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Research Progress of Virus-Induced Gene Silencing and its Application in Gramineae

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Abstract **Review Article**

With the proliferation of genome sequence information, biological science research has entered the era of big data, but how to annotate the function of genome information has become an important research goal. Virus-induced gene silencing (VIGS) is a powerful tool that has been used to study key genes in multiple plant growth and development processes. This technology uses the innate antiviral defense system of plants. And generates small interfering RNA (siRNA) in the plant by inserting the target gene fragment into the viral vector, so that the transcript of the endogenous gene of the plant becomes a target for degradation, resulting in the expression of the target gene being down-regulated. This paper systematically reviews the development, mechanism, and advantages of VIGS technology, focuses on the vectors that can be applied in grasses, and finally discusses the limitations and development trend of VIGS technology in the application of Gramineae.

Keywords: VIGS, functional genomics, apply.

1. INTRODUCTION

As large amounts of crop genomic information are published, studying and verifying the specific function of genes is an important task in the post-genome era. Loss-of-function is an important means of revealing gene function. Loss of function of genes can usually be achieved at 3 levels: the first is gene mutation (GM) at the genome level, that is, a nonsense mutation or deletion of the coding sequence of a gene, resulting in loss of gene function; the second is transcriptional gene silencing (TGS) at the transcriptional level, that is, the gene coding sequence is normal, but transcription is inhibited, making it unable to function; the third is posttranscriptional gene silencing (PTGS) at the posttranscriptional level, that is, genes can be transcribed normally, but cannot be translated normally, such as premature degradation, resulting in loss of gene function. At present, commonly used loss-of-function research methods still rely on transgenic technologies, such as creating Transfer DNA (T-DNA) insertion mutants or gene editing (Clustered Regularly Interspaced Short Palindromic Repeats) targeting mutants, inhibiting targeted genes through CRISPR, and using RNA interference (RNA). Interference, RNAi) causes post-transcriptional silencing of the gene of interest. However, a mature transgenic technology system has not yet been established for a variety of crops, so the above methods cannot be applied to the study of their gene function [1-2]. VIGS is a technology that performs reverse genetic manipulation on plants and does not rely on transgenes [3], which integrates non-transgenic systemic infection of plant viruses, the immune response of plants, and cell RNAi silencing mechanism, which can be a simple, fast, and efficient to silencing target genes, which belongs to post-transcriptional gene silencing. Compared with the gene function loss research method that relies on genetic transformation, VIGS technology has the characteristics of no need for genetic transformation and a short experimental period and is increasingly used in the study of gene function in different crops [4]. Based on the VIGS mechanism, this paper systematically elaborates on the development process of plant VIGS technology, focuses on the application status of VIGS in Gramineae, and further summarizes and discusses the limitations and prospects of VIGS, to provide a reference for VIGS technology to study the gene function, further application and development of Gramineae.

2. Development and Mechanism of VIGS Technology

VIGS technology is based on the principle of plant-based RNA interference (RNAi) and viral immune response [4]. In infected plants, vectors containing the objective gene (plasmid or viral DNA, etc.) can replicate in large quantities [5]. When the viral vector replicates and expresses in the host, under the action of RNA polymerase, double-stranded RNA (dsRNA) is produced, and dsRNA is cleaved by specific endonuclease enzymes in cells to produce 21-25 nt of small interfering RNA (siRNA), siRNA can induce messenger RNA (messenger RNA) with its homologous plant endogenous gene, mRNA) degraded and can bind to form RNA-induced silencing complex (RISC) [6, 7]. On the one hand, RISCs can specifically interact with homologous RNAs in the cytoplasm to degrade homologous RNAs, resulting in post-transcriptional PTGs; RISCs, on the other hand, specifically interact with homologous DNA within the nucleus to be modified by methylation, etc., resulting in TGS [8].

Viruses as an important vector in VIGS technology, the earliest researchers mainly focused on RNA viruses, and the crops involved mainly include Ben's tobacco, tomato, and so on. Kumagai MT *et al.*, [9] used tobacco mosaic virus (TMV) as an infection vector. It carries the cDNA antisense strand of the PDS target gene, and after infection with tobacco, leaves appear albuminized. Since then, a large number of studies have proved that the Tobacco rattle virus (TRV) is the most widely hosted virus among plant viruses. It can infect more than 400 varieties of more than 50 dicots and monocots, although many hosts are infested without causing symptoms. Liu Y *et al.*, [10] successfully infected tomato plants with the TRV virus vector, resulting in the silencing of three genes PDS, CTR1, and CTR2, and the virus could also infect tomato fruits, resulting in gene silencing [11]. Chung E [12] was the first to infect pepper leaves with TRV, and after PDS silence, chlorophyll molecules were subsequently photo oxidized and degraded, resulting in the photo bleaching of leaves, which proved that this technique could be used to study the gene function of peppers. In addition, this technology has also been successfully applied to crops such as petunias [13] and strawberries [14]. In addition to tobacco rattle virus TRV, there are many viral vectors involved in virus-induced gene silencing technology. For example, Ruiz MT *et al.*, [14] constructed potato virus X (PVX) [15] vectors with the cDNA sequence of the PDS gene of Bentham's tobacco. Plants exhibit photo bleaching after tobacco infection and can cause and ed systemic gene silencing [16]. Faivre-Rampant O *et al.*, [17] study that PVX can induce PDS gene silencing in diploid and tetraploid potatoes and the potato leaves appear photo bleaching phenomenon. This phenomenon can be observed in tissue culture seedlings and miniature potato blocks with multi-generation propagation. In addition, VIGS vectors such as Pea early browning virus (PEBV) [18] and Barley stripe mosaic virus (BSMV) have been studied and applied, VIGS technology breaks through the limitations of crop species and virus types. It is widely used to study the gene function of a variety of crops [19].

3. Applications

At present, VIGS technology has been successfully applied to plants such as *Nicotiana tabacum* L., *Arabidopsis thaliana*, *Gossypium spp*, *Lycopersicon esculentum* Mill., *Solanum tuberosum* and other plants [20]. There are three main VIGS vectors used in Gramineae, namely barley striped mosaic virus, bromemo-saicvirus (BMV), and rice tungro bacilliform virus (RTBV). The first two of these are RNA viruses and the last is a DNA virus. BSMV vectors are mainly used in grasses such as wheat, barley, oats, wild two-grain wheat, tufted wheat, and two-spike short-stemmed grass [21]. BMV vectors are mainly used in Gramineae such as rice, corn, and barley, while RTBV vectors are mainly used in rice.

BSMV is a barley-streaked mosaic virus, an RNA virus containing three parts of the genome: α, β, and γ.RNAα encodes the methyltransferase/helicase subunit of RNA-dependent RNA polymerase (RdRp), RNAβ specifies the coat protein (CP) and the three major triple gene blocking (TGB) proteins (TGB1, TGB2, and TGB3), and the polymerase (GDD) subunit of RNAγ encoding RdRp and gamma proteins. At present, BSMV vectors mainly use modified β and γ molecules as recombinant carriers. The most commonly used of these are γ molecules as carriers for recombination. Holzberg first inserted PacI and NotI clone sites downstream of gamma-b's Open Reading Frame (ORF) terminator; this makes the BSMV-VIGS system apply for the first time in Gramineae barley. Another strategy is to introduce cloning sites upstream of the gamma-b sequence. Recently, β molecules of BSMV have also been modified for use in VIGS systems. Ka11 Walek *et al.*, inserted the BamHI clone site between the ORF and polyA of BSMVβc to modify the BSMVβ molecule. In addition, the coding region of γ b gene of BSMV was transformed into a Ligation-independent-cloning (LIC) independent of the ligation reaction, so that it can meet the requirements of high-throughput cloning. In addition, Campbell *et al.*, also infected barley with recombinant vectors carrying two target genes in the β and γ of BSMV, achieving simultaneous silencing of both genes [22].

BMV is an RNA virus belonging to the genus Brome mosaic virus, which is less used in VIGS systems than BSMV. BMV, like BSMV, consists of three parts of the genome: RNA1, RNA2, and RNA3. One current strategy for modifying BMV vectors is to replace the RNA3 molecule of F-BMV with RNA3 molecules of R-BMV with more convenient restriction cloning sites. In 2006, Ding *et al.*, [23] for the first time, the separated F-BMV was transformed as the carrier of

the VIGS system, VIGS experiments were successfully carried out on rice, corn, and barley. Another remold of BMV vectors is the introduction of a nucleotide and two cloning sites in the 3' UTR region of the RNA3 molecule, successfully silencing the PDS gene in barley. In another recent experiment, Benavente *et al.*, [23] successfully achieved gene silencing in maize using BMV-VIGS technology.

RTBV is a pseudo retrovirus with double-stranded DNA belonging to the genus Gruvirus. The genome of RTBV is about 8 kb and includes four open reading frames ORFs-IV, where the function of the proteins encoded by ORFI, II, and IV remains unclear. ORF III encodes a polyprotesome including capsid protein, aspartate protease, and reverse transcription RNase H. By introducing multiple clonal sites to modify RTBV vectors, DNA virus vectors was successfully used to achieve gene silencing in rice for the first time by agrobacterium connection.

In addition, in 2022, Zhang Li *et al.*, [23] preliminarily constructed a FoMV virus-mediated gene silencing system in sorghum, which can provide a faster and more efficient research tool for the study of sorghum gene function.

4. VIGS Advantages

The advantages of VIGS for studying gene function are its ease of construction, short cycle time, and low cost. It usually takes only a few weeks to construct a recombinant vector and infiltrate the plant for functional characterization, so it can be used for functional characterization of genomic and EST sequences on a large scale [24]. VIGS has outstanding advantages for the study of difficult-to-obtain mutant crops. When the plant genome is large, the conserved DNA region of the target gene family is utilized, VIGS avoids the problem of functional redundancy by silencing multiple members of a gene family, and when the big family of proteins or gene duplications are involved, various point mutations and insertions fail to produce an appropriate phenotype [25], VIGS make homologous genes with sequence difference of 10%-20% silencing [26]. The use of VIGS to silence target genes does not require the complete coding sequence of the target gene, only a fragment of the cDNA is required to achieve silencing of the target gene [27]. In contrast, the cost of dsRNA synthesis is too high when RNAi technology is applied, which is not conducive to the promotion of products [28]. CRISPR/Cas9 technology relies on PAM sequences to enable gene editing, and editing is not possible in regions where there are no PAM sequences nearby [29]. VIGS has the advantage of integrating cDNA libraries into viral vectors for high-throughput gene screening analysis [30].

5. Limitations and Solutions of VIGS Application in Gramineae

5.1. The Silence Time is short and the Silent Effect is Unstable

First, the virus-induced gene silencing had a short silence time and an unstable silencing effect. VIGS technology silence lasts for a short time, generally, about one month, so the application of VIGS technology for plants with long growth cycles will be limited. Moreover, the effect of the VIGS silencing gene is unstable on each plant, and the virus activity can be enhanced by selecting the virus vector specific to the host plant or by modifying the vector, prolonging the action time of VIGS on the host and enhancing the silencing effect of the target gene, and exploring the conditions of specific vector inoculation may avoid the problems of the short duration of silence and unstable effect [22].

5.2. Selection of Target Genes

The length, orientation, and sequence of the target gene fragment inserted in the VIGS vector will affect the silencing efficiency. Inserting a gene fragment that is too long may result in a virus that does not move systematically, while one that is too short may not produce an effective siRNA and may be lost more quickly. When tobacco PDS gene fragments of different lengths were inserted into TRV vectors, when the length of the inserted tobacco PDS gene fragments was 200~1300 bp, a good silent phenotype could be produced. While inserting PDS gene fragments of different lengths into vectors constructed based on the PNRSV virus, it was found that photo bleaching could occur when the gene fragment length was 100-200 bp, and the inserted PDS gene fragment was lost when it was larger than 200 bp. Therefore, the length of the inserted target gene fragment has to be explored according to different viral vectors. The effect of the orientation of the target gene insertion fragment on the silencing efficiency is also an issue worthy of deeper investigation. The reverse repeat sequence of 60 bp PDS was cloned, and the forward and reverse fragments were inserted into the Foxtail mosaic virus (FoMV) vector simultaneously, and photo bleaching could occur 21 d after inoculation. Based on the vector constructed by Bean pod mottle virus (BPMV), the forward and reverse target gene fragments were inserted into the vector separately, and it was found that the reverse insertion could obtain higher silencing efficiency; while the same fragment of PDS was inserted into the vector constructed by TRSV in both forward and reverse directions and was found to produce photo bleaching, there was no significant difference in silencing efficiency. Therefore, the effect of the direction of insertion of the target gene fragment on the silencing efficiency may also depend on the properties of the viral vector itself. It may also be related to whether hairpin-loop-like dsRNA structures can be produced, but the exact mechanism has not been elucidated. In addition, the selection of the insert fragment sequence needs to consider whether the insert fragment can

produce effective siRNAs and the off-target silencing issue [31].

5.3 Off-Target Silencing Phenomenon

VIGS can lead to inhibition of the expression of non-target genes, resulting in off-target silencing, resulting in a decrease in the silencing efficiency of genes, and off-target silencing depends on many factors, such as the location of target genes, the length of dsRNA, whether the method of transforming infected plants is appropriate, and the efficiency of VIGS vector infection. In response to the above problems, when constructing VIGS vectors, SGN, siRNA-Scan, and other software can be used to select gene silencing regions and evaluate whether the resulting 21 nt siRNA can perform efficacy. Zhou *et al.*, [32] proposed an improved method of vector construction using a 70 bp gene fragment containing a 21 bp base fragment from three different genes that do not match each other, using this improved method to reduce off- target silencing of VIGS and to specifically knock out highly similar members of the gene family. Improved vectors should be accompanied by the exploration of new virus induction mechanisms. Ossowski *et al.*, demonstrated that cabbage leaf curling virus (CaLCuV) and TRV in the use of the newly developed microRNA-based virus-induced gene silencing (MR-VIGS) method in Ben's tobacco, can also reduce off-target phenomena.

6. Outlook

As a new reverse genetics technology for plant gene function identification, VIGS does not require complex mutant screening and acquisition of transgenic plants compared with gene function research methods such as transgene, gene knockout, and antisense invalidation, which is easy to operate, fast to obtain phenotype and low cost. At present, VIGS has been successfully applied to the mining of functional genes for a variety of Gramineae traits. With the continuous development of new technologies such as high-throughput sequencing and the optimization of VIGS technology, the limitations of VIGS technology have gradually been overcome. As an important technical means for the study of crop gene function in the post-genomic era, VIGS technology will accelerate the research progress of gramineae plant gene function and promote the rapid development of Gramineae crop improvement and molecular breeding.

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