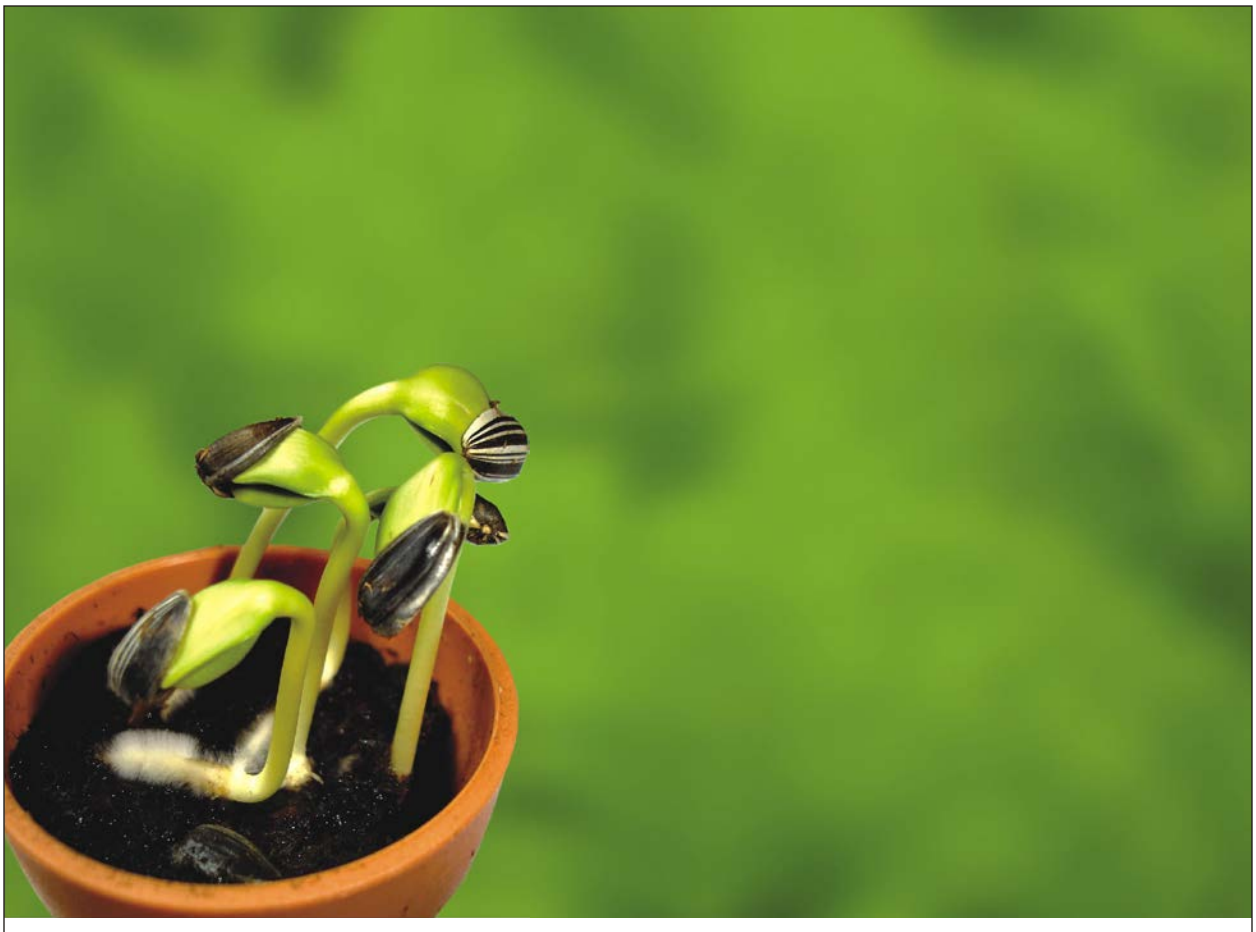

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Gene Silencing Technologies in Creating Resistance to Plant Diseases

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ABSTRACT

Plant diseases represent one of the most destructive and major problems of agricultural production. In order to sustain high quality yielding and disease resistant plants, there is need to incorporate some biotechnological practices like gene silencing technologies to the breeding program. Gene silencing is a technique used to suppress the expression of a gene and is controlled by a variety of mechanisms. Transcriptional, post-transcriptional, virus-induced (VIGS) and MicroRNA gene silencing are the different pathways. The applications of gene silencing technologies in creating artificial resistance to plants have been shown for crown gall disease, viral infections, plant nematodes, disease-resistant root stocks and others. Furthermore, the advantages of gene silencing technologies cannot be over emphasized in its use for loss of function and functional genomics analysis has been reliable, fast, easy, ensures rapid selection and can be used for a wide host range, though, the issue of suppression of non-targeted genes especially for members of a gene family, is one of the major limitations of VIGS. Finally, gene silencing technology has proven to be an effective tool for next generation of plant genomics.

Key words: Plasmids, *Agrobacterium*, siRNAs, RNAi, TGS, VIGS, miRNA

INTRODUCTION

Gene silencing is a technique used to turn down or switch off the activity of genes. It directs a natural mechanism to degrade the RNA instructions of specified gene, preventing the gene from making its protein. Certain DNA elements such as transposons, fragments of DNA that replicate within an organism's genome, can however disrupt this functioning and disable genes. To defend against such harmful elements, eukaryotic cells form inactive tightly-packed DNA called heterochromatin, whose dense structure serves to repress (silence) the expression of nearby gene sequences and protect the genome. It, therefore, suppresses the expression of introduced genes as well as endogenous genes with high homology.

Furthermore, gene silencing (also known as RNA interference, RNAi) occurs when a nucleic acid sequence bearing sufficient homology hybridizes to an RNA transcribed from a gene. The duplex formed, recognized as an aberrant RNA structure, will subsequently trigger the degradation of the homologous RNA transcript. As a consequence of this post transcriptional

homology dependent mRNA degradation, the corresponding gene is said to be "silenced" as its expression is substantially reduced. Gene silencing, however, is recognized to have a great potential utility in ascribing function to identified genes (Lacomme *et al*., 2005).

Moreover, gene silencing is an effective tool that allows the function of hundreds and thousands of genes to be tested, can silence genes throughout an organism or in specific tissues, offers the versatility to partially silence or completely turn off genes, works in both cultured cells and whole organisms and can selectively silence genes at particular stages of the organism's life cycle. The interest in gene-silencing-related mechanisms started from the early 1990s, when this phenomenon was first noted as a surprise observation by plant scientists during the course of plant transformation experiments, inwhich the introduction of a transgene into the genome led to the silencing of both the transgene and homologous endogenes (Eamens *et al*., 2008).

In the early study by Matzke *et al*. (1989) using two transfer DNA (T-DNA) vectors encoding different selectable markers which were sequentially introduced into the tobacco (*Nicotiana tabacum*) genome by *Agrobacterium* mediated transformation. He reported that the selectable marker encoded by the first T-DNA became inactive in a subset of their double-transformant population following the introduction of the second vector. The observed transgene inactivation was correlated with the methylation of the promoter sequences driving the expression of the selectable marker gene, delivered by the initial transformation event and the initiation of DNA methylation and gene inactivation was dependent on the genomic integration of the second T-DNA. The authors suggested that the substantial homology shared by the two T-DNA vectors including two copies of the nopaline synthase promoter per T-DNA insert may have initiated the methylation of the first vector.

In 1990, during the course of a transformation event to engineer petunia (*Petunia hybrida*) plant with increased transgene copies of the purple flower pigmentation gene, chalcone synthase (CHS). Additional transgene incorporated into the genome to intensify the purple coloration of the flowers brought about suppression of the homologous endogenes with different patterns of color range of intense purple, mixtures of purple and white and flowers that were completely white. Transcripts analysis of the transformed population showed that in some plant lines, both the introduced and endogenous forms of CHS gene were suppressed or turned off or silenced to differing degrees, through a homology-dependent gene silencing mechanism, they referred to as "cosuppression" (Napoli *et al*., 1990; Van der Krol *et al*., 1990). Many of the plants with entirely white or white-sectored flowers contain multiple methylated, transgene copies suggesting that CHS cosuppression is a post-transcriptional event showing some of the characteristics found in RNA-Mediated Virus Resistance (RMVR). Though, the underlying mechanisms behind these initial findings were unknown (Waterhouse *et al*., 1999).

Lindbo *et al*. (1993) showed that plants engineered to express virus-encoded sequences like the viral coat protein or a segment of viral replicase confers resistance to the virus from which the protein sequence were derived or to a closely related viral strain. They transformed tobacco plants with the gene sequence of the Tobacco Etch Virus (TEV) coat protein to provide TEV resistance. They reported that TEV could initiate replication in transformed plants, producing the typical systemic symptoms of infection but these plants were able to outgrow TEV infection approximately 3-5 weeks after the initial inoculation with TEV and they "recovered" healthy non-infected state. Recovered leaves did not support subsequent inoculations with TEV but they supported replication of the unrelated virus, Potato Virus X (PVX). Molecular analyses of the recovered tissue using nuclear run-off and northern blotting showed that introduced TEV sequences were still actively

transcribed but corresponding mRNA failed to accumulate. These observations led the authors to speculate that the gene silencing or cosuppression initiated by the transgene and viral trigger was localized to the cytoplasm and occurred at the post transcriptional level. To account for the sequence specificity of transgene-mediated TEV resistance, the authors proposed that this process is initiated by high level of RNA, above a certain threshold, in the cytoplasm and that a plant-encoded RNA-dependent RNA polymerase could be involved to generate a complementary RNA strand which identified and hybridized with the invading viral RNA to disrupt its function and cause degradation. This hypothesis has received much support in recent years and has remained the focus of study on how it is induced and maintained.

This review study aims at explaining gene silencing, discuss the various mechanisms (pathways) involved in gene silencing describe with examples, gene silencing application in creating resistance in plants and in other aspects of plant metabolism and highlight the advantages and limitations of using gene silencing technologies.

MECHANISMS OF GENE SILENCING

Gene silencing is based on mechanism involving the transgenically expressed proteins and in some others is based on RNA. Transgenes are usually introduced into the genomes of plants using *Agrobacterium tumefaciens*, a soil bacterium that transfers to susceptible plants a section of DNA called T-DNA (transfer DNA) that is carried on a resident plasmid. The single T-DNAs integrate into the genome either head-to-head, tail-to-tail or head-to-tail arrays (Preuss and Pikaard, 2003). The natural role of this mechanism is as a genetic immune system conferring protection against viruses. There may also be a genomic role of the process reflected in RNA directed methylation of transgenes.

The different mechanisms developed for gene silencing technologies include:

- Post Transcriptional Gene Silencing (PTGS) or RNA interference (RNAi)
- Transcriptional Gene Silencing (TGS)
- Virus-Induce Gene Silencing (VIGS)
- MicroRNA Gene silencing (miRNA)

Post-Transcriptional Gene Silencing (PTGS): Post Transcriptional Gene Silencing (PTGS) is a mechanism that degrades specific messenger RNAs prior to protein synthesis and thereby, reduces the expression of a specific gene (Strokes, 2000). Post Transcriptional Gene Silencing (PTGS), RNA interference (RNAi) in animals and basal eukaryotes and quelling in fungi and plants are examples of a broad family of phenomena collectively called RNA silencing (Matzke *et al*., 2001; Vaucheret *et al*., 2001; Waterhouse *et al*., 2001). PTGS is an "RNA-degradation mechanism" that shows highly specific process (Vaucheret *et al*., 2001). In Post Transcriptional Gene Silencing (PTGS), messenger RNA or messenger RNA precursors are synthesized but apparently are degraded rapidly or improperly processed or both (English *et al*., 1996; Bruening, 1998). PTGS was demonstrated for chalcone synthase by analyses of RNA synthesized by isolated nuclei, i.e., in "run-on" experiments (Atkinson *et al*., 1998). The accumulation of transcripts in the nucleus but not in the cytoplasm (English *et al*., 1996) also suggests that transcription is not altered significantly and that PTGS may occur in the cytoplasm. Plant RNA viruses which have been the usual targets of RNA-mediated resistance, replicate in the cytoplasm.

The unifying features of RNA silencing phenomena are the production of small (21-26 nt) RNAs that act as specificity determinants for down-regulating gene expression (Hammond *et al.*, 2000; Zamore *et al.*, 2000; Parrish and Fire, 2001) and the requirement for one or more members of the Argonaute family of proteins (Hammond *et al.*, 2001). The cellular and biochemical functions of Argonaute proteins families (or PPD proteins, named for their characteristic PAZ and Piwi domains) are now known and some have been characterized in details (Hock and Meister, 2008; Meng *et al.*, 2013).

Biochemical frame work for post transcriptional gene silencing/RNA silencing in plant: The biochemical framework for RNA silencing in plants and animals are similar in some ways in that small RNAs are generated in animals by members of the Dicer family of double-stranded RNA (dsRNA)-specific endonucleases (Bernstein *et al.*, 2001). Dicer family members are large, multidomain proteins that contain putative RNA helicase, PAZ, two tandem ribonuclease III (RNase III) and one or two dsRNA-binding domains. The tandem RNase III domains are believed to mediate endonucleolytic cleavage of dsRNA into small interfering RNAs (siRNAs), the mediators of RNAi. In Drosophila and mammals, siRNAs, together with one or more Argonaute proteins, form a protein-RNA complex, the RNA-Induced Silencing Complex (RISC) which mediates the cleavage of target RNAs at sequences with extensive complementarity to the siRNA (Hammond *et al.*, 2000, 2001; Zamore *et al.*, 2000).

Apart from Dicer and Argonaute proteins, RNA-dependent RNA polymerase (RdRP) genes are required for RNA silencing in Caenorhabditis elegans (Sijen *et al.*, 2001), Neurospora crassa (Cogoni and Macino, 1999) and Dictyostelium discoideum (Martens *et al.*, 2002) but likely not for RNAi in Drosophila or mammals (Schwarz *et al.*, 2002; Roignant *et al.*, 2003). In plants, PTGS initiated by transgenes that over express an endogenous mRNA also requires a putative RdRP, SGS2 (Dalmay *et al.*, 2000), although transgenes designed to generate dsRNA bypass this requirement (Beclin *et al.*, 2002). Similarly, silencing induced by viruses replicating through a dsRNA intermediate Virus-Induced Gene Silencing (VIGS) does not require SGS2 (Dalmay *et al.*, 2000).

Dicer in animals and CARPEL FACTORY (CAF, a Dicer homolog) in plants also generate microRNAs (miRNAs), 20-24 nt, single-stranded non-coding RNAs thought to regulate endogenous mRNA expression (Lee *et al.*, 1993; Lee and Ambros, 2001). miRNAs are produced by Dicer cleavage of stem-loop precursor RNA transcripts (pre-miRNAs); the miRNA can reside on either the 5' or 3' side of the double-stranded stem (Lee *et al.*, 1993; Lee and Ambros, 2001). In animals, pre-miRNAs are transcribed as longer primary transcripts (pri-miRNAs) that are processed in the nucleus into compact, folded structures (pre-miRNAs) then exported to the cytoplasm where they are cleaved by Dicer to yield mature miRNAs (Lee *et al.*, 2002). Animal miRNAs are only partially complementary to their target mRNAs, this partial complementarity has been proposed to cause miRNAs to repress translation of their targets, rather than direct target cleavage by the RNAi pathway (Hutvagner and Zamore, 2002). Plant miRNAs have far greater complementarity to cellular mRNAs and have been proposed to mediate target RNA cleavage via an RNAi-like mechanism (Rhoades *et al.*, 2002).

Zamore *et al.* (2003) reported from their study on the biochemical framework for RNA silencing in plants that the extracts of wheat germ, introduced for the study of translation and protein translocation in the 1970s (Roberts and Paterson, 1973), recapitulate many of the key features of RNA silencing in plants. Using this *in vitro* system, they show that in plants, ATP-dependent,

Dicer-like enzymes cleave dsRNA into small RNAs that have the structure of siRNAs. Unlike Drosophila embryos or mammalian cells, plants convert dsRNA into two distinct classes of siRNAs, long and short siRNAs. The short siRNAs are ~21 mers which directs posttranscriptional silencing via mRNA degradation and the ~24 mers triggers systemic silencing and the methylation of homologous DNA (Hamilton *et al*., 2002). Their inhibitor studies suggested that a different Dicer-like enzyme generates each siRNA class. They further showed that a wheat RdRP activity can synthesize dsRNA using exogenous single-stranded RNA as a template without an exogenous primer and that this dsRNA is preferentially converted into long siRNAs. Another class of small RNAs apart from siRNA found in plants are the microRNAs (miRNA) which are generated by a Dicer family member CAF, miRNAs are encoded in stem-loop precursor RNAs that are cleaved by CAF into 21-24 nt single-stranded small RNAs (Reinhart *et al*., 2002). Exogenous miRNA precursors were not faithfully processed into mature miRNAs in wheat germ extract. Instead, *in vitro* transcribed pre-miRNAs were cleaved into small RNAs too long to correspond to authentic, mature miRNAs. Perhaps the Dicer ortholog responsible for miRNA maturation in wheat, presumably wheat CAF, is absent from wheat germ extracts. In Arabidopsis, CAF transcripts that encode a protein with a nuclear localization signal have been reported, suggesting that CAF protein may be nuclear (Jacobsen *et al*., 1999). Because wheat germ extracts are essentially cytoplasm, nuclear CAF might not be present in the extract (Zamore *et al*., 2003).

Plant miRNAs differ from animal miRNAs in that there are corresponding mRNA sequences in the Arabidopsis and rice genomes with significant complementarity to miRNA sequences (Reinhart *et al*., 2002; Rhoades *et al*., 2002). The high degree of complementarity between the analyzed plant miRNAs and specific families of developmentally important plant mRNAs led to the proposal that plant miRNAs direct developmentally controlled mRNA destruction (Rhoades *et al*., 2002). That is, after the plant miRNAs are generated by the cleavage of pre-miRNAs by CAF, they enter the RNAi pathway and function as siRNAs. In contrast, animal miRNAs are thought to act as translational repressors (Ruvkun, 2001). An untested feature of this proposal is that an RNAi-like pathway in plants tolerates the three to four mismatches sometimes observed between a miRNA and its predicted mRNA target.

Wheat germ extracts contain miRNA-programmed complexes that specify endonucleolytic cleavage of corresponding target RNAs. In particular, miR165 has been proposed to down-regulate PHV and PHABULOSA (PHB) mRNA expression in Arabidopsis by an RNAi-like mechanism (Rhoades *et al*., 2002). PHV and PHB encode homeodomain-leucine zipper transcription factors implicated in the perception of radial position in the shoot tissues that give rise to leaves (McConnell *et al*., 2001). Dominant PHV and PHB mutations alter a single amino acid (glycine-→-glutamic acid) in the sterol/lipid-binding domain of the proteins, suggesting that the mutant phenotype results from a change in the function of PHV and PHB (McConnell *et al*., 2001). However, the discovery of plant miRNAs complementary to this site in PHV led to the suggestion that the molecular basis of the dominance is the persistence of PHV and PHB expression at developmental stages when these mRNAs are normally destroyed (Rhoades *et al*., 2002).

Zamore *et al*. (2003) also reported that wheat germ extracts contain an endogenous RISC programmed with a miRNA. This endogenous miRNA complex can direct efficient cleavage of the wild-type Arabidopsis PHAVOLUTA (PHV) mRNA sequence but not that of a previously described dominant PHV mutant that perturbs leaf development. The miR165/166-programmed RISC is a multiple-turnover enzyme that directs multiple rounds of target-cleavage. This finding supports the view that in plants miRNAs direct RNAi and explains the molecular basis for the dominant PHV mutation in Arabidopsis (Zamore *et al*., 2003).

Transcriptional Gene Silencing (TGS): DNA methylation and chromatin remodeling play a major role in Transcriptional Gene Silencing (TGS) and blocking gene expression (Waterhouse *et al*., 2001). In TGS, silenced transgenes coding regions and promoters are densely methylated (Kooter *et al*., 1999). It is also proposed that the increase in DNA methylation possibly induces formation of heterochromatin which is associated to TGS (Ye and Signer, 1996; Wassenegger and Pelissier, 1998; Wassenegger, 2000). DNA methylation promotes protein binding that recognizes methylated cytosine, leading to chromatin remodeling (Alberts *et al*., 2002), thus avoiding the binding of transcription factors (Kooter *et al*., 1999). In *Pinus radiata*, the extent of DNA methylation increases in mature meristematic regions when compared to juvenile regions, suggesting that methylation is involved in developmental control and aging processes (Fraga *et al*., 2002).

Heterochromatin can be defined as condensate chromosomal regions which are densely stained and known for genetic inactivity (Griffiths *et al*., 1998). Methylation, acetylation, phosphorylation and ubiquitination of core histones H2A, H2B, H3 and H4 are implicated in gene regulation (Lippman and Martienssen, 2004). These chemical modifications within histones alter the packing state of DNA between euchromatin (active DNA) and heterochromatin (inactive DNA). Histone acetylation is one factor that can destabilize chromatin structure by altering the charge composition within chromatin (Alberts *et al*., 2002). Chemical alterations within histone tails may function as signals for chromatin remodeling complexes which are responsible for regulating the accessibility of the cells transcriptional machinery to the DNA (Alberts *et al*., 2002). At least in plants there is a direct link between DNA methylation and histone methylation suggesting that they play a common role in transcriptional gene silencing (Lippman and Martienssen, 2004).

Early study suggested that TGS and PTGS (RNAi) were independent phenomenas. However, it was later discovered that viruses and transgenes originating dsRNA induced both TGS and PTGS (RNAi), suggesting that these could be alternative but not exclusive routes of regulation (Vaucheret and Fagard, 2001). RNA silencing has also been associated to de novo DNA methylation in plants (Chan *et al*., 2004). The fact that almost all DNA and histone methylation events are confined to transposons and repeats suggests a role for RNAi as a targeting mechanism for specific sequence chromatin remodeling or TGS (Lippman and Martienssen, 2004). Study carried out on *Schizosaccharomyces pombe* and *Arabidopsis* indicates that the RNA-directed DNA methylation (RdDM) signal transmitted from the cytoplasm to the nucleus is most likely siRNA (Fig. 1) (Xie *et al*., 2004). Additionally, chromatin-based silencing guided by siRNAs may act as a genomic defense system to suppress mobile genetic elements or invasive DNA (Dawe, 2003; Schramke and Allshire, 2003).

Components of the RNA interference pathway are also used in many eukaryotes in the maintenance of the organization and structure of their genomes. Modification of histones and associated induction of heterochromatin formation serves to downregulate genes pre-transcriptionally (Holmquist and Ashley, 2006), this process is referred to as RNA-Induced Transcriptional Silencing (RITS) and is carried out by a complex of proteins called the RITS complex. In fission yeast this complex contains an argonaute, a chromodomain protein Chp1 and a protein called Tas3 (Verdel *et al*., 2004). As a consequence, the induction and spread of heterochromatic regions requires the argonaute and RdRP proteins (Irvine *et al*., 2006). Indeed, deletion of these genes in the fission yeast *S. pombe* disrupts histone methylation and centromere formation (Volpe *et al*., 2002), causing slow or stalled anaphase during cell division (Volpe *et al*., 2003). In some cases, similar processes associated with histone modification have been observed to transcriptionally upregulate genes (Li *et al*., 2006).

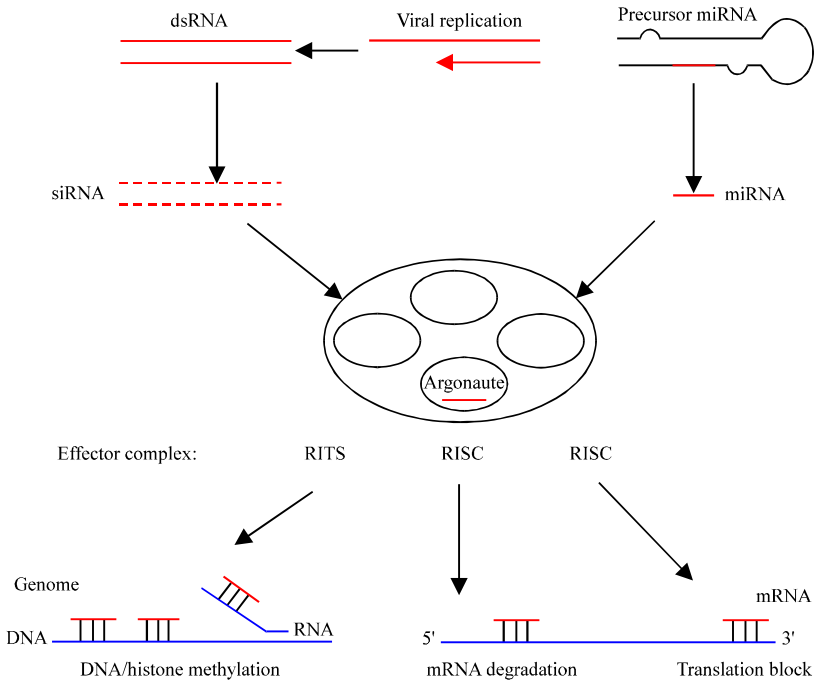

Fig. 1: Gene Silencing Techniques. Here, the Enzyme Dicer trims double stranded RNA to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-Induced Silencing Complex (RISC) which targets messenger RNA to prevent translation (Hammond *et al.*, 2000)

The mechanism by which the RITS complex induces heterochromatin formation and organization is not well understood and most studies have focused on the mating-type region in fission yeast which may not be representative of activities in other genomic regions or organisms. In maintenance of existing heterochromatin regions, RITS forms a complex with siRNAs complementary to the local genes and stably binds local methylated histones, acting co-transcriptionally to degrade any nascent pre-mRNA transcripts that are initiated by RNA polymerase. The formation of such a heterochromatin region, though not its maintenance, is dicer-dependent, presumably because dicer is required to generate the initial complement of siRNAs that target subsequent transcripts (Noma *et al.*, 2004). Heterochromatin maintenance has been suggested to function as a self-reinforcing feedback loop, as new siRNAs are formed from the occasional nascent transcripts by RdRP for incorporation into local RITS complexes (Sugiyama *et al.*, 2005).

Virus-Induced Gene Silencing (VIGS): Virus-induced gene silencing is an RNA-mediated post-transcriptional gene silencing mechanism that protects plants against foreign gene invasion by silencing or knocking down the expression of targeted gene (Anonymous, 2010). VIGS has become very popular to perform reverse genetics and can also be used for forward genetics (Robertson, 2004). This involves the *Agrobacterium* mediated cDNA construction containing fragments of endogenous gene sequences into the targeted plant. Once expressed *in vivo*, dsRNA are generated from an encoded viral polymerase as the virus replicates and spread through the plant (Robertson, 2004). These dsRNA are then targeted by DICER-like enzymes and degraded into

siRNA. In turn, the siRNA molecules provide a template for degradation of complimentary RNAs, including complimentary endogenous mRNAs, by the RNA-Induced Silencing Complex (RISC). Silencing persist until proliferation of viral RNAs is overcome by the silencing response. VIGS induction is a useful alternative to generating stable transformed plants which is often difficult and laborious and offers the ability to overcome functional redundancy by suppressing all or most members of a gene family (Burch-Smith *et al*., 2004). VIGS technology will also be a useful tool in analyzing a wide range of gene function in plants through loss of function assays (Baulcombe, 1999; Lu *et al*., 2003).

VIGS vectors are developed from viral systems that affect many plant hosts including many dicots (Robertson, 2004; Burch-Smith *et al*., 2004; Watson *et al*., 2005). VIGS technologies usually use two 35S promoters; one promoter to control the transcription of the viral transcript and the other promoter to control the vector's transcription and a self cleaving ribozyme sequence to enable rapid generation of intact viral transcripts (Kramer and Gould, 2007). The effectiveness of a VIGS vector rests firstly on the ability of the virus to replicate and accumulate to sufficient levels in the host plant to generate dsRNA molecule which initiate silencing as recent studies have shown that dsRNA generation is a limiting factor in VIGS (Lacomme *et al*., 2005). Secondly, the virus must be devoid of strong genome based Post Transcriptional Gene Silencing (PTGS)-Suppressors such as HC-Pro (potyvirus) or 2b (Cucumoviruses) (Voinnet *et al*., 1999) that protect the virus against this RNA-mediated resistance mechanism. Given these conditions, it is likely that a plant virus that is able to trigger a significant VIGS response in a host plant can also be used for VIGs in a distant susceptible host (Lacomme *et al*., 2005).

Of all the vectors already analyzed for plant's Virus-Induced Gene Silencing (VIGS) through the use of *Agrobacterium* mediated gene transfer, TRV (Tobacco Rattle Virus) has been the most efficient as it can penetrate meristematic cell with ease (Burch-Smith *et al*., 2004), be used in different hosts and in overall, causes mild viral symptoms in a wide range of susceptible hosts (Vide Database).

The conditions before using VIGS technologies include that the region of a gene to target for silencing must be carefully considered for every VIGS experiment. This is because PTGS will potentially silence any transcript containing at least 23 nucleotides identity to the targeted sequence. It is important to determine at the outset, through BLAST searches or DNA gel blot analysis, whether or not the gene to be suppressed is a member of a gene family. For single copy genes, presumably any region of the Open Reading Frame (ORF) can be used for silencing. To suppress a single gene within a family, one must carefully select regions that do not contain stretches of 23 nucleotides of exact identity. One possible approach to be considered is the use of UTR (untranslated region) sequence to discriminate between genes with highly conserved ORFs. Conversely, it may be practical to choose regions that are conserved between genes to co-suppress several members of a gene family and overcome possible functional redundancy (Lu *et al*., 2003). The second criteria depend on the environmental conditions of growth chamber. Temperature is one of the most important factors for good viral spread and effective silencing. To achieve good silencing phenotypes with TRV on tomato, temperatures at or below 21°C is required and 25°C with TRV in *N. bathamiana* (Burch-Smith *et al*., 2004).

MicroRNAs (miRNA): MicroRNAs (miRNAs) are ~22 nt endogenous RNAs that can play important regulatory roles in animals and plants by cleavage or translational repression of mRNA (Bartel, 2004; Duan *et al*., 2006). These molecules comprise one of the more abundant classes of

gene regulatory molecules in multicellular organisms. Plant MicroRNAs (miRNAs) affect only a small number of targets with high sequence complementarity while animal miRNAs usually have hundreds of targets with limited complementarity (Schwab *et al*., 2006). MicroRNAs were firstly reported in *Caenorhabditis elegans* by the discovery that the *lin*-4 gene coded a pair of small RNAs instead of a protein (Lee *et al*., 1993). Further evidence showed that these small RNA products had antisense complementarity and were responsible for the translational regulation of another gene, the *Lin*-14 (Lee *et al*., 1993; Wightman *et al*., 1993). *Lin*-14 mRNAs levels were not altered but the protein output of the gene was reduced.

In plants, miRNAs are involved in the control of leaf and flower development (Aukerman and Sakai, 2003; Emery *et al*., 2003; Palatnik *et al*., 2003; Chen, 2004). It appears that a substantial fraction of the gene regulatory molecules in plants could be RNA rather than protein transcription factors (Bartel, 2004). Plant ~22 nt long miRNAs are endogenously expressed and potentially processed from one arm foldback precursors. These molecules are also generally conserved in evolution and come from regions of the genome distinct from previously annotated genes (Reinhart *et al*., 2002). Recent data demonstrates that miRNAs are also involved in other mechanisms such as stress and environmental change response (Bonnet *et al*., 2006).

MicroRNAs and siRNAs have a shared central biogenesis and can perform interchangeable biochemical functions. Therefore, these two classes of silencing RNAs cannot be distinguished by either their chemical composition or mechanism of action (Bartel, 2004). However, some distinction can be made regarding origin, evolutionary conservation and types of genes they silence: miRNAs derive from genomic loci distinct from other recognized genes, whereas siRNA often derive from mRNAs, transposons, viruses or heterochromatic DNA; miRNAs are processed from transcripts that can form local RNA hairpin structures, whereas siRNAs are processed from long bimolecular RNA duplexes or extended hairpins; a single stranded mature miRNA is generated from each miRNA hairpin precursor molecule, whereas a multitude of siRNA duplexes are generated from each siRNA precursor molecule, leading to many different siRNA accumulating from both strands of this extended dsRNA and miRNA sequences are nearly always conserved in related organisms, whereas endogenous siRNA sequences are rarely conserved (Bartel and Bartel, 2003).

MicroRNAs in plants are probably processed by DCL1 (Dicer) in the nucleus (Papp *et al*., 2003) and its molecular pathway is exclusively linked to a dsRNA binding protein (HYL1) (Vazquez *et al*., 2004). Mature miRNA can be loaded into RISC or microRNA effector complex (miRNA), the first directs mRNA cleavage and the second is responsible for translational repression (Meister and Tuschl, 2004). Preferentially plant miRNA use RNA target cleavage instead of translational suppression and are nearly perfectly paired to their targets (Rhoades *et al*., 2002).

From the works of Niu *et al*. (2006), it has been demonstrated that the modification of plant endogenous miRNA precursors to interfere with viral mRNA sequences can confer virus resistance in *Arabidopsis thaliana*. These modified miRNA are termed artificial miRNA (amiRNA). amiRNA make an effective tool for specific gene silencing in plants, especially when several related but not identical, target genes need to be down regulated (Schwab *et al*., 2006) and this technique will open new perspectives for engineering viral resistant plants.

General experimental procedures in the construction of virus derived vectors

Construction of virus derived vectors: This is generated by cloning a short stretch of sequence (PCR fragment amplified from a cDNA template) usually 300-700 nucleotides (Preuss and Pikaard, 2003) in length using specific oligonucleotide primers incorporating tow restriction sites, respectively

at 5' and 3' termini for cloning into a vector under the control of the viral 35S promoter within a binary vector. The construct can be cloning in sense or antisense orientation. The DNA construct of the vector RNA virus is selected from the group consisting of potato virus x, tobacco mosaic virus, tobacco etch virus, tobacco rattle virus, tomato bushy stunt virus and brome mosaic virus (Lacomme *et al*., 2005). When preparing a vector to single gene within a family, one must carefully select regions that do not contain stretches of 23 nt of exact identity. Conversely, it may be practical to choose regions that are conserved between gene to co-suppress several members of a gene family and overcome possible functional redundancy (Lu *et al*., 2003).

***Agrobacterium* infection of plants:** *Agrobacterium tumefaciens* is transformed through insertion of the vector construct into the plant with a toothpick, vacuum infiltration or syringe infiltration. This *Agrobacterium* is allowed to infect the desired plants and once the symptoms of the *Agrobacterium* infection is noticed in plant, it is assumed that the vector construct must have been transferred. After 5-6 weeks, the plants are scored for loss-of-function phenotypes associated with suppression of the target gene (Kramer and Gould, 2007).

RNA extraction and cDNA synthesis: Leaves exhibiting a range of silencing phenotypes including the controls (untreated plants) are collected and flash frozen at -80°C. Total RNA was extracted from frozen control and silenced leaves using the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA). Each total RNA sample was DNase treated to remove residual genomic DNA and the concentration was adjusted to 0.3 μg μL^{-1} (Lacomme *et al*., 2005; Kramer and Gould, 2007).

Immunoblot analysis: Protein extraction and western blot analysis are carried out following the protocol of Lacomme and Cruz (1999).

RT-PCR analysis of viral transcripts and targeted gene expression: Different individual cDNA pools are prepared for each RNA sample using 3.3 μL of Dnase-treated RNA, Superscript II Reverse Transcriptase (Invitrogen) and reverse primers specific for each vector. The samples are then PCR screened (20, 25, 30 and 35 cycles) for the presence of viral RNAs using specific primers. PCR reactions are then separated by electrophoresis in a 1% agarose gel containing 0.5 mg L^{-1} ethidium bromide. DNA band intensities are UV imaged on an Alpha Innotech ChemiImager at levels below saturation and calibrated against a low DNA mass ladder (Invitrogen) using AlphaEase FC imaging software. A subset of reactions is repeated twice for accuracy and the final gene to gene ratio values are normalized to the lowest ratio (Lacomme *et al*., 2003; Kramer *et al*., 2007).

APPLICATIONS OF GENE SILENCING TECHNOLOGIES IN PLANTS

Plants have great economic importance in agriculture, medicine and industry and equally serve as a strong economic backbone of many countries (Okigbo and Mmeka, 2006). The plants products utilized include wood, cellulose, oils, fruits, resins, latex and nuts. In order to sustain high quality yielding and disease resistant plants, there is need to incorporate some biotechnological practices like gene silencing technologies to the breeding program. This will help to overcome some problems encountered which conventional breeding programs cannot solve alone. Also, gene silencing is relevant in agricultural biotechnology because stable expression of transgenes is required for the successful commercialization of genetic engineered crops.

Crown gall: Gene silencing has been used to interrupt the process of tumor formation in crown gall disease. Crown gall disease is a tumor disease caused by a bacterium *Agrobacterium tumefaciens* which have the ability to transfer its own DNA into the DNA of the plant it infects and directs the plant to produce the proteins that trigger tumor formation as a response to certain chemicals released from the wound. The tumors appear as galls or lumps of tissue near the base of the plant's stem and causes damage to the plant by blocking the transportation of nutrients and water up and down the plant stem or tree trunk. Escobar *et al.* (2001, 2002) in their study on tomatoes, *Arabidopsis thaliana* and Walnut were able to target two bacterial genes (*iaaM* and *ipt* oncogenes) that were keys to tumor formation. They used gene silencing technique with hpRNA (hairpinRNA) construct to "turn off" those genes and prevent gall from forming on the plants. The gene silencing works by interrupting or suppressing the activity of a targeted gene, preventing it from coordinating production of specific proteins. In this case, the researchers targeted the bacterial genes that cause overproduction of growth hormones. It is this hormonal overproduction that resulted in uncontrolled cell growth and gall formation. Thus, by using the gene-silencing technique, the researchers were able to produce genetically engineered plants that could still be infected by *Agrobacterium tumefaciens* but would not produce the hormones that lead to gall formation of which there were more than 50% reduction in gall formation among the genetically engineered tomato and Arabidopsis plants. Apart from lack of galls, the genetically engineered plants did not look any different than their non-transgenic counterparts.

Viral infection: Greater than 90% of all plant viruses are RNA viruses that replicate through a dsRNA intermediate. So, RNA interference in plants has evolved, in part, as a means for protection against viral infection and retrotransposon proliferation (Waterhouse *et al.*, 2001; Voinnet, 2001). Gene silencing can be used to introduce virus resistance into plants via inserting a piece of a DNA that has been modified to include one small fragment of a virus into a plant. This then effectively vaccinates the plant (CSIRO, 2004). According to Al-Kaff *et al.* (1998), plants are able to respond to pathogen attack to restrain development of a systemic infection by encoding genes which counteract the plant's RNAi surveillance system, thus evading degradation. One clear demonstration came from grafting the root and lower portion of a plant in which a reporter gene was silenced by RNAi to a shoot in which the same reporter was active. Amazingly, a silencing signal traversed the graft junction to cause silencing in the shoot (Palauqui *et al.*, 1997; Preuss and Pikaard, 2003). Likewise, infecting a reporter gene-expressing plant with a virus that included sequences matching the reporter gene caused report gene silencing far beyond the site of infection (Voinnet and Baulcombe, 1997). Thus it is thought that cells that initiate RNAi as in the response to virus infection can generate small RNAs, or some other mobile signal which traffic through the phloem throughout the plant (Cazares *et al.*, 1999) and help target related RNAs, thus protecting against a spreading RNA virus.

Disease resistant root stocks: Gene-silencing techniques have been used to produce disease resistant root stocks for growing non-transgenic crops. The root stocks would carry the disease resistance traits introduced through gene silencing. The harvested crop, however, would not be genetically altered and there would be no possibility of unintentionally transferring the foreign genes to other domestic or wild plants through pollen (Dandekar *et al.*, 2001).

Post Transcriptional Gene Silencing (PTGS): Over a decade, PTGS have been used commercially to develop plants with resistance to viral infections. Among them were the transgenic papayas (*Carica papaya*) with resistance to Papaya ringspot virus (PRSV; Fuchs and Gonsalves, 2007) and the Monsanto-produced NewLeaf Plus and NewLeaf Y potatoes (*Solanum tuberosum*) with resistance to Potato leafroll virus and PVY (www.Research.cip.org). Wang and Waterhouse (2000) used RNA silencing techniques for virus protection of barley (*Hordeum vulgare*) against *Barley yellow dwarf virus* (BYDV). They used a hpRNA (hairpin RNA)-encoding construct driven by the maize (*Zea mays*) ubiquitin promoter and 5' end of the virus which was transformed into barley to produce lines with complete immunity to BTDV.

Artificial MicroRNA (amiRNA) suppression: Niu *et al.* (2006) used artificial miRNas (amiRNAs) to control viruses by destroying their RNA within a plant cell. They used a 273-bp sequence of the Arabidopsis miR159a pre-miRNA transcript expressing amiRNAs against the viral suppressor genes P69 and HC_Pro to provide resistance against turnip mosaic virus infection, respectively. In addition, a dimeric construct harboring two unique amiRNAs against both viral suppressors conferred resistance against these two viruses in inoculated Arabidopsis plants. Using a different amiRNA vector to target the 2b viral suppressor of the *Cucumber mosaic virus* (CMV), a suppressor that interact with and blocks the slicer activity of AGO1 was also shown to confer resistance to CMV infection in transgenic tobacco. There was a strong correlation between virus resistance and the expression level of the 2b-specific amiRNA (Qu *et al.*, 2007; Eamens *et al.*, 2008).

Plant nematodes: Plant parasitic nematodes, such as the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes which cause significant damage to important crops such as legumes, vegetables and cereals have been controlled using RNA silencing technologies. This was carried out in *C. elegans*, using two approaches. The first relies on targeting plant genes that are involved with the infection process and the second approach targets essential genes within the nematode. *Heterodera schachtii* induces syncytial feeding structures in the roots of host plants and this requires the up-regulation of such transporter genes to facilitate increased nutrient flow to the developing structure. Targeting these genes and down-regulating them with RNA silencing resulted in a significant reduction of female nematode development (Hoffman *et al.*, 2008). RNA silencing can be induced in *C. elegans* by feeding it dsRNA, so it is reasoned that expressing hpRNAs containing sequences of vital nematode genes in the host plant might deliver dsRNA to a feeding nematode to incapacitate or kill it, indeed, tobacco plants transformed with hpRNA (hairpin RNA) constructs against two such root knot nematode genes have shown such an effect: the target mRNAs in the plant parasitic nematodes were drastically reduced and the plants showed effective resistance against the parasite (Fairbairn *et al.*, 2007).

The second approach relies on the phenomenon that hpRNA encoded in a plant can induce RNA silencing in a nematode that feeds upon it. A study by Baum *et al.* (2007) has described how this approach can be used against herbivorous insect pests with much less intimate feeding associations. They fed western corn rootworm larvae on artificial diets supplemented with specific dsRNAs, to screen a large number of genes for effective targets and identified 14 whose knockdown by dsRNA killed the larvae. Transforming maize with an hpRNA against one of these genes, a subunit of the midgut enzyme vacuolar ATP, gave protection against western corn rootworm infestation at a level that was comparable to that provided by the *Bacillus thuringiensis* (Bt) toxin transgene. Indeed,

the hope that this approach might provide a backup for Bt protection in crops like cotton (*Gossypium hirsutum*) and maize, in which insects are continuing to develop resistance to Bt, is an alternative strategy.

Parasitic weeds: Furthermore, gene silencing technologies have been designed against a parasitic weed, broomrape (*Orobanche* spp.) which causes a heavy damage to numerous crops, reducing both crop yield and quality. These root parasites are difficult to control with chemical, cultural, biological and with conventional means. The seeds can remain viable in the soil for many years (Aly, 2010). According to ISB news report (Aly, 2010), a new strategy has been developed to enhance host resistance to *Orobanche* based on parasite-induced expression of a selective *sarcotoxin* IA polypeptide (Hamamouch *et al*., 2005). Hamamouch and colleagues were able to generate genetically engineered tobacco plants expressing a cecropin peptide (*sarcotoxin* IA) under the inducible control of the HMG2 promoter. While, having no obvious effect on the host plant growth and development, transgenic plants show enhanced resistance to the parasitic weed *Orobanche*, with reduced *Orobanche* biomass and increased host biomass, compared to non-transgenic controls. Aly (2010) also reported that regulation of mannitol in *Orobanche* by Mannose 6-Phosphate Reductase (M6PR) is a process essential for water and nutrient uptake from its host by engineering a potential host (tomato) to produce a systemic signal to effect silencing of the metabolic activity in the parasite. Using an inverted repeat technique for gene silencing of M6PR, through a gene construct containing a specific fragment from *Orobanche aegyptiaca* M6PR mRNA in the host, it confer resistance to the host plant against the parasite (Aly *et al*., 2009).

OTHER APPLICATIONS OF GENE SILENCING TECHNOLOGIES IN PLANTS

The examples described above are all RNA silencing-based strategies that protect the plant from pest or pathogen attack but another widely embraced use of RNA silencing technology has been for reshaping metabolic pathways. For example, RNA silencing has been used to improve the human health attributes of cottonseed oil. Cotton is the world's sixth largest source of vegetable oil but the oil profile has relatively high levels of palmitic acid which, although providing stability at the high temperatures used in deep frying, also gives it low-density lipoprotein cholesterol-raising properties in humans. Oils that are low in palmitic acid and rich in either oleic acid or stearic acid have thermostability without the associated low-density lipoprotein cholesterol-raising properties. Liu *et al*. (2002) have used hpRNA constructs to silence the CE949 and CE9412 desaturases which catalyze the biosynthesis of these fatty acids and have obtained plants that produce seed oil that is much more suitable for human consumption. In a similar vein, modification of the starch composition of wheat (Triticum aestivum) destined for human consumption in affluent countries, by altering its amylose-amylopectin ratio, has the potential to reduce the incidence of cardiovascular disease and colon cancers. Regina *et al*. (2006) have used hpRNA constructs to silence an isoform of a starch-branching enzyme to produce a high-amylose transgenic wheat line which if widely adopted in western countries could have significant public health benefits (Eamens *et al*., 2008).

RNA silencing technology also has many important nonfood applications, such as altering photosynthetic pathways in algae to give increased bioreactor performance (Mussgnug *et al*., 2007) and reshaping the morphine pathway in poppies (Papaver somniferum) to increase the yield of pharmaceutically significant compounds (Allen *et al*., 2004). An interesting application in the medical therapeutic arena has been in engineering plant-produced antibodies. Monoclonal

antibodies are widely used in the therapeutic treatment of cancer, autoimmune and inflammatory diseases and plant-based production of these antibodies is becoming increasingly popular. However, plant-directed glycosylation of the Fc region of an antibody may compromise its ability to mediate effector functions and may also be immunogenic. To combat these potential problems, RNA silencing has been used in the algal Lemna production system to silence two endogenous glycan-transferase activities, resulting in the production of therapeutic antibodies with glycosylation homogeneity which improves not only the antibody's safety but also its functionality (Cox *et al*., 2006).

Ironically, the plant RNAi application most likely to be the next commercial reality is one that delivers aesthetic rather than nutritional, medical or environmental benefits to man kind. It has been a long quest to produce a blue rose but it has now been achieved with the help of RNA silencing. Roses lack an enzyme for the biosynthesis of dihydromyricetin, an intermediate compound required for the production of delphinidin-based anthocyanins, the major constituents of violet and blue flowers. When the gene encoding this enzyme in Viola was transferred to roses, its expression resulted in the generation of transformed plant lines with purple petals, because one of the rose enzymes involved with the conversion of dihydromyricetin into delphinidin also converts other intermediate compounds into red and yellow pigments. However, silencing this gene using RNAi and introducing the homologous gene from Iris gave transformed rose plants that bore flowers with pure blue hues never seen before (Katsumoto *et al*., 2007).

RNA interference has been used for applications in biotechnology, particularly in the engineering of food plants that produce lower levels of natural plant toxins. Such techniques take advantage of the stable and heritable RNAi phenotype in plant stocks. For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pest (Sunilkumar *et al*., 2006). Similar efforts have been directed towards reduction of the cyanogenic natural product linamarin in cassava plants (Siritunga and Sayre, 2003).

Gene silencing techniques have been applied in making selection in the F1 generation. The dominant nature of RNAi makes it an important means of knocking down expression of targeted genes in plant hybrids. Classically, the investigation of gene function in hybrids has been problematic. Because the majority of mutations are recessive, out-crossing a recessive mutant in order to form an F1 hybrid results in rescue of the mutation due to the wild-type gene contributed by the second parent, thus masking any phenotype of interest. Selfing the F1 hybrid to produce F2 individuals are alike due to segregation and random assortment of the other entire gene from both parents, thus obscuring the unique genetic constitution of the F1 hybrids. The problem is further exacerbated in inter-species hybrids which are often sterile, such that the F1 provides the only generation that can be studied. For these reasons, RNAi technology is ideally suited for studying the role of particular genes in first generation hybrids, as only one parent needs to carry the RNAi-inducing transgene. Based on these conditions, the use of RNAi to explore the role of chromatin modifying genes in stabilizing gene expression patterns and in finding novel phenotypes in newly formed hybrids can be achieved using this approach (Preuss and Pikaard, 2003).

ADVANTAGES AND LIMITATIONS OF GENE SILENCING TECHNOLOGIES

Advantages:

- This method could be effective against a broad spectrum of a pathogen's species and have implication for the control of other plant parasites and pathogens
- It is reliable, reduce labor, lower expenses, easy, increase cropping choices and eliminate the need for chemicals that may be harmful to the environment
- It could be used for functional analysis of plant genes through loss-of-function of genes
- It generates rapid phenotype that plant transformation is not needed (Lu *et al*., 2003)
- There is no need to screen large populations to detect the function of a specific gene, only a single plant is enough to follow phenotype with targeted silencing. Therefore, repeating the experiment is easy and time effective
- Host range wideness of viral vectors is the other versatility of the approach. For instance TRV can infect spinach, beet, potato and tobacco naturally. Hence, TRV-based VIGS is applied to *Nicotiana benthamiana*, tomato, Arabidopsis, chilli pepper, opium poppy, aquilegia vulgaris
- VIGS system is particularly useful on plants which are difficult or impossible to transform
- VIGS can be applied to the genes associated with embryonic development or essential housekeeping functions in plant (Ding *et al*., 2006)
- It has the ability to target multiple gene family members with a single RNAi-inducing transgene
- The gene knock downs due to RNAi are dominant and the insertional or other loss-of-function mutations are recessive. The dominant aspect of RNAi allows the knock down of genes in polyploid genomes that contain four or more orthologs and are thus refractive to traditional mutagenesis. Likewise, orthologs can be knocked down in F1 hybrid in which the RNA-inducing transgene is introduced through only one of the parents. So, the dominance of RNAi allows one to save time by eliminating the additional generations needed to identify individuals that are homozygous for recessive loss-of-function alleles (Preuss and Pikaard, 2003)

Limitations:

- Complete loss-of-function by VIGS might not be achieved. Generally, 75-90% down regulation in the expression level of the targeted gene is accomplished. Unfortunately the low level of gene expression can be enough to produce functional protein and phenotype in silenced plant. Some of viral infections can cause symptoms on plant that might mask the phenotype caused by the phenotype. This might be minimized by TRV vectors as TRV-VIGS system produces mild symptoms
- Since, the system relies on sequence information, it can only be used for specific gene silencing if only the sequence information is known
- The approach also depends on pathogen-host interaction, so pathogen infection may manipulate host function and alter development and morphology. There should be positive control in all VIGS assays to mark the effect of viral inoculation on silenced plant
- VIGS might suppress non-targeted gene in silenced plant cell or tissue

CONCLUSION

Finally, successful application of gene silencing depends on high target specificity and silencing efficiency. VIGS shows much promise as a tool for gene function studies and for high throughput

functional genomics in plants which can be used in the next generation plant genomics. When this gene silencing technologies are incorporated into plant breeding, problems associated with plant diseases and different metabolic processes (like high cyanide, lignin, palmitic acid content) will be addressed.

Moreover, gene silencing technology (especially VIGS) requires sequence information. This is a limiting factor to some plant species without EST (Expressed Sequence Tag) database, though some of this species can be characterized with EST databases from closely related species (Lu *et al*., 2003). Constructing libraries for uncharacterized species is necessary. When this is done, large-scale screens that could be applied for large number of different hosts can commence.

Also, VIGS which might suppress non-targeted gene in silenced plant cell or tissue should be addressed before the next genomic era.

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