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Virus-induced gene silencing and its application in plant functional genomics

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Virus-induced gene silencing is regarded as a powerful and efficient tool for the analysis of gene function in plants because it is simple, rapid and transformation-free. It has been used to perform both forward and reverse genetics to identify plant functional genes. Many viruses have been developed into virus-induced gene silencing vectors and gene functions involved in development, biotic and abiotic stresses, metabolism, and cellular signaling have been reported. In this review, we discuss the development and application of virus-induced gene silencing in plant functional genomics.

virus-induced gene silencing, gene function, gene expression

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In recent decades, abundant plant genome information has become available through genome sequencing and expressed sequence tag (EST) analysis. In the post-genome era, a major challenge is to determine gene functions. However, even in the model plant *Arabidopsis*, less than 10% of predicted genes have been identified and characterized functionally [1]. To knockout or reduce gene expression, traditional approaches for high-throughput reverse-genetic gene function screening include chemical induced mutation, random mutation, and T-DNA insert mutation. However, these methods may be hindered in the studies of non-model plants because of their large genome size, low transformation efficiency, and lack of a clear genetic background.

Compared with traditional methods, virus-induced gene silencing (VIGS) is a powerful, recently developed approach for plant loss-of-function assay that can overcome the above-mentioned problems [2,3]. A recombinant virus for VIGS, delivering a fragment of plant gene into plant cells, can induce plant defense mechanism to silence both the targeted endogenous plant gene and the virus, through post-transcriptional gene silencing (PTGS). Gene functions can then be characterized by the sequence-specific phenotypes [2,4]. Combined with the abundant plant genome and EST sequences, VIGS will significantly accelerate the gene function identification in various plant species. More than 30 VIGS vectors have been developed, and these vectors have been widely used to uncover the functions of genes involved in basic cellular functions, metabolic pathways, development biology, plant-microbe interaction, and abiotic stress (Table 1) [5,6]. In this review, we will discuss the recent advances in the application of VIGS in plant functional genomics.

1 Establishment and development of VIGS

In 1997, van Kammen [7] first mentioned the term "VIGS'' to describe the phenomenon of recovery from virus infection. Since then, the term "VIGS" has been used exclusively

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for the technique of using recombinant viruses to knockdown expression of endogenous genes [2,8]. In the early stages, most of the VIGS systems were based on RNA viruses. In 1995, Kumagai *et al*. [9] inserted a fragment of *phytoene desaturase* (PDS), a key enzyme of the carotenoid biosynthetic pathway, into the Tobacco mosaic virus (TMV) [9]. When this recombinant virus was inoculated into *Nicotiana benthamiana*, a blench phenotype in the leaves was observed and this phenomenon was caused by reduction in endogenous PDS mRNA. In 1998, similar results were obtained using another RNA virus, Potato X virus (PVX), carrying a fragment of the *PDS* cDNA [8]. Thus, VIGS is considered to be a useful technique for suppressing endogenous gene expression and uncovering plant gene functions. In 2001, a novel VIGS vector was established based on Tobacco rattle virus (TRV). TRV was demonstrated to induce more efficient silencing of transgenes and endogenous genes [10]. TRV could spread more vigorously throughout the entire plant, including meristem tissue, and the symptoms induced by TRV are much milder compared with other viruses [10]. The TRV vector has been widely used in gene function studies of tomato, tobacco, *Petunia hybrida*, chili pepper, *Arabidopsis*, and cotton plants (Table 1).

DNA viruses, such as geminiviruses, have also been successfully modified into VIGS vectors. In 1998, Robertson *et al.* used TGMV as a vector to determine whether episomal DNA could induce silencing of homologous, chromosomal genes. They modified the TGMV DNA-A component into a vector and induced silencing of the two marker genes, the *sulfur* (su), a magnesium chelatase required for chlorophyll formation, and the firefly *luciferase* (luc) gene [11]. Subsequently, VIGS vectors were developed based on the DNA viruses African cassava mosaic virus (ACMV) and Cotton leaf crumple virus (CLCrV), which have been used for functional genomic studies in cassava and cotton plants [12,13].

Subsequently, a novel VIGS vector was developed based on an RNA satellite virus, the U2 strain of TMV (STMV). The RNA satellite virus silencing vector was able to obtain pronounced and severe knockout phenotypes for 13 targeted endogenous genes involved in various biochemical pathways and expressed in different plant tissues [14]. The advantages of STMV as an excellent candidate VIGS vector are its small genome size and high replication rate in plants.

Tao *et al*. and Huang *et al*. reported induction of VIGS based on Tomato yellow leaf curl China virus (TYLCCNV) betasatellite (DNAβ) and Tobacco curly shoot virus (TbCSV) alphasatellite (DNA1) [15,16]. They demonstrated that these modified satellite vectors could effectively suppress *GFP* in transgenic *N. benthamiana* and endogenous *Su* in plants, when co-agroinoculated with their helper viruses. These modified satellite vectors have been used to induce gene silencing in *Nicotiana* spp., *Solanum lycopersicum*, and *Petunia hybrida* plants [15–19].

VIGS vectors have not only been applied in dicotyledonous plants, but also in monocotyledonous plants. In 2002, VIGS was established in barley and wheat using Barley stripe mosaic virus (BSMV), where certain genes related to resistance against leaf rust or powdery mildew were characterized in barley [20]. Ding *et al*. [21] also showed that a modified VIGS vector based on Brome mosaic virus (BMV) could be used for the analysis of gene function in rice and maize.

Table 1 Various VIGS vectors and plant species used for gene silencing

Virus vector	Plant species and tissue for inoculation	Inoculation method	References
RNA virus			
Apple latent spherical virus (ALSV)	*Nicotiana* spp., *Arabidopsis thaliana*, Apple, Pear, *Solanum lycopersicum*, *Cucumis* spp.: leaf; *Leguminaceae*: leaf; beanpod: seed	*Nicotiana* spp. and *Arabidopsis thaliana* with sap inoculation; soybean, apple and pear with particle bombardment into seedlings using extracted RNA from the virus infected plants	[22–24]
Brome mosaic virus (BMV)	*Oryza sativa, Zea mays*, *Hordeum vulgare*, *Festuca arundinacea*, *N. benthamiana*: leaf	Rub inoculation of *in vitro* transcripts for other crops and vacuum infiltration for rice	[21,25,26]
Barley stripe mosaic virus (BSMV)	*Brachypodium distachyum*: root; Barely, *Avena* species and *Triticum aestivum*: leaf	Rub inoculation of *in vitro* transcripts	[20,27–30]
Bean pod mottle virus (BPMV)	*Glycine max*: root, leaf and shoot; *Phaseolus vulgari*: leaf	Rub inoculation of *in vitro* transcripts and particle bombardment	[31–33]
Cucumber mosaic virus (CMV)	*G. max*: leaf and seed	With rub inoculation of *in vitro* transcripts in *N. benthamiana* and then inoculation of soybean plants with extract sap	[34]
Cymbidium mosaic virus (CymMV)	*Phalaenopsis orchids*: flower	Rub inoculation of *in vitro* transcripts	[35]
Potato virus X (PVX)	*N. benthamiana*: leaf; *Solanum tuberosum*: leaf, root and tuber	Agro-inoculation	[8,36,37]
Potato virus A (PVA)	*N. benthamiana*: leaf	Rub inoculation of *in vitro* transcripts and particle bombardment	[38]
Pea early browning virus (PEBV)	*Lathyrus odorata*: leaf, shoot, root, flower and beanpod; *Medicago truncatula*: leaf	Agro-inoculation	[39–41]
Poplar mosaic virus (PopMV)	*N. benthamiana*: leaf	Agro-inoculation	[42]

(*To be continued on the next page*)

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Virus vector	Plant species and tissue for inoculation	Inoculation method	References
Plum pox virus (PPV)	*N. benthamiana*: leaf	Agro-inoculation	[43]
Sunn-hemp mosaic virus (SHMV)	*N. benthamiana* and *Medicago truncatula*: leaf	Rub inoculation of *in vitro* transcripts	[44]
Tobacco mosaic virus (TMV)	*N. benthamiana*: leaf	Rub inoculation of *in vitro* transcripts	[45,46]
Tobacco necrosis virus A (TNV-A)	*N. benthamiana*: leaf	Agro-inoculation	[47]
Tomato bushy stunt virus (TBSV)	*N. benthamiana*: leaf	Rub inoculation of *in vitro* transcripts	[48]
Tobacco rattle virus (TRV)	*Aquilegia vulgaris*: flower; Opium or California poppy: leaf and flower; *N. benthamiana*: leaf, shoot, flower and root; *S. lycopersicum*: leaf, shoot, flower and fruit; *A. thaliana*: leaf, shoot, flower and silique; *Capsicum* spp.: leaf and fruit; *Petunia hybrida*: leaf and flower; *Solanum* spp.: leaf; *Gossypium* spp.: leaf; *Jatropha curcas L.*: leaf; *Thalictrum dioicum*: leaf and flower	Agro-inoculation	[10,49–63]
Turnip yellow mosaic virus (TYMV)	*A. thaliana*: leaf, shoot, flower and silique	Rub inoculation of *in vitro* transcripts	[64]
DNA virus			
Abutilon mosaic virus (AbMV)	*N. benthamiana*: leaf	Agro-inoculation	[65]
African cassava mosaic virus (ACMV)	*Manihot esculenta* and *N. benthamiana*: leaf	*N. benthamiana* with agro-inoculation and *Manihot esculenta* with particle bombardment	[13]
Beet curly top virus (BCTV)	*Spinacea oleracea* and *S. lycopersicum*	*Spinacea oleracea*: with particle bombardment and *S. lycopersicum* with agro-inoculation	[66]
Cabbage leaf curl virus (CaLCuV)	*A. thaliana* and *N. benthamiana*: leaf	*A. thaliana* with particle bombardment and *N. benthamiana* with agro-inoculation	[19,67]
Cotton leaf crumple virus (CLCrV)	*Gossypium* spp.: leaf, flower and boll	Particle bombardment and agro-inoculation	[12]
Grapevine virus A (GVA)	*N. benthamiana* and *Vitis vinifera*: leaf	Agro-inoculation	[68]
Pepper huasteco yellow vein virus (PHYVV)	*Capsicum* spp: fruit	Particle bombardment	[69]
Rice tungro bacilliform virus (RTBV)	*O. sativa*: leaf	Agro-inoculation	[70]
Tomato golden mosaic virus (TGMV)	*N. benthamiana*: leaf	Particle bombardment	[71]
Tomato leaf curl virus (ToLCV)	*N. benthamiana* and *S. lycopersicum*: leaf	Agro-inoculation	[72]
RNA satellite virus			
Satellite of tobacco mosaic virus (STMV)	*N. tabacum*: leaf and flower	Rub inoculation of *in vitro* transcripts	[14]
Satellite DNA			
Tomato yellow leaf curl China virus (TYLCCNV) Betasatellite	*S. lycopersicum*: leaf, shoot, fruit and root; *Nicotina* spp.: leaf; *Petunia hybrida*: leaf and flower	Agro-inoculation	[15,17,18]
Tobacco curly shoot virus (TbCSV) Betasatellite	*N. benthamiana*: leaf	Agro-inoculation	[73]
TbCSV Alphasatellite	*S. lycopersicum* and *Nicotina* spp.: leaf; *Petunia hybrida*: flower	Agro-inoculation	[16,19]

2 Methodology and development of VIGS

In a VIGS system, to suppress expression of an endogenous plant gene, a fragment of the gene to be silenced should be cloned and inserted into the VIGS vector and then inoculated into plants. The VIGS phenotype can be subsequently observed. Generally, to increase the efficiency of silencing, the VIGS system should be optimized.

First, the size of the inserted fragment of target endogenous gene may affect the efficiency of VIGS. Most VIGS vectors have the capacity to carry a fragment of length between 150 and 800 bp. VIGS vectors may fail to induce gene silencing if a fragment of more than 1500 bp is inserted. Although some studies showed that a 23 bp insertion was able to induce VIGS, fragments of 200–350 bp in length is usually chosen for VIGS to obtain higher silencing efficiency. Furthermore, some studies found that the orien-

tation of the inserted gene fragment was also an important factor that could affect the efficiency of VIGS, with higher silencing efficiency being induced by a reverse oriented insertion compared with that of a forward oriented insertion. However, it is not applicable to all vectors. For example, the efficiency of the TYLCCNV DNAβ vector is the same whatever the orientation of the inserted fragment [19]. Additionally, the silencing efficiency could be significantly enhanced if the target fragment was constructed as a hairpin structure [74].

Selection of the target gene is important for VIGS. Evidence has shown that an improper gene fragment might induce off-target silencing, producing an inaccurate phenotype [75]. Many candidate fragments can be selected for silencing of a specific gene. However, if the target gene belongs to a gene family, some sequences may have conserved domains among different genes in the gene family, and the fragment of the target gene may have more than 23 bp that is homologous to other genes in the gene family resulting in the degradation of non-target genes. Therefore, a more specific fragment needs to be chosen. Generally, a fragment from UTR region is a good choice. On the other hand, the conserved domains should be chosen to avoid functional complementation by genes from the same family; in this case all the genes in the family are silenced [76].

The efficiency of gene silencing may be affected by different inoculation methods. The common methods used for inoculation are agro-infiltration, rub-inoculation with RNA transcripts, and particle bombardment (Table 1). For some viruses, susceptible plants will be inoculated firstly to multiply the virus, and then the sap or the virus RNA extract will be used to inoculate target plants. High silencing efficiency was reported using agrodrench, a method of watering the plant roots with agro-inocula directly [60]. Liu *et al*. [58] successfully induced the TRV vector into tomato by spraying a TRV agro-culture using an airbrush. Ding *et al*. reported that efficient gene silencing could be obtained by vacuum agro-infiltration in plants that are hard to inoculate by conventional methods. For fruits, direct injection with an agro-culture produces a more obvious silencing phenotype than inoculation of cotyledons or seedlings [53,77]. Some studies showed that efficient silencing could be induced by injecting plucked tomato, strawberry and bilberry fruits with an agro-culture containing VIGS vector [78–80], which is useful for studying gene functions during the post harvest stage. Co-inoculation of viral suppressors with VIGS vectors may also improve the silencing efficiency. When plants were inoculated with a mixture of VIGS vector and a gene-silencing suppressor, higher accumulation of virus in local inoculated cells induced a higher efficiency of silencing in systemic leaves. With the establishment of more and more new virus inoculation methods, VIGS will be applicable to more plant species.

Finally, environmental factors of plant growth will affect the efficiency of gene silencing. At higher temperatures, the accumulation of virus is significantly reduced, which impairs the efficiency of virus induced silencing. On the other hand, lower temperatures lead to higher virus concentration and silencing efficiency. For TRV vectors, tomato plants should be kept at less than 21°C. Lower temperature and humidity will increase silencing efficiency [12,54]. However, for some vectors, temperature is not so important, for example, both DNAβ and DNA 1 vectors can induce highly efficient silencing from 22 to 32°C [15,18].

3 Validation of gene functions in different plant organs or tissues via VIGS

The high efficiency of VIGS has led to its increasing use in uncovering the functions of hundreds of plant genes involved in defense response pathways, plant development, and metabolism. Recent progress in gene function identification by VIGS is detailed below.

3.1 Genes involved in the defense response to pathogens, insects, and abiotic stresses

Plants grow in an environment surrounded by a diversity of microbes and abiotic stresses. A highly effective defense system has been evolved to resist potential attack by biotic and abiotic stresses. Previous studies have determined the functions of diverse plant genes involved in virus-, bacteria-, fungi-, and insect-resistance and stress responses.

In the study of plant resistance to virus infection, the most successful examples of using VIGS to uncover gene functions in defense response pathway was the *N* gene against TMV and *Rx* gene against PVX. Up to now, a number of genes have been identified, such as *NRG1*, *NbCA1*, *NbCAM1*, *NbrbohB*, *RAR1*, *EDS1*, *NPR1/NIM1*, *MEK1*, *MAPKK*, *NTF6*, *MAPK*, *WRKY/MYB* transcription factors, *COI1* and *CTR1* genes [18,59,81–84]. The power of VIGS as a tool in reverse genetics is further manifested by the subsequent studies of the roles of *BECLIN-1* and *NRIP1* in *N*-gene and *RanGAP2* in *Rx*-gene induced programmed cell death (PCD) [85–87]. Silencing of *BECLIN-1* by TRV in *N. benthamiana* plants containing the N gene showed an unrestricted PCD response upon TMV infection [86]. *NRIP1*, which can directly interact with both the *N* gene and the 50 kD helicase (p50) of TMV, is involved in pathogen recognition, and is required for *N* gene-mediated complete resistance to TMV [85]. The interaction of *Rx* and *RanGAP2* in *N. benthamiana* or potato is required for extreme resistance to PVX, where *RanGAP2* is part of the *Rx* signaling complex [87]. In addition, a number of host genes involved in virus replication and movement in plants have been identified by VIGS [17,88–93].

VIGS has also been applied to study plant resistance against fungi [94]. A series of host genes involved in *Cladosporium fulvum*-tomato resistance have been charac-

terized [95]. *NRC1* and *SlPLC4,* and *ACIK1* and *NbCA1* genes have been proved to be necessary for *Cf-4/Avr4* and *Cf-9/Avr9* interactions, respectively, to elicit plant resistance [84,96–98]. Using a VIGS assay, Liu *et al*. [99] showed that *CITRX* is a negative regulator of the *Cf-9/Avr9* induced hypersensitive response (HR). Furthermore, *LeMPK1*, *LeMPK2*, and *LeMPK3* are activated during the *Cf-4/Avr4*-induced HR [100]. Additionally, some genes related to defense responses and basal resistance against powdery mildew fungus [101,102], wheat leaf rust fungus [29,103], *Colletotrichum destructivum*, *C. orbiculare* [104], and tobacco blue mold fungus [105] have been identified in dicotyledonous or monocotyledonous plants.

Applications of VIGS in studies of plant resistance against bacteria have been reported. Many new genes in the pathway of the *Pto*-mediated defense response against *Pseudomonas syringae* have been discovered in tomato, i.e., *NbCA1*, *NPR1*, *TGA1a*, *TGA2.2*, *MEK1*, *MEK2*, and *NTF6* [84,106]. Recently, the function of COR, a virulence factor of *P. syringae*, was revealed by VIGS. In addition, the roles of *SGT1* and *SlALC1*, two key genes in the coronatine/jasmonate (JA) pathway, and inducible NO synthase (iNOS) in resistance against *P. syringae* have been uncovered [107]. In tobacco plants, the important roles of *NbHSP90c-1*, *NbHSP70c-1*, *WIPK*, and *SIPK* genes in non-host resistance to *P. cichorii*, and *SGLP* in resistance against *Ralstonia solanacearum*, have been demonstrated by VIGS [108–110]. In a study searching for genes required for defense responses against *Xanthomonas campestris* in plants, *Bs4*, *SGT1*, *CaCYP450A*, *CaRING1*, *SlMKK2* and *SlMPK2* genes have been characterized [111–115].

VIGS has also been successfully used to investigate the resistance of plants to nematode and herbivore attack, such as aphids and *Manduca sexta*. Some genes have been found to have important roles in resistance against nematodes and aphids, such as *SlWRKY72a*, *HSP90*, *LeMPK2*, *LeMPK1*, *LeMPK3*, *SlSERK1*, and *SGT1* [116–120]. Suppressing *threonine deaminase* (TD) and *germin-like*, *jasmonate-resistant4* (JAR4) by VIGS confirmed that these genes have important roles in activating plant defenses to *M. sexta* [121,122]. *MPK1* and *MPK2*, which are essential components of the systemic signal pathway, were shown by VIGS to be required for successful defense against herbivorous insects [123]. Using VIGS, *MKK1*, *MEK2*, and *BAK1* have been demonstrated to be involved in herbivore *M. sexta*-induced responses by regulating JA biosynthesis [124,125].

VIGS was used to identify genes that mitigate stress. *late embryogenic abundant 4* (lea4) was shown to be involved in mitigating moisture stress [126]. *SlGRX1* was reported to regulate abiotic tolerance against oxidative, drought, and salt stresses [127]. In pepper, *CaOXR1* was found to play roles in tolerance to high salinity and osmotic stress [128]. In tobacco plants, *NbPHB1* and *NbPHB2,* two subunits of prohibitins, were found to have a crucial role in mitochondrial biogenesis and protection against stress and senescence in plant cells. *NaHD20* has a role in responses to dehydration [129,130]. In addition, VIGS has been used to study water deficit-induced genes in peanut [131].

3.2 Plant development related genes

VIGS is a transient assay for suppressing gene expression; therefore, VIGS facilitates the study of genes whose loss of function could be lethal to plants. Up to now, many development-related genes have been characterized by VIGS. Recently, a study on the flowering of the opium poppy using VIGS indicated that *PapsAG-1* has a role in stamen and carpel identity; however, the homologous gene, *PapsAG-2*, while displaying redundancy in these functions, has a distinctive role in the development of the septae, ovules, and stigmas [132]. In tobacco and *Petunia hybrida* plants, many flower development related genes, such as flowering time determine genes (*FCA* and *FY*), floral organ identity genes (*AP3* and *DEFICIENS*) [40,133,134] and flower development genes (*NbMADS4-1*, *NbMADS4-2*, *PhPHB1* and *PhPHB2*) [135,136] have been identified by VIGS. In a study of leaf and shoot development, Kang *et al*. [137] showed that the silencing of the *NbBPS1* gene resulted in growth retardation, abnormal leaf development, and cell death. This phenotype is different from the case of the *Arabidopsis bps* mutant. Bouvier *et al*. [138] used VIGS to suppress the expression of *SAMT1* in *N. benthamiana*. The severe growth retardation phenotype in silenced plants suggested that this methylation-related protein has an important role in plant development. The plant vascular development gene (*RPN9* [93]), *Retinoblastoma-related gene* (RBR [139]), a plant root development gene [140] and some genes in meristem, such as *Dt1* [141] and *ML1* [140] have been functionally characterized by VIGS. These results suggest that VIGS is one of the most powerful tools for the analysis of genes whose loss-of-function mutants cause embryonic and seedling-lethality [4–6].

3.3 Cellular functions and metabolism

VIGS has been used to study plant cellular functions and metabolic pathways, such as biotin, enzyme biosynthesis, and organic identity. Burton *et al*. and Held *et al*. used PVX and BSMV vectors, respectively, to study the function of *Cellulose synthase* (CesA) [142,143]. VIGS was also used to determine the genes involved in the biosynthesis of capsaicinoids (*AT3*, *Comt*, *pAmt*, and *Kas*) [69,144], D-apiose (*UDP-D-apiose/UDP-D-xylose synthase*, and AXS1) [145], flavin [146], *histone H3* [147], and key proteins in the RNA silencing pathway, such as Argonaute1- and Argonaute 4-like genes [148]. In addition, genes involved in the regulatory functions of PCD have been recently identified using VIGS, for example, the mitochondrial-associated hexokinase *Hxk1* gene [149], 20S proteasome, the 19S regulatory complex of the 26S proteasome [150] and a regulatory gene

of PCD (*CDC5* [151]). VIGS has also been used to characterize cellular functions of genes involved in chloroplasts and mitochondria biogenesis [129,150,152–157], plastid biogenesis [138,150], peroxisome biogenesis [158], alkaloid biosynthesis [159–161], isoprenoid biosynthesis [162,163], ascorbic acid biosynthesis [164], sterol biosynthesis [36,165], and membrane biogenesis [166].

4 Pros and cons of VIGS

Compared with other genomic techniques, VIGS has several advantages: (i) VIGS is much faster. A notable characteristic of VIGS is that it can induce loss-of-function phenotype of a specific gene in a short period. Therefore, the gene function can be characterized quickly, obviating the tedious process of plant regeneration [5,6]. (ii) Plant transformation is avoided, which means that studies of gene function in plants that are difficult to transform (e.g., cotton and soybean) would be more productive once the VIGS system is established. (iii) VIGS permits the study of genes that are vital for plant viability. VIGS can be used at the seedling or early development stages, and has been proved a powerful tool in the analysis of genes whose mutations cause embryonic and seedling-lethality. VIGS is the only technique that permits the study of such plant genes that are involved in plant development. (iv) The expression of multiple genes with functional redundancy can be silenced simultaneously through VIGS using conserved domains. On the other hand, a specific region can be used for VIGS if just one gene among a gene family is intended to be silenced. (v) It allows quick comparison of the functions of homologous genes among different plant species at the same time, producing more accurate gene function identification [5,6].

VIGS also has some disadvantages or limitations. For example, (i) in most cases, the expression of gene cannot be completely inhibited through VIGS. Although the expression of the target gene is reduced, the residual expression of the target gene can be enough for its function. Therefore, for those genes, the loss-of-function phenotype cannot be observed through VIGS. (ii) VIGS requires prior knowledge of target gene sequence information. The efficiency of silencing may be compromised by redundant genes, unless the full genome or sufficient EST sequences are available. (iii) Genes expressed during germination or the early seedling stage cannot be analyzed by VIGS, because VIGS is usually performed on adult plants and most of the VIGS phenotype is not inherited. (iv) The efficiency may vary and the phenotype of VIGS is not very stable. Results may not be consistent among different experiments or different plants. To resolve this problem, it is common to use a marker gene that shows a visible silencing phenotype as a positive control.

5 Conclusion and outlook

Over the last 15 years, VIGS has been successfully used to discover and confirm gene functions in many plants, including both dicotyledonous and monocotyledonous plants. Further understanding of the mechanism of gene silencing and development of vectors for VIGS will lead to more plant species being studied by newly constructed VIGS systems, especially those that are hard to analyze by conventional approaches. Recently, more plant genomes have been sequenced, and new molecular biology techniques have been established for VIGS. For example, artificial miRNA silencing vectors have been used in VIGS, and a VIGS cDNA library was constructed using the gateway system [135,167,168]. With further technical improvements, VIGS will continue to be widely used in plant functional genomics.

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