Advances in the Application of Virus-Induced Gene Silencing in Plants

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Abstract

VIGS (Virus-induced gene silencing) can effectively silence target genes at the RNA level to investigate their functions. The virus vectors used for silencing are divided into three categories, DNA virus, RNA virus, and satellite virus, which can be used to silence the target genes in different species based on existing research. The genes used as markers for silencing system identification include PDS and PCNA. VIGS can be applied to some plants, in particular cash crops and fruit trees, to study their disease-resistance genes and genes related to growth and development to provide a basis for improving crop characteristics.

Keywords

VIGS, Application, VIGS Vector, Marker Genes

1. Introduction

Traditional methods to study plant gene function include transgenic technology, gene knockout, the gene induced overexpression, and RNAi technology. These research methods all have certain limitations, such as long research cycle, the need for genetic transformation and low conversion efficiency, which limit the rapid and efficient application of these methods in the study of plant gene function [1] [2]. However, virus-induced gene silencing (VIGS) is a rapid and efficient tool for plant functional genomics research because it permits knockdown of genes-of-interest and observation of elicited phenotypes within 3 to 4 weeks [3]. VIGS is a post-transcriptional gene silencing technique [4] and has been used as a method to study resistance genes and functional genes of many cash crops.
The molecular mechanism of VIGS is shown in Figure 1; the fragment of the target gene is inserted into the viral vector, the constructed viral vector is introduced into Agrobacterium, and then the plant is infected with Agrobacterium. After the virus enters the plant cells, it will through replication and transcription using various raw materials in the plant cells to form a double-stranded dsRNA. The double-stranded dsRNA in the cells will then be recognized by Dicer analogs of specific RNAase III family endonucleotides and small interfering (si)RNA of 19 - 24 nt will be cut. siRNA binds to RNase in plants as a single strand to form an RNA-induced silencing complex. In the cytoplasm, the mRNA of the target gene will bind to the complex, which leads to the degradation of the mRNA of the target gene. The infected plant would have the same phenotype as a deletion mutation of the target gene, and thus the function of the target gene could be inferred based on the change in the trait [5] [6] [7] [8].

In different RNA-induced silencing systems [9], VIGS can be used for transient transformation and has advantages, such as simple and routine use in a single generation without requiring a long time to screen many transgenic plants, over methods that require permanent modification [1] [2] [10]. Therefore, VIGS is considered to be a powerful reverse genetics technique for studying the function of plant genes [11] [12]. It has been successfully developed and applied in several dicotyledonous plants and some monocotyledonous plants [13] and has been widely used to identify genes related to abiotic stress tolerance [14] [15].

2. Types of Virus Vectors and Marker Genes

Viruses used to carry the target gene fragment are divided into DNA viruses, RNA viruses, and satellite viruses (Table 1). The first VIGS vectors, derived from tobacco Mosaic virus (TMV) [16], potato virus (PVX) [17] [18], and tobacco bell virus...
Table 1. Types of virus vector [62] [63].

| Virus vector                        | Virus genus    | Silenced hosts                                                                 |
|-------------------------------------|----------------|--------------------------------------------------------------------------------|
| **RNA virus**                       |                |                                                                                 |
| Barley stripe mosaic virus          | Hordeivirus    | Barley, Wheat                                                                  |
| Bean pod mottle virus               | Comovirus      | *G. max*                                                                       |
| Brome mosaic virus                  | Bromovirus     | Barley, rice, maize                                                            |
| Pea early browning virus            | Tobravirus     | *P. sativum*                                                                   |
| Poplar mosaic virus                 | Carlavirus     | *N. benthamiana*                                                               |
| Potato virus X                      | Potexvirus     | *N. benthamiana, S. tuberosum*                                                  |
| Tobacco mosaic virus                | Tobamovirus    | *N. benthamiana, N. tabacum L.                                                 |
| Tobacco rattle virus                | Tobravirus     | Arabidopsis, *N. Benthamiana, Tobacco                                           |
| **DNA virus**                       |                |                                                                                 |
| African cassava mosaic virus        | Begomovirus    | *N. Benthamiana*                                                               |
| Cabbage leaf curl virus             | Begomovirus    | Arabidopsis                                                                    |
| **Satellite virus**                 |                |                                                                                 |
| Satellite tobacco mosaic virus      |                | *N. tabacum L.*                                                                 |

(TRV) [19] [20], were originally used for silencing the genes of *Nicotiana benthamiana* and *Solanum lycopersicum*.

2.1. RNA Virus Vectors

BSMV (*Barley striped mosaic virus*) is an orthomolecular RNA single-stranded virus, consisting of three RNA chains: α, β, and γ [21]. RNA α of BSMV ND18 encodes RNA polymerase (RdRp)-dependent methyltransferase/helicase subunits, and RNA β encodes special envelope proteins (CP), and three major proteins essential for virus movement between cells (TGB1, TGB2, and TGB3). RNA γ encodes RdRp polymerase subunit (GDD) and γb proteins, which are involved in viral pathogenesis, long-distance movement and suppression of host RNA silencing defenses. When wheat leaves are infected, symptoms are irregular stripes of dark green and light green. In addition, it appears as yellow and white stripes in early stage new leaves, along with decreased plant height, reduced pistil fertility, and dry seeds [3].

BMV (*Brome mosaic virus*) is a tripartite, single-stranded, positive-sense RNA virus [22]. BMV genomic RNA1 and RNA2 encode 1a and 2a proteins, respectively; both are required for virus replication [23] [24] [25]. BMV genomic RNA3 is dicistronic, encoding a 3a protein required for virus cell-to-cell movement in the plant and a capsid protein (CP) necessary for virion formation and virus movement in the plant [26] [27] [28] [29]. The CP is translated from a subgenomic RNA (RNA4). For both reported BMV vectors, foreign gene fragments are inserted into genomic RNA3 [30] [31] [32] [33].

TRV (*Tobacco rattle virus*) is a soil-borne virus belonging to the RNA virus
family. The sedimentation coefficient of TRV virus is 296 - 3065 and 115 - 2455 S, and the virion length is 180 - 215 and 46 - 114 nm. Virions are straight rods; there are two kinds of virions: long virions (190 - 210 nm × 25 nm) and short virions (40 - 80 nm × 20 - 25 nm), and the ratio of long virions to short virions is 1:2 [34]. The virus genome contains two RNA strands, RNA1 and RNA2. RNA1 encodes RNA polymerase genes, and cysteine-rich proteins, and RNA2 encodes capsid proteins and enzyme cleavage sites of cloned target genes; the virus particles are extremely stable. Two chains of RNA1 and RNA2 constitute binary vectors, so TRV-induced gene silencing requires simultaneous action of RNA1 and RNA2 [19] [35]. RNA 1 encodes sufficient protein for replication and movement within host plants. RNA 2 encodes proteins that allow virions to form and spread between plants, mediated by nematodes [36].

2.2. DNA Virus Vectors

African cassava mosaic virus (ACMV) is a single-stranded (ss)DNA virus, belong to the Begomovirus genus [37]. Geminiviruses are small, circular DNA viruses that can be inoculated into plants using Agrobacterium or microprojectile bombardment of plasmid DNA [38]. The genome of ACMV is composed of DNA-A and DNA-B, which are approximately 2.7 kb; both are required for systemic infection of plants. It is necessary for replication, and transcriptional regulation of viral gene expression to DNA-A and DNA-B are almost identical in homologous areas containing cis-acting elements. The two-part ACMV genome encodes eight proteins responsible for the viral life cycle and movement among host plants. The multifunctional replication initiator protein (Rep) is essential for the initiation of rolling circle replication (RCR) of both DNA A and DNA B. Rep can also act as a transcription inhibitor, causing hypersensitivity and virus resistance in plants. ACMV infection can induce antiviral RNA silencing defenses, affect siRNA production, disturb microRNA biosynthesis, and cause abnormal developmental phenotypes in plants [37].

2.3. Satellite Tobacco Mosaic Virus

STMV (Satellite tobacco mosaic virus) is a natural two-component system that isolates virus replication and movement from silencing-inducible components. This configuration can enhance the stability of the system. Owing to the small genome of STMV (single tRNA of 800 - 1300 bases), satellite virus RNA has a high replication efficiency and accumulates to high numbers in infected plants. In addition, because the genome is small, it is easy to obtain the infectious clones needed for vector construction. Because the satellite virus RNA does not have a cap and does not polyadenylate in vivo, there is no need to protect the satellite virus transcript through laborious and expensive in vitro end modification until it has been inoculated. Satellite viruses can alleviate symptoms caused by helper viruses (Roossinck et al. 1991) and allow sensitive screening for phenotypic changes in knockout plants [39].
3. Marker Genes for VIGS

VIGS will lead to the occurrence of genotypic phenotypes in plants after the silencing of endogenous target genes, and genes that determine the success of the silencing system are called marker genes (Table 2).

PDS (phytoene desaturase) genes serve as a convenient visual indicator for VIGS [5]. Plants that successfully silenced PDS include barley, wheat, soybean, rice, tobacco, and Arabidopsis thaliana [40] [41] [42] [43]. The silenced plants showed photo-bleaching. After the silencing system was stable, the phenotype of the gene defect could be observed more readily in the plants (Figure 2).

PCNA (proliferating cell nuclear antigen), a highly conserved processing factor for DNA polymerase δ, is required for DNA replication and repair and is highly expressed in dividing cells. When PCNA was silenced, young leaves continued to expand, forming cabbage like clusters at the apical meristems. Leaves were often misshapen with truncated basipetal growth and little or no petiole development (Figure 3).

Table 2. Types of marker gene [63].

| Gene | Function | Silence phenotype |
|------|----------|------------------|
| PDS  | phytoene desaturase | Carotenoid synthesis related enzymes | Leaf blade bleaching |
| pcna | Proliferating cell Nuclear antigen | Factor of DNA polymerase δ | Meristems stop growing |
| ChlH | magnesium chelatase | Enzymes involved in chlorophyll synthesis pathways | Leaves bright yellow |
| ChsA | chalcone synthasa | Enzyme of anthocyanin synthesis pathway | A part or whole of a design or color that turns white |
| su   | Sulfur gene | Magnesium ion chelase in chlorophyll synthesis pathway | superior leaf |
| rpIIa| RNA polymeraseII-a | RNA synthesis | Leaves twisted and whitened |
| gfp  | green fluorescent protein | Fluorescence occurs under ultraviolet excitation | The leaves appear red under ultraviolet light |
| luc  | luciferase | Fluorescence occurs under ultraviolet excitation | There was no fluorescence under ultraviolet light |

Figure 2. PDS silencing phenotype. (a) Tomato leaf infected with TRV virus only. (b) PDS gene silenced in tomato using TRV construct. Photobleached tissue indicates regions of silencing [1].
Figure 3. VIGS of NbPCNA caused abnormal leaf development and growth retardation [37].

4. Application of VIGS in Plants

The phenotype of a gene defect after utilization of VIGS technology is readily apparent, so it is more widely used in research of crop disease-resistance genes. After silencing the relevant resistance genes using VIGS technology, inoculation of bacteria will occur and the associated stress will be imposed. The phenotype of the plant can clearly be observed. In addition, some environmental stresses and the emergence of crop diseases may lead to the decline of cash crop yield, so functional research on related resistance genes is very important for improving the characteristics of affected crops.

4.1. Studies on the Function of Disease-Resistance Genes

In the study of resistance genes related to leaf rust, Scofield et al. silenced wheat varieties Lr21, RAR1, SGT1 and HSP90 by BSMV-VIGS and found that these genes played an important role in the lr21-mediated resistance pathway [44]. In the study of resistance to powdery mildew, Wang et al. used BSMV-VIGS to silence Hv-LRR in *Haynaldia villosa* and analyzed its role in resistance to powdery mildew [45]. Várallyay et al. used the BSMV-VIGS system to construct the BSMV: PDS: Mlo vector has realized the labeled gene with the target gene silence system, promoted the VIGS system more widely used [46]. MLO, a transmembrane protein, plays a negative regulatory role in inhibiting plant defenses in uninfected tissues. It protects against cell death and the response to biotic and abiotic stress. In this study, wheat plants showed broad-spectrum powdery mildew resistance at the individual level after the lack of expression of this functional protein. Delventhal et al. used BSMV-VIGS system-silenced MLO in barley. High resistance to powdery mildew was also found [47]. Further, the resistance gene of barley powdery mildew was studied. Hein et al. used VIGS to confirm the requirement for Sgt1, Rar1, and Hsp90 genes in the Mla13-mediated resistance response to powdery mildew in barley, as previously observed in other R gene-mediated responses in dicot species including *Arabidopsis* and *Nicotiana* [48].

4.2. Investigation of Abiotic Stress Resistance Genes

In the study of abiotic stress resistance genes, Zhang et al. (2018) used VIGS and
iTAQ and found that TaGRP2, CDCP, and Wcor410c potentially play vital roles in conferring osmotic-cold tolerance in bread wheat. Tavakol (2017) silenced AetDreb2, a member of the ERF family, which accelerates plant wilting and reduces RWC under dehydration conditions. Further, silencing of the ERF109 gene confirmed its influence on tolerance to salt stress in Arabidopsis [49]. In addition, two new members of the ERF family in pepper (CaPTI1) and P. somniferum (PsAP2) affected the regulation of defense-related genes and caused a significant reduction in the AOX1 level through VIGS [50] [51]. Zhang et al. (2018) used TRV-VIGS to elucidate the effect of ghb1-1 on salt resistance of cotton seed. BSMV-VIGS was utilized to silence the SAMS, SAMDC and γ-ECS genes, and their relationship with drought resistance was analyzed. Silenced plants were compared to normal plants after drought stress. Silenced plants were more susceptible to the effects of drought stress, and the SAMS, SAMDC and γ-ECS genes were associated with drought resistance and water saving functions. These results showed that the S-adenosine methionine metabolism pathway in wheat drought resistance and water saving has potential value [52]. Kuzuoglu-Ozturk et al. (2012) used BSMV-VIGS to confirm that Atg8-silenced plants exposed to osmotic stress had decreased Atg8 expression levels in comparison to controls. Thus, Atg8 is a positive regulator in osmotic and drought stress responses.

4.3. Study of Genes Related to Growth and Development

The function of TaRSR1 in starch synthesis of wheat grains was explored using VIGS by Liu et al. (2016) In addition, in research related to the Wheat root length gene of COI1 [53], VIGS of the P23k gene led to abnormal leaf development, asymmetric orientation of main veins, and cracked leaf edges caused by mechanical weakness. Further, the involvement of P23k in the synthesis of cell wall polysaccharides and contribution to secondary wall formation in barley leaves were confirmed [54]. In plants, phospholipase D (PLD) is a key component of signal transduction in diverse pathways, including programmed cell death, senescence, and responses to biotic and environmental stresses. This research using BSMV-BASED VIGS found that the PLD gene was related to environmental stress responses [55]. Further, Ding et al. (2006) used VIGS technology to silence actin protein in rice. In addition, the function of TaRSR1 in starch synthesis in bread wheat used VIGS [56]. Bread wheat (Triticum aestivum L.), one of the major staple crops in the human diet, is an essential component of global food security [57]. This research can help improve crop varieties. In the study of tomato fruits, by silencing LeACS2, LeCTR1, and LeEILs, their role in fruit development was discovered [20]. This research also promoted studies related to fruit development using VIGS.

5. Future Prospects

VIGS is a reverse genetic method for studying the function of plant genes. It uti-
lizes RNA-mediated post-transcriptional gene silencing and acts as an antiviral
defense system in plants. VIGS, as a tool, has the advantages of being simple to
perform, highly efficient, and independent of genetic transformation in compar-
ison with traditional technology and is suitable for analysis of large-scale gene
function and lethal phenotype gene analysis, and so on [58]. However, this tech-
nique still has many limitations, such as limited inoculation methods between
different plants and difficulty in observing characteristics of some genes after
deletion. Still, the rapid and efficient characteristics of this technique cannot be
ignored. In recent years, geminivirus vectors that can infect many plant species,
including wheat, have been developed for exogenous protein expression. For
other viral systems, inserted genes have limited cargo capacity in the proto-
viral system. However, the insertion of larger foreign genes is possible, which involves
the suppression of important genes encoding motor proteins and shell proteins,
thereby eliminating intercellular movement or plant-to-plant transmission and
inhibiting virus replication [59]. In addition, after the deletion of genes related
to nutrient growth and resistance to diseases and insect pests and environmental
stress, plants can show clearly observable characteristics, which also is favorable
for the study of the functions of these genes.

Recently, the expression of GFP and iLOV and co-expression with the target
gene has been studied. In addition, there have been studies on the expression ef-
effects of GFP with different fragment sizes in hosts carrying the VIGS vector [7]
[21]. Cheuk and Houde (2017) changed the components of BSMV to confirm
that different amounts of components had different cargo capacities [60]. This
research can allow changes in virus vectors, so that they can carry at least two
gene fragments, which would permit more gene functions to be determined. Thus,
when we build vectors, we can put a target gene and a marker gene in the vector
or two genes that can produce phenotypes, and then silence and study them. In
this way, we can use VIGS technology to study more gene functions.

For inoculation methods, the characteristics of plants studied at different growth
stages are also limited owing to the existence of virus transmission time in many
current methods. Zhang et al. (2017) used vacuum and co-cultivation agroinfil-
tration of germinated seeds, which can greatly advance the growth stage of silenced
plants, and is useful in studying the characteristics of plants at the seedling stage
[61].

VIGS technology plays a major role in improving the characteristics of some
crops and fruit trees, which promotes the development of cash crops.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.
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