

Virus Induced Gene Silencing Optimization in Plants: Things to be Considered

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Abstract

Study of biological processes is mostly limited to model plant species possessing considerable advantages like small genome size, tractability for genetic studies, ease of use, short generation time, and consequently availability of immense genetic resources. Discoveries from model species are extremely valuable but not enough for improvement of agronomic characteristics of economically important plants mainly due to divergence of mechanisms through evolution. Transient techniques are emerging as powerful tools for functional studies in diverse plant species and for validation of discoveries made in model species. Virus Induced Gene Silencing (VIGS), a transient reverse genetics tool, is extensively being used for performing rapid loss-of-function experiments in plants. Several of the advantages of VIGS including its suitability for high throughput studies will extend functional studies to diverse plant species, contributing to our understanding of unique biological processes. One of the main factors hindering even wider application of VIGS is its requirement for specific conditions with each species. This manuscript reviews the available information in the literature regarding efforts invested in several plant species and points out the key factors to be considered optimizing for achievement of efficient gene knock-down phenotypes in novel plant species.

Keywords: gene splicing, knock-down phenotype, VIGS, virus

Introduction

Recent advancements in sequencing technologies have resulted in rapid accumulation of sequenced genomes, currently pending assignment of functions to their sequences. Unlike model plant species, like *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), barrel clover (*Medicago truncatula*) and *Physcomitrella patens*, many economically important plants are still devoid of comprehensive genomic resources necessary for functional genomics studies. These resources include efficient transformation protocols, central repository of overexpression, mutant, and recombinant inbred lines, genetic maps of chromosomes, and various online genetics and genomics databases. Consequently, our current mechanistic understanding of the basic biological processes is mostly based on studies conducted in model plant species. Within plant families, similarities of genes and their physical organization on the chromosomes has already made “translational genomics” from

model plants to crop species possible (Krutovsky et al., 2004). However, this transfer has not always been successful (especially across families) mainly due to extensive genome rearrangements, gene duplications, and divergence of certain mechanisms during evolution (Yan et al., 2003). Such cases necessitate studies to be directly conducted on crop species of interest.

Most economically important plants are not easily amenable to genetic manipulation, due to difficulties in plant regeneration and genetic transformation. Thus transient gene manipulation techniques represent useful alternatives. Among transient gene knock-down approaches, Virus Induced Gene Silencing (VIGS) (Ruiz et al., 1998) is particularly valuable due to rapid generation of phenotype by bypassing the need to generate time-consuming transgenic lines, its ease of preparation, cost effectiveness, ability to overcome functional redundancy as well as embryo lethality, and feasibility to high-

throughput functional analysis (Lu et al., 2003; Burch-Smith et al., 2004). VIGS exploits plant's natural post-transcriptional gene silencing (PTGS) antiviral mechanism to knock down endogenous plant genes. In brief, viruses are engineered to include portion of plant gene of interest in their genomes. Furthermore, the modified copy of the viral genome is inserted into a binary vector system, transformed into an *Agrobacterium tumefaciens* strain, and delivered into the plant via agro-inoculation. Upon delivery in plant, the T-DNA of *A. tumefaciens* carrying the engineered virus sequence gets integrated in the plant genome and is transcribed by the host machinery. The virus infection and spread activates plant PTGS as part of its natural antiviral mechanism to eliminate the foreign entity (Ding, 2010; Szittyá and Burgyan, 2013). Besides agro-inoculation, other virus delivery methods have been developed as well (discussed below). RNA silencing is ubiquitously triggered by double-stranded RNA molecules generated as an intermediate step during virus replication which are recognized by host DICER, an RNase-like enzyme, and chopped into 21–25 nucleotide sequence of small interfering RNAs (siRNAs). The siRNAs are then loaded onto RNA-induced silencing complex (RISC) which guides specific cleavage or suppression of complementary target sequences at post-transcriptional level. In the process, endogenous plant gene transcripts homologous to the inserted sequence in the viral vector (VIGS-vector) are degraded as well. For more details about the mechanism I refer readers to recent reviews of the topic by (Meister, 2013; Wilson and Doudna, 2013).

Some of the disadvantages of VIGS that limit its application include down-regulation of gene expression levels compared to other loss-of-gene function approaches, patchiness of silencing resulting in a mosaic of silenced and non-silenced tissue, requirement for optimization with each new species, and variability among replicates in terms of silencing efficiency (Burch-Smith et al., 2004). An additional potential disadvantage of VIGS includes risk of off-target gene silencing which represents a general pitfall of the RNAi

technology. According to computational predictions, about 50–70% of gene transcripts in Arabidopsis plants were expected to have potential off-targets when used as silencing trigger for PTGS with up to 50% of the predicted off-target genes tested in plants were actually silenced when tested experimentally (Xu et al., 2006). Thus it is necessary to ascertain the specificity of VIGS by choosing highly specific trigger sequences using the available computational tools like BLAST and siRNA Scan (<http://bioinfo2.noble.org/RNAiScan.htm>). For more details, readers are referred to a book chapter by Senthil-Kumar and Mysore (2011). Despite these limitations, VIGS has been successfully used in number of plant species for which genetic transformation is difficult to achieve (discussed below). Moreover, efficacy of VIGS was expanded to characterize genes in different plant parts like leaf (Liu et al., 2002), root (Bhattarai et al., 2007; Atamian et al., 2012), flower (Liu et al., 2004), seed (Yamagishi and Yoshikawa, 2009), and fruit (Fu et al., 2005).

Utilization of VIGS as a powerful reverse genetics tool with many other plant species awaits development of optimized protocols. This manuscript describes the various factors affecting VIGS efficiency that need to be optimized towards establishment of VIGS system in novel plant species.

Establishment of compatible plant-virus interaction

Compatible plant-virus interactions result in immense viral replication and its systemic spread to uninfected tissues. Thus the reliability and effectiveness of VIGS of endogenous plant genes depends on suitable combination of plant species and virus vector. Over the years, number of RNA and DNA viral sources has been engineered as VIGS vectors with both specific and broader applicability and characteristics (For comprehensive list see Lange et al., 2013; Scofield and Nelson, 2009). Only handful of these vectors is derived from DNA viruses, with the majority representing modified positive-strand

RNA viruses (Lange et al., 2013). A few of these DNA or RNA viruses require a helper virus (also known as Satellite DNA or RNA) for infection (Zhou and Huang, 2012). Hence a rich repertoire of VIGS vectors provides valuable resources for selecting the most suitable for a given plant species.

Most of the available VIGS constructs have been developed for functional genomics studies in dicotyledonous plants. The available systems for VIGS in monocots are based on *Brome mosaic virus* (BMV) (Ding et al., 2006), *Barley stripe mosaic virus* (BSMV) (Holzberg et al., 2002), and *Rice tungro bacilliform virus* (RTBV) (Purkayastha et al., 2013). *Tobacco rattle virus* (TRV)-based VIGS system (Liu et al., 2002) is the most widely used in core eudicots, with reported successful gene down-regulation in some basal eudicots and monocots (Becker and Lange, 2010). The main TRV characteristics include a reported host range of over 60 plant species from 12 families, generation of very mild interfering viral symptoms, and efficient spread throughout the plant including meristematic tissues (Burch-Smith et al., 2004). Consequently, TRV has been successfully used to silence genes in several plant parts or tissues, namely root (Valentine et al., 2004), leaf (Liu et al., 2002), flower (Ratcliff et al., 2001) and fruit (Fu et al., 2005). Therefore, unless the plant of interest is not a host for TRV, TRV-based vector is recommended as the first choice for testing effective induction of silencing in a novel plant species. BSMV-based vectors are emerging as powerful system for VIGS experiments in monocotyledon plants and represent a good starting point for a novel monocot species.

Marker genes

For rapid and visual evaluation of VIGS efficiency, the *phytoene desaturase* (PDS) gene marker, encoding an enzyme involved in the carotenoid biosynthesis pathway, is widely used. The silencing of PDS produces a photobleaching phenotype (Ratcliff et al., 2001; Liu et al., 2002). The *Magnesium Chelatase* subunit I (ChII) gene is

another visual marker for silencing that has been used as in its absence; chlorophyll is damaged producing a characteristic photo-bleaching of the affected tissue (Kjemtrup et al., 1998). Being a single copy gene in most plants analyzed so far, PCR amplification and cloning of PDS should be relatively easy. Although VIGS using PDS gene from closely related plant species has been successful, the use of PDS sequence from the same test species is recommended to explore the full potential and make accurate evaluations.

Suitable Agrobacterium strain

It has been known for many years that susceptibility to infection by *A. tumefaciens* strains (referred hereon as Agrobacterium) varies among different plant species and cultivars. Recent studies demonstrated the complexity of Agrobacterium host range which relies on multiple interactions within both the bacterium and host-plant factors (Gelvin, 2003; Lacroix and Citovsky, 2013). The influence of vector-Agrobacterium combination on the frequency of successful stable transformation as a result of optimal T-DNA integration has been extensively demonstrated (Shrawat and Lorz, 2006). For instance, in pigeon pea and rice, Agrobacterium strain LBA4404 was more compatible in giving higher frequency of transformants in all the genotypes tested (Shrawat and Lorz, 2006; Surekha et al., 2007) while Agrobacterium strain EHA 105 was found to be the most efficient with *Chlamydomonas reinhardtii* (Pratheesh et al., 2012). Since early steps of VIGS mechanism are similar to that of stable RNAi lines, choice of Agrobacterium is crucial for highly efficient gene knockdown.

The Agrobacterium strains used in VIGS include GV3101, EHA105, GV2260, LBA4404 with the former two being the most common. GV3101-TRV combination has been successfully used for efficient endogenous gene down-regulation in tomato (*Solanum lycopersicum*) (Bhattarai et al., 2007), origami (*Aquilegia coerulea*) (Sharma and Kramer, 2013), pepper (*Capsicum annuum*) (Wang et al., 2013), cotton (*Gossypium hirsutum*)

(Gao and Shan, 2013), tobacco (*Nicotiana tabacum*) (Zhang et al., 2013b), *Nicotiana benthamiana* (Hayward et al., 2010), *Gladiolus hybridus* (Zhong et al., 2013b), *Petunia hybrida* (Jiang et al., 2011), *Cysticapnos vesicaria* (Hidalgo et al., 2012), madagascar rosy periwinkle (*Catharanthus roseus*) (Liscombe and O'Connor, 2011), eggplant (*Solanum melongena*) (Liu et al., 2012), and *A. thaliana* (Burch-Smith et al., 2006). EHA105 strain has been used in combination with RTBV vector in *O. sativa* (Purkayastha et al., 2013), DNA β - and DNA1-based vector in tobacco, tomato, and *Petunia* (Zhou and Huang, 2012), BSMV-based vector in *N. benthamiana*, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), *Brachypodium distachyon* (Yuan et al., 2011), and *Grapevine virus A* (GPA)-based vector in grape (*Vitis vinifera*) and *N. benthamiana* (Muruganatham et al., 2009).

Despite the aforementioned successes with different *Agrobacterium* strain, vector, and plant combination performing direct comparison will be difficult as studies have been conducted under different conditions. However, reliable conclusions can be drawn from the work of few research groups that have addressed this issue in detail. In *Gossypium barbadense*, the three *Agrobacterium* strains tested (GV3101, LBA4404 and EHA105) showed no significant difference in the number of the plants with the VIGS phenotype (Pang et al., 2013). The same was observed in opium poppy (*Papaver somniferum*), where using *Agrobacterium* strains GV3101 and EHA105 experiments suggested that both strains were equally efficient at silencing. However, the expression data revealed stronger suppression of PDS transcript levels using TRV-based vector delivered to the plant via *Agrobacterium* EHA105 (5.3- to 7.5-fold average reduction) compared with GV3101 (3.2- to 4.4-fold average reduction) (Hileman et al., 2005). Taken together, data from systematic and controlled experiments based on quantification of gene knock-down efficiency is necessary for making accurate conclusions. It is recommended to test more than one *Agrobacterium* strain as part of VIGS

optimization effort with analysis of transcript downregulation using qPCR.

Choice of plant cultivar

Genetic differences among the cultivars of the same species may result in differences in their susceptibility to virus infection and consequently silencing efficiency (Kaloshian, 2007). This has been demonstrated in wheat, where among 12 cultivars tested, Zak, CS, and Eltan showed the maximum intensity of photo-bleaching upon PDS silencing (Bennypaul et al., 2012). Similarly, testing the effects of CHS silencing on a range of purple-flowered commercial *Petunia* cultivars showed significant variations in the silencing phenotype (Jiang et al., 2011). Solanaceous plants like tomato and tobacco are no different. In most tobacco cultivars, photobleaching upon PDS silencing occurred only weakly or was limited in leaves except in leaves of the cultivars Samsun and Samsun NN (Zhang et al., 2013b). Cultivar difference in terms of silencing efficiency was also reported in pepper (Wang et al., 2013), Arabidopsis (Jupin, 2013), soybean (Zhang et al., 2013a), *G. hybrida* (Deng et al., 2012), and tomato (Jiang et al., 2008). The genetic bases for these differences are not completely understood. However, recent transcriptomic and proteomic analyses demonstrated the extraordinary complexity of the pathogenic process requiring many host susceptibility factors (Pallas and Garcia, 2011). Thus possible factors contributing to the observed variation include differences in movement of either the virus or the silencing signal between cells and lack of compatible susceptibility determinants in the target plant.

Temperature conditions

Temperature conditions during plant growth after inoculation have profound effect on the efficiency, uniformity, and spread of gene silencing (Jiang et al., 2011). Early studies on *Agrobacterium* showed optimal T-DNA transfer occurred at 19°C and transfer was not seen at temperatures above 28°C (Fullner and Nester, 1996). In contrast, PTGS in *Drosophila* expressing

inverted repeat was shown to be temperature-sensitive as well, but with phenotypic consequences seen at 29°C, but not at 22°C (Fortier and Belote, 2000). Recently, increased transmission of viral pathogens by disease vector mosquitoes observed during cooler temperatures was correlated with loss of RNA silencing in those mosquitoes resulting in increased susceptibility (Adelman et al., 2013), suggesting that low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. Consistently, RNA silencing-mediated plant defense was demonstrated to be temperature dependent where activation of RNA silencing and the amount of siRNAs gradually increase with rising temperature (Szittyta et al., 2003). In contrast, recent investigation of PTGS in Arabidopsis mediated by antisense sequences (A-PTGS) and sense sequences (S-PTGS) showed that high temperature inhibits S-PTGS while low temperature inhibits A-PTGS (Zhong et al., 2013a).

Robust silencing results were obtained depending on plant species at temperatures 19–25°C. After virus inoculation plants were maintained at 20–22°C for Arabidopsis (Jupin, 2013), 23–25°C for cotton (Gao and Shan, 2013), 20–25°C for soybean (Zhang et al., 2013a), 25°C for apple (Sasaki et al., 2011). Experimental analysis in pepper showed that 18°C reduced the efficiency of silencing and the silencing phenotype was optimal at 22 °C (Wang et al., 2013). In tomato, optimal silencing was evident at 22°C where both at lower and higher temperatures, efficiency and strength of the phenotype was reduced (Jiang et al., 2008). In contrast, Fu et al. (2005) showed that conditions of low temperature (18°C) enhanced the silencing of PDS throughout inoculated tomato plants. These controversial results could be explained by the different plant varieties used by each group. For BSMV-based VIGS in different wheat tissues the temperature regimen of 22°C day/18°C night was found optimal. At a day temperature of 20°C, photo-bleaching was less intense and covered less leaf area as compared with that at 22°C (Bennypaul et al., 2012).

Interestingly, it has been shown that compared with other vectors, Satellite vector (DNA β and DNA1)-induced gene silencing is insensitive to high temperature, and VIGS efficiency was not significantly different between 22°C and 32°C (Zhou and Huang, 2012). Whether this is due to difference in the PTGS mechanism activated by Satellite viruses or is a result of other factors awaits further investigation.

Altogether, the available information suggests that in general different temperatures should be used to have both efficient T-DNA integration and PTGS. Consequently, it is recommended to grow plants under cold conditions within the 2-3 days post infiltration followed by exposure to higher temperatures for efficient PTGS activation and siRNA production.

Efficient VIGS-vector delivery

As mentioned earlier, successful virus infection is instrumental for efficient silencing of endogenous plant genes. Several approaches have been developed for delivery of VIGS vectors into plants ranging from simple rub inoculation to sophisticated gold-coated particle bombardment. Choice of method depends on plant species, viral vector, and growth stage, which play important roles in the intensity of silencing.

Syringe-infiltration method represents one of the earliest Agrobacterium delivery approach in which the mouth of 1-mL needleless syringe is placed on the abaxial side of the leaf and Agrobacterium culture is injected into the leaf with minimal pressure. This approach is most efficient and extensively used for functional characterization in *N. benthamiana* as it is fast, easy and at the same time provides adequate Agrobacterium delivery into the plant (Liu et al., 2002). The suitability of this approach has been demonstrated in other plant species like tomato (Ekengren et al., 2003; Velasquez et al., 2009), Arabidopsis (Burch-Smith et al., 2006), *Gerbera hybrida* (Deng et al., 2012), pepper (Wang et al., 2013) and others although with lower efficiency

of silencing compared to *N. benthamiana*. Plants or tissues that are difficult to infiltrate, such as soybean (*Glycine max*), eggplant, and pepper, syringe infiltration based vector delivery is inefficient resulting in low efficiency silencing. This is partly due to the inherent leaf morphology, presence of trichomes and other waxy compounds of the leaf surface. This limitation was to some extent overcome by submerging the plant completely inside *Agrobacterium* solution followed by vacuum application to force the bacterium into the leaf. This approach (known as vacuum infiltration) was used in opium poppy (Hileman et al., 2005), *Arabidopsis* (Burch-Smith et al., 2006), *Jatropha curcas* (Ye et al., 2009), origami (Sharma and Kramer, 2013), and *G. hybridus* (Zhong et al., 2013b). Unfortunately, some of those research groups do not report data regarding the use of syringe infiltration (if any) which prevents us from making conclusions at this point. However, results from experiments conducted with *G. hybrida* (Deng et al., 2012) and tomato (Bachan and Dinesh-Kumar, 2012) showed greater efficiency of PDS silencing with vacuum infiltration compared to syringe infiltration. However, one of the main disadvantages of vacuum infiltration approach is its suitability to older plants as younger seedlings often have low survival frequency. This sometimes limits its application as plant age has profound effect on silencing efficiency (discussed below). Recently vacuum infiltration of TRV has been shown to efficiently infect tomato sprouts with very high survival rate. Moreover, this highly efficient sprout vacuum-infiltration (SVI) method was successfully applied to germinated pepper, eggplant, and *N. benthamiana* seedlings (Yan et al., 2012). Another advantage of the SVI-method is its applicability to functional characterization during early stages of vegetative growth (1-3-week-old seedlings) since silencing was observed on the first true leaves of diverse solanaceous species compared to silencing phenotypes development when plants are at least 5–6 weeks old with regular vacuum infiltration. This suggests that the SVI-based VIGS approach might

be applicable for studies of important seedling behaviors during establishment (Yan et al., 2012).

Another method called particle bombardment or biolistic inoculation technique was developed that overcomes the seedling lethality of vacuum infiltration and is suitable for infection of younger seedlings at cotyledon stage. Moreover it is particularly useful in those plants with which vacuum infiltration is difficult like monocots. This method employs a “gene gun” to blast particles coated with viral nucleic acid into the plant. Biolistic inoculation of gold-coated *Apple latent spherical virus* (ALSV) RNAs to apple (*Malus domestica*) and soybean cotyledons resulted in high infection efficiency as it is generally difficult to achieve a highly efficient infection rate of ALSV vectors in this species using other methods (Yamagishi and Yoshikawa, 2013). This method was also used for *Euphorbia mosaic virus* (EMV)-based VIGS vector to silence genes in *N. benthamiana* and pepper (Villanueva-Alonzo et al., 2013), *Bean pod mottle virus* (BPMV)-based vector in soybean or common bean (*Phaseolus vulgaris*) plants (Zhang et al., 2013a), ALSV based vector in pear (*Pyrus sp.*) (Sasaki et al., 2011), and BSMV-based vector in wheat and barley (Holzberg et al., 2002; Scofield et al., 2005). Recently it has been shown that use of a different vector can efficiently infect cotton plants known to be difficult to get infected by syringe infiltration. In this study, particle bombardment and agroinoculation methods using *Cotton leaf crumple virus* (CLCrV) resulted in similar silencing efficiencies (Tuttle et al., 2012). This means that using the appropriate virus, syringe infiltration can replace biolistic inoculation. The major disadvantage of this technique is involved in construction of infective clones and the use of expensive equipment which also requires specific operational expertise. Recently development of an inexpensive homemade gun was reported which might increase the use of this technique (Tuttle et al., 2012).

Rub inoculation with sap of vector-infected leaves is another approach. In this case, viral

vectors are first delivered via agroinfiltration into the leaves of *N. benthamiana*, a plant susceptible to both *Agrobacterium* and the virus vector. Sap is then extracted from the infiltrated leaves and used to inoculate the desired plant species. This system is gaining significant interest in the VIGS community due to its relative simplicity and low setup costs and applicability for high throughput studies. High efficiency down regulation of three genes was demonstrated in the monocots, wheat, barley, and *B. distachyon* using BSMV VIGS vector using rub inoculation of *N. benthamiana* sap comparable to silencing efficiencies obtained by biolistic delivery of BSMV (Yuan et al., 2011). Rub-inoculation approach has also been used to deliver in vitro transcripts from BSMV vectors in wheat (Scofield and Brandt, 2012) and *Turnip yellow mosaic virus* (TYMV)-derived circular DNA vector harboring 80 bp synthetic oligonucleotides corresponding to inverted-repeat fragments of the target gene (Jupin, 2013). Rub inoculation of sap from agroinfiltrated *N. benthamiana* approach bypasses the T-DNA integration step. The success of this technique relies on the availability of high titer of infectious viral particles which will lead to efficient plant infection. However, inefficient infection has been documented as well mainly due to the inability of virus particles to circumvent the plant's physical barriers after being applied on the leaf surface, although the use of abrasives partly resolved this problem.

Syringe-injection inoculation method has been tested as well. This method uses a clinical syringe to inoculate about 50 µl of the bacterial suspension at the meristematic region located at the crown region of the plant as documented in rice (Purkayastha et al., 2013), injection into the phloem of stem as in tomato, tobacco and petunia (Zhou and Huang, 2012), and leaf or inflorescence injection in *Phalaenopsis* sp. (Hsieh et al., 2013). Syringe-injection inoculation onto the spikes at heading stage was tested as well. However, the results showed inconsistent silencing compared to spike-rub inoculation (Ma et al., 2012).

A high-pressure spray method was successfully used to silence genes in tomato (Liu et al., 2002) and eggplant (Liu et al., 2012). The high-pressure spray method achieved better silencing effect and higher efficiency in eggplant compared with the syringe method (Liu et al., 2012). Other available methods include Agrodrench which involves exposing the crown of the plant to *Agrobacterium* (Ryu et al., 2004) and pinch wounding method in which the stem is pinched just below the apical meristem and the youngest leaf pair (Liscombe and O'Connor, 2011).

Only handful of careful experiments has been conducted addressing the suitability of the different methods in a given species via side by side comparisons under identical experimental conditions. In opium poppy, among direct injection into leaves, misting, agrodrench, and vacuum infiltration approaches tested, only the later was successful (Hileman et al., 2005). Similarly, among the syringe infiltration, mechanical inoculation with circular or linearized plasmids, vacuum infiltration and rub inoculation of plant sap methods tested with BSMV only the later was successful in wheat and barley (Yuan et al., 2011). In another study, testing Agrodrench, vacuum infiltration, syringe infiltration, and pinch wounding method, demonstrated consistent silencing using the pinch method in *C. roseus* (Liscombe and O'Connor, 2011).

Altogether these results express the need for more comparative assays in this matter. Having more data from diverse plant species and multiple methods tested will be valuable in revealing insights that can be used in making educated decisions regarding the method to be used in a novel plant species that will most likely work. Therefore, until general patterns established, finding the most appropriate infection method will be based on trial and error.

Insert size

While it has been reported that the PDS fragments of 192–1,304 bp can be used successfully to induce VIGS with TRV-based

vector in *N. benthamiana*, longer fragments (1661bp) gave little or no silencing (Liu and Page, 2008). This may be due to impairment of viral replication and movement or loss of insert from the recombinant vector. With BSMV-based VIGS system, fragment sizes 120-500 bp in length are preferred as shorter fragments are less effective for silencing (Scofield et al., 2005), while longer fragments are less stable in BSMV in planta (Cakir et al., 2010). With *Cymbidium mosaic virus* (CymMV)-based vector the silencing effect of different size inserts (1498, 1133, 758, 334 and 81 bp) was evaluated with Phalaenopsis flower. Surprisingly, the results showed that the shortest insert fragment produced the highest gene-silencing efficiency, with no significant differences in silencing effect or mRNA level between 81- and 334-bp fragments (Hsieh et al., 2013). Similarly, with *rbcS* gene in apple, the 51, 102, and 201 bp sequences could suppress *rbcS* mRNA at the same level. However extensive analysis undertaken by Yuan et al. (2011) with different sizes of inserts targeting different genes in three monocot species by BSMV vector concluded that 300 and 400 bp HvPDS fragments and 200 or 400 bp TaPDS fragments elicit similar levels of photobleaching in barley and wheat leaves, respectively. In additional comparisons, the 102 bp BdPDS fragment was less effective than the 303 bp or 402 bp and the extent of silencing in wheat was substantially more efficient with the 250 bp and 300 bp TaChlH inserts than the 547 bp insert. These results indicate that sequences ranging from 200 to 400 bp provide effective silencing with BSMV VIGS in these three species (Yuan et al., 2011). Collectively, it seems that the gene silencing is specific for the cognate gene fragments used to induce silencing which differs among different plant species.

Plant developmental stage

The developmental stage of a plant at the time of inoculation is a known critical factor for VIGS (Burch-Smith et al., 2004; Hileman et al., 2005; Deng et al., 2012). Thus it is necessary to investigate the effect of the growth stage of

agroinoculated plants on gene silencing efficiency. In general viral infection is more efficient during younger developmental stages with better intensity of silencing phenotype.

Young seedlings, cotyledon up to 4-leaf-stage depending on species, have been commonly used for biolistic, syringe inoculation, and spray based infection of plants resulting in efficient gene silencing in trees (apple and pear) (Sasaki et al., 2011), tobacco (Zhang et al., 2013b), *N. benthamiana* (Bachan and Dinesh-Kumar, 2012), soybean and common bean (Zhang et al., 2013a), eggplant (Liu et al., 2012), and Petunia (Jiang et al., 2011). Detailed investigation of the effect of the growth stage of agroinoculated pepper plants on gene silencing efficiency in pepper showed infiltration into the cotyledons of 2 true leaf stage plant was the best (Wang et al., 2013). PDS was effective in *G. barbadense* seedlings inoculated from the expanding cotyledon stage to the one- to two-leaf stage, 90–100% of which exhibited photobleaching symptoms suggesting that younger plants are better for TRV-based silencing in this species (Pang et al., 2013).

Similar age seedlings were found applicable for vacuum infiltration of origami (Sharma and Kramer, 2013), tomato (Bhattarai et al., 2007), and Arabidopsis (Bachan and Dinesh-Kumar, 2012). Even younger stages in some plants were suitable like *G. hybridus* cormels (Zhong et al., 2013b) and tomato sprouts of about 0.5–1 cm in length (Yan et al., 2012). However for some species the survival rate was considerably reduced with younger seedlings as in the case of *G. hybrida* (Deng et al., 2012) and opium poppy (Hileman et al., 2005). With rub and clinical syringe infection methods different developmental stages yielded efficient silencing as evident in wheat (Ma et al., 2012), rice (Purkayastha et al., 2013), Arabidopsis (Jupin, 2013), tobacco, tomato, and Petunia (Zhou and Huang, 2012) suggesting that age of plant is more forgiving with these two approaches.

Inoculum concentration

To add yet another layer of complexity, studies have shown that in some plant species, the concentration of Agrobacterium affects the efficiency of silencing. Optimization efforts in pepper demonstrated that optical density OD₆₀₀ concentration (OD) 1.0 yields most intensive silencing and that OD 2.0 is much less efficient while OD 3.0 kills the plants (Wang et al., 2013). In contrast, OD 1.5 or above exhibited the greatest effect on VIGS in *G. barbadense* (Pang et al., 2013). Surprisingly, increasing the density of the Agrobacterium inoculum from OD 1.0 to OD 4.0 showed no difference on *G. hybrida* silencing efficiency (Deng et al., 2012). Taken together we can conclude that the requirement in plants varies considerably in terms of optimum inoculum concentration for efficient VIGS.

Other factors

There are other factors contributing to VIGS process in plants as evident by the variability of the response. Since these factors have not been reported frequently, they will be described briefly as reliable conclusions cannot be made at this point. Presence of a poly(A) tail at the 3'-terminus of inserted fragments for PDS gene in *N. benthamiana* resulted in reduced silencing using TRV-based vector (Liu and Page, 2008) but not in case of CymMV-based vector in *Phalaenopsis* flower (Hsieh et al., 2013). Regarding the position of the gene to target for silencing, silencing efficiency was reported to be generally higher for the 3' region of the target gene with BPMV-based vector in soybean (Zhang et al., 2013a). Similarly, targeting the 3'-end of GFP showed the most consistent and dramatic reduction of GFP fluorescence and GFP mRNA expression (Juvala et al., 2012). Although, position of the VIGS fragment relative to the full length PDS cDNA had a minor effect in silencing efficiency in *N. benthamiana* although avoiding the extreme 5' and 3' ends might possibly increase silencing efficiency (Liu and Page, 2008). The orientation of the fragments in the RNA2 vector does not seem to affect the efficacy

of silencing in *Petunia* (Jiang et al., 2011). High light intensity resulted in more efficient phenotypes at least with PDS silencing in *G. barbadense* as among the seedlings grown under high light conditions (300 and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 90% and 97.5% of plants, respectively, displayed photobleaching at 2 weeks post-inoculation, compared with only 8% of those grown under low light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (Pang et al., 2013). Moreover, photobleaching under long-day conditions was significantly higher than those grown under short-day conditions with this species (Pang et al., 2013).

Conclusion and future perspective

VIGS is a powerful functional genomics tool for rapid targeted down-regulation of plant genes of interest. Due to its moderately high throughput nature, ease of application, and cost effectiveness, VIGS allows rapid prescreening of candidate genes prior to the use of other, more time-consuming techniques to assess gene function, such as stable transformation. Data from literature summarized in this manuscript demonstrates the dynamic interplay among plant variety, growth stage, infection method, vector choice and other factors such as environmental conditions in the establishment of successful gene silencing. It is, therefore, important to invest a substantial amount of effort to optimize the different factors prior to conducting VIGS studies in a novel plant species. Once these factors are optimized, it is fairly simple to obtain reproducible levels of silencing plants (Wang et al., 2013).

TRV has been widely used for gene silencing in diverse dicotyledonous plant species while in monocots, BSMV holds similar promise as selective BSMV strains have been shown to infect diverse monocot species and can possibly be applied more widely to other crop species (Yuan et al., 2011). These two vectors will be ideal starting point for testing amenability of a plant for VIGS, provided that these viruses have been reported to infect the given plant species in nature. In addition, the development of new

reliable VIGS vectors for additional plant species will be very useful to meet possible specific requirements for a range of species currently being sequenced under 1000 Plant Genome Project (www.onekp.com/samples/list.php). As a general guideline following choice of vector, it is recommended to start with testing different varieties to identify the most VIGS-sensitive cultivar under two different temperature conditions (18°C and 24°C) and using GV3101 and EHA105 *Agrobacterium* strains as these three factors have been shown to be very critical and have variable requirements among the different plant species tested so far. The next step of the optimization process should focus on identification of the appropriate infection method and plant developmental stage combination. As a final step, other factors can be considered optimizing including inoculum concentration and position of the gene targeted if necessary.

The successful application of VIGS in future sequenced plant species will result in relatively quick exploration of their genome and in depth functional analyses. Greater knowledge of gene functions obtained via VIGS will considerably benefit improvement of agriculturally important traits (yield, quality, and resistance) by both conventional breeding and transgenic approaches, explain unexplored evolutionary relationships, and advance our understanding of many novel biological processes of utmost importance.

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