressed different genes using various viral vectors which resulted in a decrease in expression of respective mRNA explaining the loss-of-function due to active mRNA silencing [5, 6]. The term 'VIGS' was coined by Van Kammen depicting the marvel of recuperation from viral infection [7]. These few initial reports opened up an avenue for the development of new VIGS vectors which resulted in a rapid assessment of gene function. Since then, various research groups are exemplifying the great potential of the VIGS approach regularly.

2. Virus-Induced Gene Silencing

VIGS is a natural antiviral immunity mechanism which acts as a first line of defense against invading RNA and DNA viruses. This technology is similar to post-transcriptional gene silencing (PTGS) and uses the recombinant viral vectors carrying a target gene fragment [8]. The recombinant vectors are introduced into the plant systems either mechanically or by agrobacterium infiltration. The delivered recombinant viral RNA replicates inside the cell via a double-stranded RNA (*ds*RNA) intermediate. which acts as a principal inducer for siRNA system. The generated siRNAs guide respective RNase complex and ultimately silence the targeted homologous gene in a sequence-specific manner [9].

As a functional genomics tool, VIGS is gaining an immense popularity higher than other related methods like transposon-tagging, T-DNA insertion mutagenesis, and RNA interference (RNAi) due to its simpler methodology. Since the method requires no stable transformation, the results could be obtained within a fairly short span of time. Furthermore, it also provides a high throughput characterization of lethal genes which might not be possible if using any other parallel techniques. VIGS is successfully used in rapid silencing of one or more genes simultaneously. Even ESTs could be used in this method as there is no need of full length sequences. Further, the approach results in an incomplete suppression of gene expression leading to the milder symptoms and phenotypes which are easy to observe [10]. Additionally, VIGS allows secondary amplification of the RNAi signals which helps in overcoming the effects of insertional inactivation of the gene and is the main subject of this book chapter.

However, there are some limitations like host range, restriction in insert size, unavailability of virulent clones and difficulties in meristem invasion. Furthermore, the VIGS vector needs to be disarmed to avoid the development of any disease symptoms [9]. Among currently available various vectors, *Tobacco rattle virus* (TRV) based VIGS vector is the widely used tool for functional characterization of different genes due to the aforesaid advantages.

3. Tobacco Rattle Virus

The limitations of previously used VIGS vectors, lacking various essential properties like wide host range, mild symptoms of infection, higher inoculation efficiency, dedicated meristem penetration, easy analysis of symptoms in subtle phenotypes, higher silencing efficiency, multiplex intensiveness and generation of uniformly silenced phenotypes, were overcome by developing TRV-based VIGS vectors [4,12-16].

The *Tobacco rattle virus* (TRV) is a member of the genus Tobravirus (Family-Virgaviridae) [17]. It is a bipartite, rod-shaped, positively sensed single-stranded pathogenic RNA (+ssRNA) plant virus which affects the genetic vigor, phenotypic appearance and yield of over 420 plant species of 50 different families including potato, tobacco, false goat's beard, bleeding heart, barbados nut, cucumber, goosefoot, black pepper, coral bells, horny goat weed, sword lily, celery, hyacinth, common marigold, rose, tulip, periwinkle, globe artichoke, daffodil, common bean, tepary bean, beets, bilberry and spinach. The stubby-root nematode *Trichodorus* and *Paratrichodorus* act as a vector for TRV which can be transmitted mechanically or through seeds [18]. Geographically the virus is found in Europe, New Zealand, and North America geographically and have two RNA genomes i.e. RNA1 and RNA2 respectively [15,17].

The RNA1 genome varies from 6.8 - 6.9 kb in different isolates and is larger than RNA2. This genome is chiefly responsible for the production of four proteins – 134 kDa *methyltransferase-helicase* protein, 194 kDa *RNA-dependent RNA polymerase (RdRp),* 29 K

movement protein (MP) and 16K protein **(Figure 1)**. The first two, 134 kDa helicase and 194 kDa RdRp, proteins are translated directly from the RNA1 genome while the other two 29K MP and 16K proteins are translated by subgenomic RNAs (sgRNAs). Recently, Ferna´ndez-Calvino and colleagues reported that the 16-kDa cysteine-rich protein (CRP) binds to Argonaute-4 and interferes with the *de novo* formation of both miRNA and siRNA-guided RISCs [19]. The smaller genomic TRV-RNA2 genome contains three genes. At the 5' proximal end the coat protein (CP) gene is translated directly from the subgenomic RNA. After the *CP* gene, there are two non-structural *2b* and *2c* genes which play a critical role in the transmission of viral genomes via trichodorid nematodes. It is hypothesized that these nematode interacting proteins by forming a bridge between the internal surface of the vector nematode feeding apparatus and virus coat protein [15,17].

4. TRV-VIGS: A New Tool in the Chest for VIGS

At present, the scientific literature is repleted with the studies related to functional characterization of a gene using tobacco rattle virus based induced gene silencing (TRV-VIGS). PubMed (http://www.ncbi.nlm.nih.gov/pubmed) search using keywords "TRV" and "VIGS" in the title and abstract field alone resulted in nearly 1282 research articles. A closer inspection of the search revealed more than 100 original articles which involved the combination of two. The TRV-VIGS has become one of the most extensively applied method for gene functional characterization. A short description of research articles published on TRV-VIGS in various plant species is given in **Table 1**.

This approach is now among the first choice to be utilized for the functional studies of any uncharacterized gene and has been rapidly adapted for high-throughput analysis. It is a fast, efficient and relatively inexpensive approach for both forward and reverse genetics. Further, it has been frequently used in many members of Solanaceae [4,12,20], Rosaceae [21], Brassicaceae [14] etc and standardized for *Arabidopsis thaliana* [14], *Nicotiana benthamiana* [4], *Solanum lycopersicum* [12], *Gossypium spp*. [22], *Capsicum annuum* [20] and *Fragaria ananassa* [21]. This gene silencing approach has also been applied to study the plant–pathogen, plant-herbivore, plant-symbiont interactions, evolution, plant development and abiotic stresses [10,23-25] **(Figure 2)**. The range of plant species amenable to TRV-VIGS approach is continuously increasing day-by-day with the refinement in inoculation techniques [16] and improvement in new TRV viral vectors [4,12,14,26] and protocols [27].

Earlier, the researchers used to deliver the infectious TRV-transcript copies, generated by *in vitro* transcription of viral RNA using a linearized plasmid DNA template, directly to the plants by mechanical inoculation on leaves for gene silencing [3, 28]. Later on, an alternative-agroinoculation method was devised for the same purpose [9,11,16,21,22,26,27, 29].

In this system, both TRV RNA 1 and TRV RNA 2 genomes are cloned between the

left and right border of the 'pTRV1' and 'pTRV2' vectors respectively by gateway or restriction based cloning method. For the latter one, the nematode interacting genes were replaced with multiple cloning sites which facilitate the insertion of desired gene cassettes (candidate genes or cDNA libraries). Further, the pTRV1 and pTRV2 are transformed into two different *Agrobacterium* cultures which are then mixed and infiltrated into leaves, roots, flowers, seedlings or other parts of the plants. Many infiltration methods have been devised to facilitate the delivery of TRV RNA components [4,12,14]. After the introduction in plant cells, the viral genes are expressed and replicated *in vivo*. The replication of a positive, single-stranded RNA genome leads to the generation of double-stranded RNA genome which are responsible for the production of siRNAs resulting in the systemic silencing signals. As a result, the endogenous mRNA of the concerned gene, homologous to the sequence inserted within MCS of pTRV2, gets silenced in newly developed leaves, roots, flowers and other parts of the plant [4] **(Figure 3)**. The process of gene silencing continues till the plant responses against silencing overcome and defend the viral RNA proliferation. To monitor the extent and duration of the silencing process, various visible reporter genes are used by inserting them into the viral genomes. The most frequently used reporter genes in TRV-VIGS are *Phytoene desaturase* (PDS) [3] and *Proliferating cell nuclear antigen* (PCNA) [29].

These vectors are widely adopted due to the specific advantages of TRV vector. TRV invades growing meristematic parts unlike any other viruses and shows milder symptoms [4]. TRV vectors are enabled for silencing homologs using gene fragments from different plant species and are amenable to multiple types of gene cloning [30]. There has been various methods developed for inoculation [4,12,14]. The TRV mediated gene silencing is able to work in multiple plant tissues and parts ranging from leaves to roots and works even in reproductive organs too [30,31] **(Table 1)**. Further, it overcomes the problem of lethal expression of transgenes in seedlings, embryos or other plant organs, which is a major problem in normal transgenics, due to its transient expression [30]. Further, TRV-VIGS silences genes for more than months and transmi it to the progeny seedlings [31]. Recently, modified TRV RNA1 component also evoked silencing without inoculation of TRV RNA 2 component [26]. This system has also been used in monocots to silence the PDS gene [16]. This report opened a gateway to explore the full potential of TRV-VIGS approach in other monocots and dicots.

5. Recent Advances in TRV-VIGS

The TRV-VIGS system was among the first RNA virus based VIGS system [4]. early investigations using TRV-VIGS were performed with the wild tobacco *N. benthamiana* and tomato [4,12,13]. In an effort to generate a more useful set of tools to understand the influence on and responses of genes, Burch-Smith and co-workers used TRV-based VIGS vector in *Arabidopsis* and optimized the vector delivery and effectiveness in this model plant [9]. Hartl and co-workers investigated the effect of *Leucine Aminopeptidase* (LAP) silencing in *Sola-*

num nigrum using TRV vector and performed herbivore feeding assays [23]. They found that silencing of *LAP* increased the *Manduca sexta* larval mass which fed on TRV-*Sn*LAP silenced plants. Using this technique, they demonstrated the role of LAP in defense against herbivore [23].

Pei and co-workers used TRV-VIGS silencing to knock-down *GST* gene in resistant tomato plants of *Solanum habrochiates* [32]. The TRV-ShGST silenced plants showed a sharp decline in H_2O_2 accumulation and hypersensitive reponse (HR), making plants susceptible to *Oidium neolycopersici*. Hidalgo and co-workers performed TRV-VIGS in *Cysticapnos vesicaria* for silencing of *PDS* and *FLO* genes using *Agrobacterium tumefaciens* strain GV3101. This gene silencing resulted in a strong photobleaching of green parts and affected the floral organ identities i.e. floral symmetry and floral phyllotaxis respectively [33]. Choi and Hwang silenced the *PO2 gene* in chili pepper using this methodology which resulted in an increase in susceptibility to osmotic stress. Additionally, they observed strong photobleaching and huge chlorophyll loss in silenced leaves [34]. In a similar experiment, Virk and colleagues used TRV-VIGS methodology to silence the *SlMPK4* gene in tomato [35]. The silencing of *MPK4* gene resulted in an early wilting and reduced tolerance of tomato plants to drought stress. Wang and co-workers optimized the TRV-VIGS protocol for pepper (*Capsicum annuum*) and also investigated the multiple factors which affect TRV-VIGS efficiency [27].

Deng and co-workers reported a modification of TRV RNA1 by inserting heterologous gene fragments in *16K* gene position [26]. They observed a less efficient silencing of *PDS* gene by TRV RNA1 vector in *N. benthamiana* revealing the importance of *16K* gene. Later, Tian and co-workers used *Green Fluorescent Protein* (GFP) gene tagged to the *coat protein* gene of TRV2 [36]. They traced only green fluorescent tissues that carried the virus and had undergone silencing. Similarly, Lim and Lee down regulated the expression of *MLO2* gene in *Capsicum* under drought stress using TRV-based VIGS methodology [37]. They observed a sharp decline in the MDA levels of silenced plants.

Recently, Vega-Arreguín and co-workers utilized the TRV-VIGS framework in *N. benthamiana* to understand the non-host type of plant resistance against the devastating pathogen *Phytophthora capsici* mediating in part by *I2R* gene family members [38]. More recently, Zhang and co-workers reported the application of TRV-VIGS approach in monocot wheat and maize using a novel infiltration solution containing Tween 20, cysteine and acetosyringone [16]. They successfully silenced both *PDS* and *MLO* genes in both plants (Table 1). They also observed that the omission of any component of infiltration solution hampered the TRV-VIGS efficiency several folds. This report expanded the experimental host range of the TRV-VIGS.

6. Recent Advances in TRV Mediated Delivery of SSNs

Over the decade, many research groups used the TRV-based vectors to silence the en-

dogenous genes in plants and exploited the delivery property of TRV based vectors to deliver the site-specific nucleases (SSNs) [39-43]. As a result, TRV has been established as a promising vector for delivery of genetic engineering reagents. At first, Marton and co-workers used the TRV-based expression system to deliver ZFNs and produced mutant plants [39]. The TRV systemically infected *N. tabacum* and *Petunia hybrida*. They observed virus ZFN-mediated targeted mutagenesis in regenerating tissues and newly developed buds due to the activation of a mutated reporter gene *DsRed2* and recovered mutated plants in both *N. tabacum* and *P. hybrida.*

Later, Honig and co-workers developed a TRV vector for the expression of a site-specific meganuclease in *N. alata* and observed the efficient, heritable mutations in d*ihydroflavonol 4 - reductase* (*DFRa*) [40]. They reported a reduced purple pigmentated phenotype in flower petals due to inactivation of *DFRa* gene. The mutations were also heritable in the M1 progeny. Few of these mutations were also inherited up to two further generations.

Similarly, Ali and co-workers developed a TRV-mediated gRNA delivery system for genome editing in *N. benthamiana* [41]. The developed system was multiplexing amenable and bypasses the transformation requirement. Also, it showed regeneration of each user-defined target sequence. They generated Cas9-overexpression (Cas9-OE) transgenic lines, and used *Agrobacterium* to deliver gRNA with binding specificity for the reporter genes-PDS and PCNA by needleless syringe agroinfiltration. Recently, Ali et al. [42] reported the TRV-mediated CRISPR/Cas9 system persistent activity up to 30 d post-agroinfection and no off-target activities.

Further, Aman and colleagues engineered the TRV RNA2 to transiently and systemically express crRNAs against *HC-Pro*, CP and GFP target in *N. benthamiana* leaves [43]. They used CRISPR RNAs (crRNAs) to test the functionality of CRISPR/pCas13a with the TuMV-GFP virus *in planta* transient and stable assays.

7. Figures

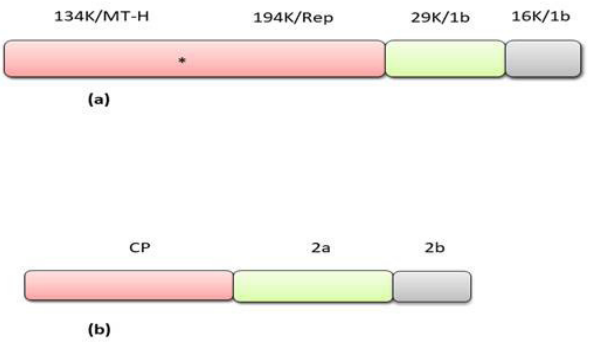

Figure 1: Schematic representation of TRV RNA1 and (a) TRV RNA2 genome organization. ORFs are boxed. Protein sizes are in kDa, indicated by K. MT, Methyl-transferase domain Do it everywhere H, helicase domain Rep, RNA-dependent RNA polymerase CP, Virus coat protein, 2b and 2c, non-structural proteins. Asterisks denote a leaky translation termination codon.

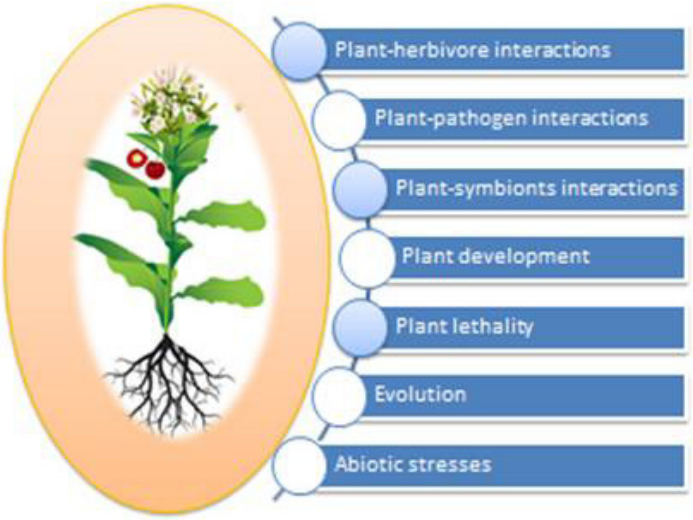

Figure 2: Application of TRV-VIGS approach in functional genomics to study and understand plant–pathogen, plant-herbivore and plant-symbionts interactions along with plant development, plant lethality, secondary metabolite synthesis, evolution and abiotic stresses.

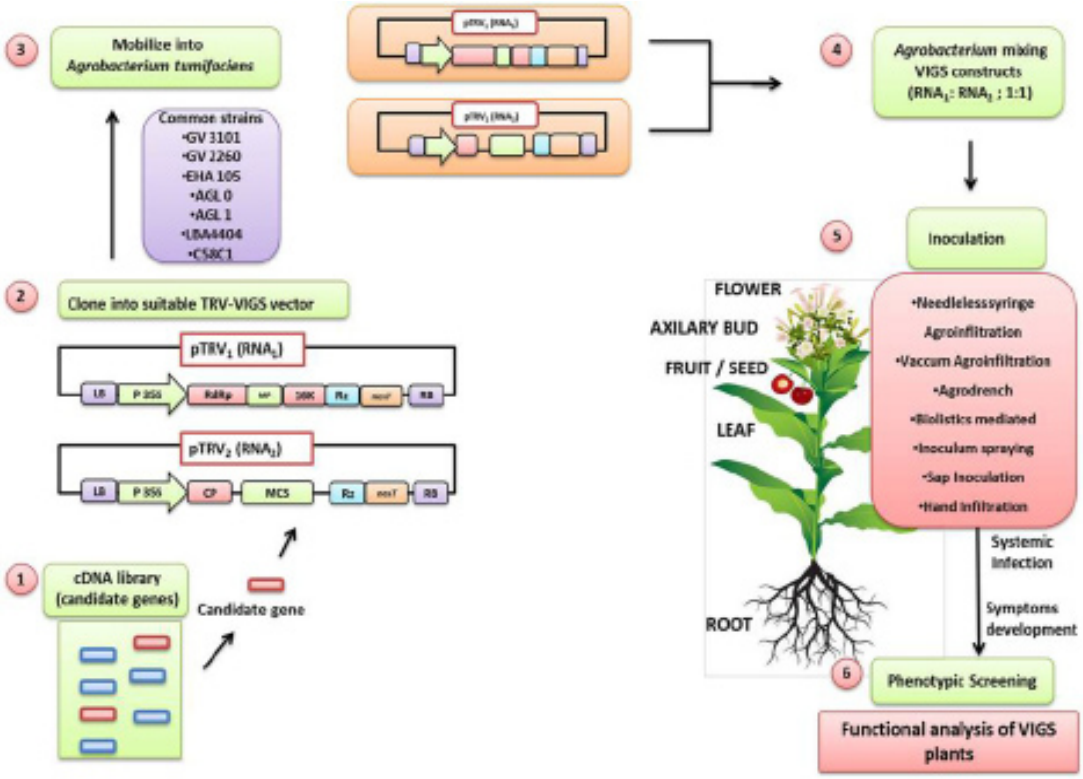

Figure 3: Schematic representation of various stages in TRV-based the VIGS vector method for silencing of endogenous. (1) A candidate gene is identified and isolated from the cDNA library. The isolated gene is cloned into the MCS of TRV RNA2-based VIGS vector by using conventional restriction digestion/based or gateway-based, ligation independent cloning. Along with this, the viral RNA1-based cassette (harboring genes for movement protein etc.) is cloned in another T-DNA *expression* system. These two binary vectors (TRV-RNA1 and TRV-RNA2) are mobilized into different independent *Agrobacterium* cells. The two different *Agrobacterium* cultures harbouring both RNA1 and RNA2 are mixed in 1:1 ratio and inoculated into different parts of the target host plant by different methods like needleless syringe agro-infiltration, vacuum agroinfiltration, agrodrench, spraying inoculum of the *Agrobacterium tumefaciens* cell suspension, sap inoculations using extracts of agroinfiltrated *N. benthamiana* leaves, biolistic-mediated inoculations of infectious transcripts generated from the two binary constructs *in vitro* etc. (6) The infiltration leads to systemic infection and the development of specific symptoms. Phenotypic attribute to target gene silencing are usually observed in 3–4 weeks followed by molecular assessment as well as testing for viral titer.

8. Tables

Table 1: List of different plant species being used for TRV-VIGS approach.

S.No	Family	Plant name	Plant part Inoculated	References
1	Actinidiaceae	Kiwifruit (*Actinidia arguta*)	Fruit	[44]
2	Amaranthaceae	Spinach (*Spinacia oleracea*)	Leaf	[45]
3	Apocynaceae	Devil pepper (*Rauwolfia tetraphylla*)	Plantlets	[46]
4		Madagascar periwinkle (*Catharanthus roseus*)	Seedling, Leaf	[47]
5	Asteraceae	Globe artichoke (*Cynara cardunculus*)	Cotyledons	[48]
6		Chrysanthemums (*Dendranthema grandiflorum*)	Leaf	[36]
7		Gerbera (*Gerbera hybrida*)	Leaf, Scapes	[49]
8	Brassicaceae	Thale cress (*Arabidopsis thaliana*)	Roots, Leaf	[14,50]
9		Black mustard (*Brassica nigra*)	Cotyledons	[51]
10	Euphorbiaceae	Barbados nut (*Jatropha curcas*)	Leaf, Seedlings	[52, 53]
11		Tung tree (*Vernicia fordii*)	Plantlet	[54]
12	Iridaceae	Sword lily (*Gladiolus hybridus*)	Cormels	[55,56]
13	Lamiaceae	Sweet Basil (*Ocimum basilicum*)	Cotyledons	[57]
14	Linaceae	Flax (*Linum usitatissimum*)	Plant crown, Leaf	[58]
15	Malvaceae	Cotton (*Gossypium sp.*)	Cotyledons, Leaf	[22,59]
16	Nyctaginaceae	Four o'clock flower (*Mirabilis jalapa*)	Tuber, Seedlings	[60]
17	Nyssaceae	Happy tree (*Camptotheca acuminata*)	Leaf	[61]
18	Orobanchaceae	Egyptian broomrape (*Phelipanche aegyptiaca*)	Leaf	[62]
19		Purple witchweed (*Striga hermonthica*)	Leaf	[11]
20	Onagraceae	Slender clarkia (*Clarkia gracilis*)	Seedlings	[63]
21	Papaveraceae	California poppy (*Eschscholzia californica*)	Cotyledons	[64]
22		Opium poppy (*Papaver somniferum*)	Leaf	[65]
23	Piperaceae	Jaborando-Manso (*Piper colubrinum*)	Leaf	[66]
24	Poaceae	Wheat (*Triticum aestivum*)	Seeds	[16]
25		Maize (*Zea mays)*	Cut seeds	[16]
26	Ranunculaceae	Colorado-blue columbine (*Aquilegia coerulea*)	4-6 leaf stage	[67]
27		Columbine plant (*Aquilegia vulgaris*)	Seedlings	[68]
28		Meadow-rue (*Thalictrum sp.*)	Root, Seedlings, Tubers	[69]
29	Rosaceae	Strawberry (*Fragaria ananassa*)	Fruit, Leaves, Axils	[21,70]
30		Apple (*Malus domestica*)	Fruit, Leaf	[71,72]
31		Crabapple (*Malus sp.*)	Fruit, Leaf	[73]
32		Siberian crabapple (*Malus xiaojinesis*)	Seedlings	[74]
33		Peach (*Prunus persica*)	Leaf, Fruit	[75]
34		Chinese white pear (*Pyrus bretschneirdi*)	Fruit	[76]
35		Birch-Leaved Pear (*Pyrus betulaefolia*)	Leaf	[77]
36		Rose (*Rosa sp.*)	Branches, Flower Stem, Sprouts (Grafted)	[78, 79]

37	Rutaceae	Pummelo (*Citrus grandis*)	Seedlings	[80]
38	Salicaceae	Poplar tree (*Populus euphratica*)	Seedlings	[81]
39	Sapindaceae	Litchi (*Litchi chinensis*)	Fruit, Stem	[82]
40		Chinese flowering chestnut (*Xanthoceras sorbifolia*)	Seedling	[83]
41	Solanaceae	Chilli Pepper (*Capsicum annuum*)	Leaf, Cotyledons	[20,84]
42		Jimsonweed (*Datura stramonium*)	Leaf	[85]
43		Jasmine tobacco (*Nicotiana alata*)	Leaf	[86]
44		Coyote tobacco (*Nicotiana attenuata*)	Leaf	[87]
45		Tobacco (*Nicotiana benthamiana*)	Leaf, 4-6 leaf stage seedling	[4,13, 26, 38,88]
46		Bolivian tobacco (*Nicotiana glutinosa*)	Cotyledon	[89]
47		American tobacco (*Nicotiana tabacum*)	Leaf	[90]
48		Petunia (*Petunia hybrida*)	Leaf, Stem	[91,92]
49		Dutch eggplant (*Solanum aculeatissimum*)	Cotyledon	[93]
50		Jerusalem cherry (*Solanum pseudocapsicum*)	Cotyledon	[94]
51		Tomato (*Solanum lycopersicum*)	Leaf, Seedling, Cotyledons	[95]
52		Eggplant (*Solanum melongena*)	Sprout	[96]
53		Potato (*Solanum tuberosum*)	Tuber, Leaf	[97]
54		Wild potato (*Solanum venturii*)	Leaf	[98]
55		Ashwagandha (*Withania somnifera*)	Sprout	[99]
56	Valerianaceae	Lattughella (*Fedia graciliflora*)	Leaf	[100]

9. Conclusions

In plants, the expression and regulation of the genes serve as a key regulatory mechanism in defense, development and adaptation to stress. The VIGS approach has enormous potential to be exploited as a tool to understand the gene function and regulation. Among all VIGS systems developed, Tobacco Rattle Virus (TRV) based VIGS vectors are the most widely used ones. Considering all the refinements in inoculation techniques and modifications in TRV viral vectors, TRV-VIGS have mostly been used as a tool for gene function studies and high-throughput functional genomics in plants. TRV-VIGS vectors have provided the effective and extreme trustworthy results with expanded host range. These vectors have also been used to deliver SSNs. In future, it is expected that further growth in knowledge will surely help in innovating advanced designs to enhance the robustness of TRV based vector technology.

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