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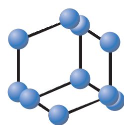
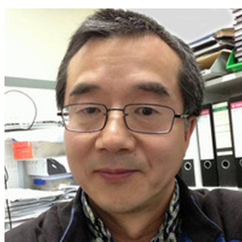


RNA Silencing in Plants: Mechanisms, Technologies and Applications in Horticultural Crops

Qigao Guo[1], Qing Liu[2,*], Neil A. Smith[2], Guolu Liang[1] and Ming-Bo Wang[2,*]

[1]College of Horticulture & Landscape Architecture, Southwest University, Chongqing, 400716, China; [2]Commonwealth Scientific and Industrial Research Organisation Agriculture (CSIRO), ACT 2601, Australia



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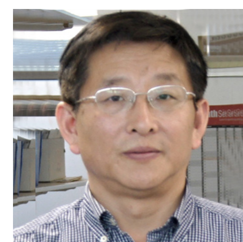

M.-Bo Wang

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Abstract: Understanding the fundamental nature of a molecular process or a biological pathway is often a catalyst for the development of new technologies in biology. Indeed, studies from late 1990s to early 2000s have uncovered multiple overlapping but functionally distinct RNA silencing pathways in plants, including the posttranscriptional microRNA and small interfering RNA pathways and the transcriptional RNA-directed DNA methylation pathway. These findings have in turn been exploited for developing artificial RNA silencing technologies such as hairpin RNA, artificial microRNA, intrinsic direct repeat, 3' UTR inverted repeat, artificial trans-acting siRNA, and virus-induced gene silencing technologies. Some of these RNA silencing technologies, such as the hairpin RNA technology, have already been widely used for genetic improvement of crop plants in agriculture. For horticultural plants, RNA silencing technologies have been used to increase disease and pest resistance, alter plant architecture and flowering time, improve commercial traits of fruits and flowers, enhance nutritional values, remove toxic compounds and allergens, and develop high-value industrial products. In this article we aim to provide an overview of the RNA silencing pathways in plants, summarize the existing RNA silencing technologies, and review the current progress in applying these technologies for the improvement of agricultural crops particularly horticultural crops.

Keywords: Double-stranded RNA, Hairpin RNA, miRNA, Plants, RNA silencing, siRNA, Virus.

1. RNA SILENCING PATHWAYS IN PLANTS

RNA silencing is an evolutionarily conserved mechanism in eukaryotes. It is induced by double-stranded RNA (dsRNA) or hairpin structured RNA (hpRNA), involving common factors including Dicer or Dicer-like (DCL) and Argonaute (AGO) family proteins [1-3]. In the basic RNA silencing pathway, dsRNA or hpRNA is processed by a Dicer or DCL protein into 20-24 nucleotide (nt) small RNA (sRNA) duplex with 2-nt 3' overhangs at both ends. One strand of the sRNA duplex is incorporated into an AGO forming an RNA-induced silencing complex (RISC). The sRNA molecule guides the RISC to the complementary region of single-stranded RNA, and the AGO protein then cleave the RNA at the nucleotides corresponding to the central region (usually nt. 10-11) of the sRNA [1-3].

The RNA silencing pathway has greatly diversified in plants to cope with different functional requirements [1, 4]. According to the source of dsRNA or hpRNA precursor andthe functional target of sRNAs, RNA silencing in plants can be classified into 4 overlapping but functionally distinct pathways: microRNA (miRNA) pathway, trans-acting small interfering RNA (tasiRNA) pathway, RNA-directed DNA methylation pathway, and exogenic RNA silencing pathway. Associated with the diversification of RNA silencing pathways, plants have evolved multiple RNA silencing factors. For instance, the model plant Arabidopsis encodes four DCLs, six RNA-dependent RNA polymerases (RDRs), and ten AGOs, plus several other factors.

1.1. The miRNA Pathway

miRNAs are 20-24-nt sRNAs derived from genetic loci known as MIR genes [5]. Like protein-coding genes, MIR genes are transcribed by RNA polymerase II to generate primary miRNA transcript (pri-miRNA). The pri-miRNA forms an imperfect "fold-back" stem-loop or hairpin structure due to the existence of intra-molecular sequence complementarity, which is processed into a short "stem-loop" precursor (pre-miRNA) by DCL1 in the nucleus with the assistance of the dsRNA-binding protein DRB1 or HYL1 [6-8]. The pre-miRNA molecule is further processed by DCL1 in the nucleus to generate a 21-nt imperfect RNA duplex comprised of mature miRNA (guide strand) and miRNA* (miRNA passenger stand). The 3' terminal nucleotides of the

*Address correspondence to these authors at the Commonwealth Scientific and Industrial Research Organisation Agriculture, Clunies Ross Street, Canberra ACT 2601, Australia; Tel: + 61-2-62465197/+61-2-62464919; Fax: +61-2-62465000; E-mail: ming-bo.wang@csiro.au; qing.liu@csiro.au

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RNA duplex are methylated at the 2'-O-hydroxyl group by the RNA methylase HUA ENHANCER1 (HEN1), which is suggested to protect the miRNA:miRNA* duplex from degradation [9]. miRNA:miRNA* duplexes are exported into the cytoplasm where the mature miRNA is loaded onto an AGO protein (primarily AGO1) to form RISC. Unlike animal miRNAs that normally target the 3' untranslated region (UTR) of mRNAs with imperfect sequence match to inhibit protein translation, plant miRNAs in general have high levels of sequence complementarity with their target mRNA, and direct sequence-specific RNA cleavage [10]. The primary target genes of miRNAs in plants are regulatory genes such as transcription factor genes. MiRNA therefore play a key role in plant development.

1.2. The Trans-acting siRNA Pathway

Like miRNAs, tasiRNAs are a class of 21-nt sRNAs derived from non-coding transcript transcribed by RNA polymerase II from genetic loci known as TAS genes [11-13]. tasiRNA biogenesis is initiated by specific miRNAs that direct the cleavage of TAS precursor RNA. The miRNA cleavage fragments of TAS transcript are converted to long dsRNA by RDR6, which is then processed by DCL4 into 21-nt siRNAs with 21-nt phasing starting from the miRNA cleavage site [13-17]. To initiate tasiRNA production, the size of miRNAs is important, with only 22-nt miRNAs being found to trigger tasiRNA biogenesis [18, 19]. The 21-nt class of miRNAs or 21-nt artificial miRNA variants of the ta-siRNA-inducing 22-nt miRNAs, are unable to trigger ta-siRNA production [18]. Like miRNAs, tasiRNAs are methylated by HEN1 [9] and interact with either AGO1 or AGO7 to direct the degradation of target mRNAs. In Arabidopsis, ta-siRNAs are found to target auxin response factors involved in phase transition from juvenile to reproductive stages [20].

A large number of tasiRNA-like siRNAs, collectively known as phased siRNAs or phasiRNAs, have been identified in both Arabidopsis and other plant species [21-24]. These phasiRNAs are the same as the originally defined ta-siRNAs, being 21-nt in size and requiring 22-nt miRNAs, AGO1, RDR6 and DCL4 for biogenesis. RNA from both protein-coding genes and non-coding sequences such as transposon and repetitive DNA can serve as template for phasiRNA production. The functions of these phasiRNAs have yet to be fully understood. However, phasiRNAs derived from leucine-rich repeat (NB-LRR) disease resistance genes have been suggested to play a role in controlling the dynamic stress responsive expression pattern of plant defence genes [22, 25]. Furthermore, phasiRNAs from transposable elements (TEs) identified in the vegetative nucleus of pollen grains, in dedifferentiated plant cell cultures and in DNA methylation mutants, are proposed to act as a back-up mechanism to post-transcriptionally silence TEs, allowing TEs to evade long-term heterochromatic or transcriptional silencing [24].

1.3. The RNA-directed DNA Methylation (RdDM) Pathway

The RdDM pathway is unique to plants and mediates *de novo* DNA methylation and transcriptional silencing in the nucleus [26-29]. RdDM is directed by 24-nt siRNAs, which is generated by a combined function of the plant-specific RNA polymerase IV (PolIV), RDR2, and DCL3. In brief, PolIV transcribes methylated and highly repetitive DNA to generate aberrant RNA and RDR2 converts this single-stranded RNA (ssRNA) into dsRNA, which is subsequently processed by DCL3 into 24-siRNAs that are also methylated at the 3' hydroxyl group of the terminal nucleotides by HEN1 [9]. The 24-nt siRNAs are loaded onto AGO4 to form RISC, a process involving both nuclear and cytoplasmic steps [30]. This AGO4-siRNA complex then interacts with long non-coding RNA transcribed from target DNA by another plant-specific RNA Polymerase V (PolV) to recruit other factors including Domains Rearranged Methylase2 (DRM2), resulting in direct *de novo* DNA cytosine methylation. *De novo* cytosine methylation at the symmetric CG and CHG (H stands for A, C or T) contexts can be maintained during DNA replication by Methyltransferase1 (MET1) and Chromomethylase3 (CMT3), respectively. However, *de novo* cytosine methylation at the non-symmetric CHH contexts cannot be maintained during DNA replication and therefore depends entirely on RdDM. Recently a non-canonical RdDM mechanism is unveiled that is induced by 21-nt siRNAs [31, 32]. The principal function of RdDM is to silence TEs and repetitive DNA to maintain genome stability. Indeed, 24-nt siRNAs are also known as repeat-associated siRNAs or ra-siRNAs as most of these siRNAs are derived from TEs and repetitive DNA in the plant genome.

1.4. RNA Silencing Induced by Exogenic Nucleic Acids

RNA silencing can be induced in plants by invading nucleic acid molecules. In particular, the term "exogenic RNA silencing" used in this review refers to RNA silencing induced by sense transgenes and viruses. The RNA silencing phenomenon was first observed in studies on sense transgenes, which showed that a transgene designed to overexpress a pigmentation enzyme in petunia is not only self-silenced but also causes the silencing of the endogenous counterpart, resulting in the loss of pigmentation in the flowers [33, 34]. Furthermore, the first evidence indicating RNA as the inducer of gene silencing also came from studies on sense transgene-mediated virus resistance in plants, where the expression of virus-derived transgenes induces sequence-specific RNA degradation leading to virus resistance [35]. Exogenic RNA silencing overlaps with the endogenous siRNA and RdDM pathways. In fact, most of our understanding on these endogenous siRNA silencing pathways has come from studies using transgenes and viruses as models.

1.4.1. Sense Transgene-induced RNA Silencing

Sense transgenes can be silenced both transcriptionally and post-transcriptionally, which often occurs when transgenes are integrated into the plant genome as multiple-copy repeats [1, 36]. The exact mechanisms for both transcriptional (TGS) and post-transcriptional (PTGS) gene silencing have yet to be fully elucidated. TGS is in general associated with DNA methylation at promoters of transgenes, which is likely to be induced by RdDM. Indeed, artificial expression of long hpRNA targeting a transgene promoter can induce DNA methylation at the promoter and TGS of the transgene [37]. It is possible that multiple-copy transgene repeats can

be recognized by PolIV and RDR2 to generate 24-nt siRNAs triggering RdDM. Alternatively, read through transcription across multiple transgene repeats can generate promoter transcript that can in turn result in 24-nt siRNAs and RdDM.

PTGS of a sense transgene requires RDR6, DCL4, SGS3 and AGO1 [4, 38], and therefore resembles the tasiRNA pathway. Two aspects of PTGS, transitivity and systemic movement, both involve 21-nt siRNA production from regions outside the primary target site [39-42], indicating that tasiRNA-like secondary siRNAs are an important component of PTGS. While the tasiRNA pathway is initiated by miRNAs, the primary inducer of PTGS remains a mystery. It has been proposed that “aberrant RNA” transcript derived from transgenes is an effective trigger of PTGS [43, 44], presumably by serving as template for RDR to produce dsRNA, but the exact nature of “aberrant RNA” remains unknown. Alternatively, some intrinsic stem-loop structures of a transgene transcript may be processed directly by DCLs [45]. Furthermore, it has been proposed that read through transcription through multiple-copy inverted-repeat transgene insertions can generate long hpRNA [46]. siRNAs processed from these primary dsRNA or hpRNAs could either induce full-scale PTGS themselves, or more likely, trigger the production of tasiRNA-like secondary siRNAs leading to full-scale PTGS. Interestingly, transitive silencing of transgenes requires DCL2, which is responsible for processing 22-nt siRNAs [41]. It is possible that DCL2-derived 22-nt siRNAs are capable of inducing secondary siRNA production by RDR6, SGS3 and DCL4 in the same way as 22-nt miRNAs in triggering tasiRNA biogenesis.

1.4.2. Anti-viral RNA Silencing

Infection of plants with any type of viruses or subviral agents is associated with the accumulation of viral sRNAs, which are processed from double-stranded viral RNA by the same host plant siRNA biogenesis machinery and direct the degradation of single-stranded viral RNAs using the same AGO proteins [47, 48]. Viruses are therefore both an inducer, and target of the siRNA pathways. In fact, RNA silencing has been regarded as a natural antiviral defence mechanism in plants [36].

siRNAs derived from RNA viruses are processed primarily by DCL4 but also by DCL2, resulting in 21-nt size class being usually the most dominant siRNAs followed by the 22-nt size class. dsRNA formed between plus and minus-strand viral genomic RNAs, and stem-loop structures formed within single-stranded viral genomic RNA, have both been suggested as a precursor for viral siRNAs [49, 50]. However, recent studies using sRNA deep sequencing indicates that viral siRNA accumulation requires host-encoded RDRs [51-55], suggesting that viral siRNA biogenesis may also resemble the endogenous tasiRNA pathway.

All size classes of viral siRNAs, namely 21, 22, and 24-nt siRNAs, are associated with the infection of DNA viruses [49, 56, 57]. For DNA viruses, genome replication and RNA transcription occur in the nucleus of the host plant cells, which may explain why the nuclear RdDM factor DCL3 also participates in processing DNA virus siRNAs generating the 24-nt size class. These 24-nt viral siRNAs can direct RdDM to the DNA virus genome, leading to DNA methylation of viral gene promoters and TGS of the viral genes [58, 59]. Thus, both the PTGS and TGS siRNA pathways are involved in plant defence against DNA viruses.

To survive the host anti-viral RNA silencing, plant viruses have evolved counter-defence mechanisms by encoding multi-functional proteins that can suppress RNA silencing [47, 60-62]. These viral suppressors of RNA silencing (VSRs) inhibit RNA silencing at various steps of the pathways, such as binding siRNA duplexes to prevent RISC formation and inducing degradation of AGO1. The non-coding subviral RNAs, namely viroids and viral satellite RNAs, have evolved stable secondary structures and/or specific subcellular localization to resist RNA silencing-mediated RNA degradation [63-65].

2. RNA SILENCING TECHNOLOGIES IN PLANTS

Since the discovery of dsRNA-induced gene silencing in 1998 [46, 66] and subsequent elucidation of the various sRNA pathways in plants, a number of transgene or virus-based gene silencing technologies have been developed to artificially induce RNA silencing in plants. These technologies, particularly the hpRNA and artificial miRNA technologies, have been widely used in gene function studies and genetic engineering for crop improvement in plants. Here we summarize these technologies based on the RNA silencing pathways through which these technologies function.

2.1. The Basic RNA Silencing Pathway - Hairpin RNA (hpRNA) Transgene

Transgenes designed to express long self-complementary hairpin RNA (hpRNA) was first demonstrated to be highly effective at inducing RNAi in plants in 1998 [46, 67]. hpRNA transgenes have since been widely used to silence genes and viral RNAs in plants [68]. A typical hpRNA construct is comprised of a sense and an antisense sequence of a portion of target gene mRNA as inverted repeats, and these inverted repeats are separated by a non-complementary spacer region (Fig. **1-A**). The spacer sequence is mainly used for stabilizing the hpRNA construct during cloning in bacterial cells, as perfect inverted-repeat DNA is highly unstable in bacteria. In addition, using a spliceable intron as spacer has been shown to improve RNA silencing efficiency in plants [69]. The sense and antisense sequences in the transcribed RNA are complementary to each other and form a dsRNA arm. Therefore, hpRNA transgene-induced silencing should not require RDRs to generate dsRNA. hpRNA is processed primarily by DCL4 to generate 21-nt siRNAs, but DCL2 and DCL3 also participate in the processing of hpRNA resulting in low levels of 22 and 24-nt siRNAs. Interestingly, hpRNA expressed either by an RNA polymerase III promoter [70], or residing inside an intron [71], is shown to be processed into predominantly 24-nt siRNAs, indicating that nucleolar-localized hpRNA is specifically targeted by DCL3. It is unclear if secondary siRNA amplification is also involved in hpRNA transgene-induced silencing. It is possible that target mRNA, or unprocessed hpRNA, can serve as template for RDRs to generate secondary siRNAs that can strengthen or reinforce hpRNA transgene-induced silencing. hpRNA-derived siRNAs, presumably the 24-nt size class, can induce RdDM to the hpRNA transgene itself. It remains

unclear if this RdDM can affect the transcriptional activity of the hpRNA transgene, as the methylation is targeted to the transcribed region but not the promoter. Interestingly, an hpRNA transgene induce stronger target gene silencing in RdDM mutant plants than in wild-type plants [72], suggesting that hpRNA transgenes are likely to be subject to RdDM-caused transcriptional self-silencing. Strategies to minimize TGS, particularly any spread of RdDM-caused DNA methylation to the promoter of an hpRNA transgene, are expected to improve the stability of hpRNA transgene-induced silencing. hpRNA transgenes are usually designed against mature mRNA sequences of target genes, but intron-targeting hpRNA transgenes have been shown to induce effective silencing [73], suggesting that hpRNA-induced silencing at least partly occurs in the nuclei.

hpRNA constructs are relatively difficult to prepare due to the inverted-repeat structure. A number of cloning vectors have therefore been developed to facilitate the preparation of hpRNA constructs, such as pHannibal and pHellsgate vectors [74, 75]. In addition, a method based on rolling-circle DNA replication by ϕ29 polymerase has been established to prepare genomewide hpRNA libraries [76, 77].

2.2. The miRNA Pathway - Artificial miRNA (amiRNA)

Following the understanding of miRNA biogenesis and miRNA precursor structures in Arabidopsis, artificial miRNA (amiRNA) is developed as an alternative technology for silencing genes in plants [78]. Basically, an amiRNA construct is made by replacing the miRNA and miRNA* sequences of a natural miRNA precursor with corresponding target gene sequences; in the modified amiRNA precursor, the stem-loop structure of the original miRNA precursor is maintained (Fig. **1-B**). The miRNA strand of an amiRNA construct consists of the sequence complementary to target mRNA, while the sequence of the miRNA* strand is designed to maintain the miRNA:miRNA* duplex structure of the native miRNA precursor [78]. Thus, amiRNA transgenes use the endogenous miRNA pathway to silence genes. amiRNA constructs are relatively easy to prepare in comparison to hpRNA constructs. The main advantage of the amiRNA technology over hpRNA transgenes is that silencing is directed by a single sRNA species targeting a short 21-nt region, and is therefore less likely to have off-target effects. However, amiRNA design involves careful sequence selection to meet the requirement for efficient AGO binding. Furthermore, amiRNA-mediated silencing does not involve secondary siRNA amplification, making it possible that amiRNAs might be more effective at silencing low abundance transcripts than high abundance ones.

2.3. The tasiRNA and phsiRNA Pathways – Artificial tasiRNAs

In the tasiRNA pathway, a non-coding RNA transcript is targeted by a 22-nt miRNA, and the cleavage fragments serve as template for RDR6 to generate dsRNA which is processed by DCL4 to 21-nt phased siRNAs. The miR173-targeted TAS1 transcript has been modified to express artificial tasiRNAs (atasiRNA), which are shown to be effective at inducing silencing of selected endogenous genes [79, 80]. The atasiRNA constructs can be made by replacing several of the 21-nt native phased siRNA sequences of the TAS1 transcript with an antisense target gene sequence of the same length. In addition, constructs in which the miR173 target site is retained but the bulk of the TAS1 sequence is replaced with a relatively long target gene sequence has also been shown to be effective at inducing silencing (Fig. **1-C**). However, substituting the miR173 target site with the target sequences of the 21-nt miR167 or miR171 abolishes the silencing effect of the atasiRNAs, confirming that 22-nt miRNAs are the necessary trigger. Like amiRNA constructs, atasiRNA constructs are relatively easy to prepare in comparison to hpRNA constructs. A recent study showed that tasiRNAs can induce DNA methylation at their corresponding TAS gene loci [81], implying that, like hpRNA transgenes, atasiRNA transgenes could potentially be subject to RdDM-mediated transcriptional self-silencing.

2.4. Three Additional PTGS-based Gene Silencing Technologies – Direct Repeat, 3' UTR Inverted-Repeat, and Terminator-less Transgenes

2.4.1. Intrinsic Direct-repeat Transgene

A transgene containing a two-copy tandem repeat sequence in the transcribed region was previously shown to induce stronger silencing than the simple sense and antisense transgenes, although at a lower efficiency than the hpRNA transgene [67]. A more recent study showed that transgenes with 3 or 4-copy intrinsic direct repeats (Fig. **1-D**) induce potent gene silencing with almost 100% efficiency in both Arabidopsis and maize [82, 83]. Direct-repeat transgenes are not expected to produce dsRNA directly, so how they induce potent gene silencing remains unclear. The silencing is associated with the accumulation of siRNAs, and has therefore been suggested to be PTGS, although the exact size of the siRNAs has not been determined. The intrinsic direct repeats appear to be capable of inducing transitive silencing: when a sequence with no homology to the direct repeats is transcriptionally fused at the 3' end, the transgene can induce silencing against both the target of the direct repeats and that of the non-homologous fusion sequence [82, 83]. This suggests that siRNAs derived from the direct repeats can induce secondary siRNA production from the downstream sequence.

2.4.2. 3' UTR Inverted-repeat (IR) Transgene

Another transgene structure that has been demonstrated to induce efficient silencing in tomato and Arabidopsis consists of a sense target gene sequence followed by an IR of the nopaline synthase (nos) terminator [84]. In this type of construct, a target gene sequence, that has no homology to the nos terminator sequence, is cloned in front of the nos IR that is arranged in an antisense-sense configuration separated with a spacer (Fig. **1-E**). Like the direct-repeat transgenes, the underlying mechanism of gene silencing induced by 3' UTR IR transgenes remains unclear. However, siRNAs can be detected from the target gene sequence upstream of the nos IR sequence, suggesting that tasiRNA-like secondary siRNAs are induced by the downstream IR structure. The antisense nos terminator sequence, and the 5' part of the sense nos terminator sequence are both transcribed which can form an hpRNA structure. It is possible that siRNAs processed from this hpRNA structure target 3' region of the fusion transcript, which in turn triggers 3' to 5' transitive spread of siRNAs resulting in target gene silencing [84].

2.4.3. Terminator-less Transgenes

Another distinct transgene structure has been found to induce effective gene silencing in plants. In this transgene structure, a 35S-HPT-nos (hygromycin resistance selectable marker gene) cassette is inserted either inside or in front of a target gene sequence, with no transcription terminator at the 3' end of the target gene sequence (Fig. **1-F**) [85]. Another promoter can be placed in front of the 35S-HPT-nos cassette, but adding a terminator sequence after the target gene sequence abolishes silencing. Like the direct repeat and 3' UTR IR constructs, the mechanism by which the terminator-less transgene induces silencing remains unclear although siRNAs are also detected in the silenced plants suggesting a PTGS nature. It is suggested that unpolyadenylated transcript is a trigger of silencing [85]. However, in this transgene structure, transcript of the target gene sequence can only be generated from read through transcription, namely from incomplete transcription termination by the nos terminator of the 35S-HPT-nos cassette. Thus, in addition to being unpolyadenylated, read through transcript may have other distinct features conducive for triggering silencing.

2.5. The RdDM Pathway – Promoter-targeting Transgenes

TGS in plants are usually associated with DNA methylation at gene promoters. TGS of transgenes could be inherited without the presence of the initial silencing inducer, making TGS an attractive strategy to stably and permanently silence a gene. The RdDM mechanism has therefore been explored for developing efficient TGS technologies. However, while promoters of transgenes can be readily methylated and inactivated by siRNAs derived from viruses or hpRNA transgenes [37, 86], those of endogenous genes have proved to be relatively recalcitrant to RdDM-mediated TGS, although cytosine methylation can be induced [4, 87]. It has been suggested that histone modifications at the target promoters are required to cause TGS [87]. Nevertheless, a small number of endogenous promoters have been successfully silenced using hpRNA transgenes [88, 89]). Furthermore, a recent study shows that transgenes expressing unpolyadenylated antisense transcript of endogenous promoters (Fig. **1-G**) can induce efficient TGS of the endogenous genes, which is associated with both the accumulation of 24-nt siRNAs and repressed chromatin at the target promoters [90]. hpRNA transgenes without terminator is also shown to induce TGS, but sense transgenes are not effective. These results indicate that at least some endogenous promoters can be transcriptionally silenced by RdDM. Standard hpRNA transgenes generate predominantly 21-nt siRNAs, and are therefore not ideal for inducing RdDM. hpRNA expressed from a Pol III promoter [70], or residing inside an intron (Fig. **1-H**) [71], is processed predominantly to 24-nt siRNAs. It would be interesting to investigate if these types of hpRNA constructs can be used to trigger TGS in plants.

2.6. The Anti-viral RNA Silencing Pathway - Virus Induced Gene Silencing (VIGS)

Infection of plants with any viral and subviral agents is associated with the accumulation of siRNAs corresponding to all regions of viral genomes. The natural function of these viral siRNAs is to guide the degradation of viral RNA and methylation of viral DNA, inhibiting virus replication. However, the same viral siRNAs can also direct silencing against host gene transcript if sequence homology exists between viral genomes and host genes, as exemplified by the silencing of the chlorophyll biosynthetic gene *CHLI* by siRNAs derived from the Cucumber mosaic virus Y-satellite RNA [91, 92]. The antiviral defence RNA silencing mechanism has therefore been used to develop virus-induced gene silencing (VIGS) technologies [93]. In VIGS, a target gene sequence is inserted into a viral vector, and the recombinant virus is then used to infect plants, causing target gene silencing. VIGS is rapid, and does not require plant transformation, which is particularly advantageous for silencing genes in plants that are recalcitrant to transformation. Both DNA and RNA viruses, as well as viral satellites, have been used to generate VIGS vectors. However, there are some limitations for VIGS. For instance, most plant viruses have a relatively narrow host range, so each VIGS vector can only be applied in a limited number of plant systems. Furthermore, not all viruses can be easily modified to generate an infectious clone, and only those that do not cause severe symptoms can be used for making VIGS vectors. Another limitation with VIGS is that most plant viruses replicate in somatic tissues but are excluded from reproductive tissues, making it difficult to silence genes in all plant tissues. Nevertheless, VIGS technologies have been successfully used in analysing gene functions in a variety of plant species.

3. EXAMPLES OF RNA SILENCING APPLICATIONS IN HORTICUTURAL PLANTS

RNA silencing technologies, particularly the hpRNA transgene technology and more recently the amiRNA technology, have been widely used to study gene function, generate pathogen and pest resistance, and improve other agronomical traits by manipulating the expression of metabolic pathway genes in a variety of model and crop plant species. In this review, we focus on the examples of RNA silencing applications in horticultural plants, particularly in pathogen and pest resistance and crop improvement by metabolic engineering.

3.1. Enhancement of Resistance to Biotic Stresses

Biotic stresses caused by viral, bacterial, and fungal diseases as well as insects, and nematodes are severe constraints to crop productivity. Viruses are particularly difficult to control as they use a variety of strategies to multiply and to spread both through and between plants. Virus-resistant potato (*Solanum tuberosum* L.) plants transformed with vectors simultaneously expressing both the sense and antisense transcripts of the viral helper-component proteinase (*HCPro*) gene was one of first examples showing complete immunity to potato virus Y (PVY) [46]. Commercial varieties of potato highly resistant to three strains of PVY have been developed by expressing hpRNA derived from the 3' terminal part of the coat protein gene of PVY [94]. More recently, transgenic tomato plants resistant to potato spindle tuber viroid (PSTVd) were obtained by expressing hpRNA from PSTVd sequences [95]. In some cases, virus-resistant plants generated through expressing virus coat proteins were in fact also mediated by the RNA silencing mechanism. For example in

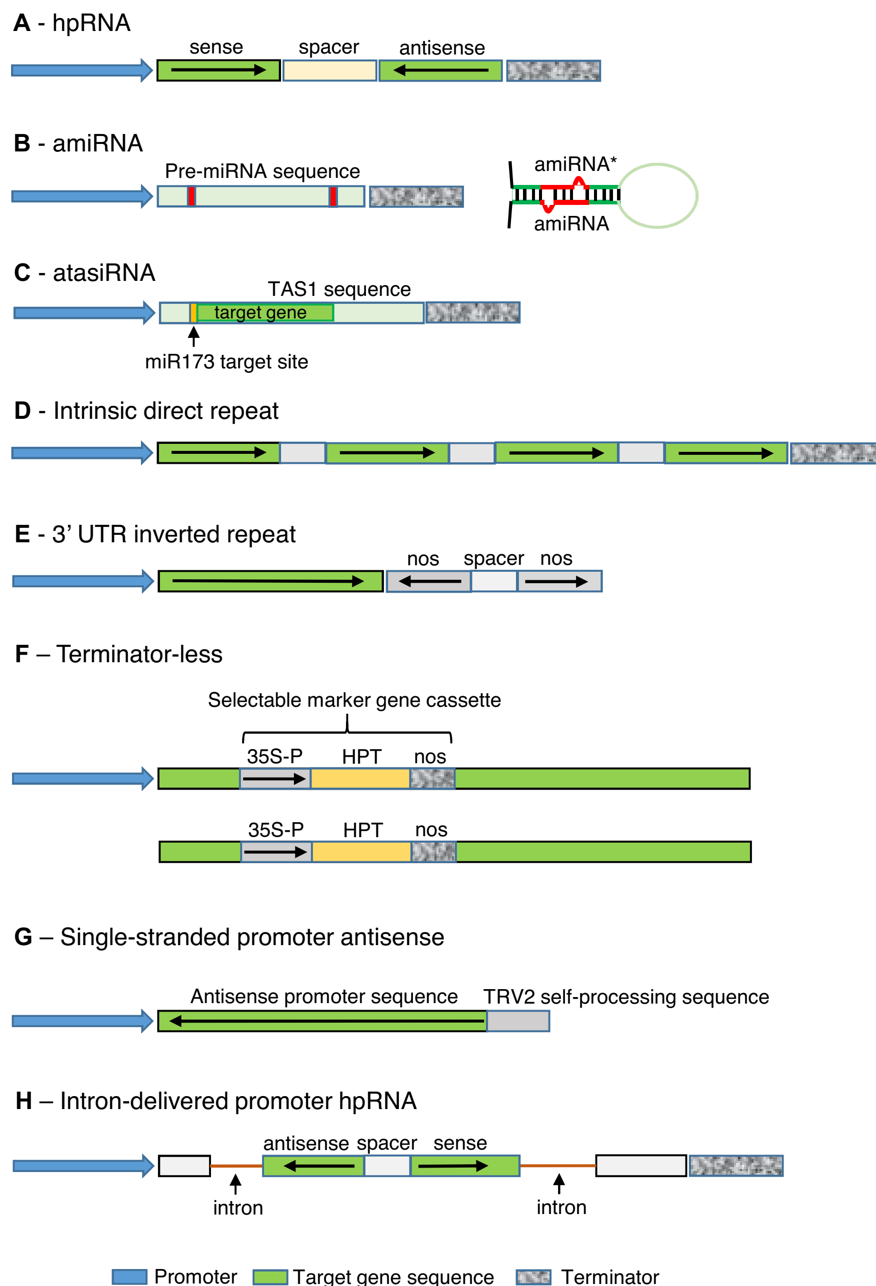

Fig. (1). Schematic diagrams of the different types of RNA silencing constructs discussed in this review. The target sequence is shown in green, except for the amiRNA construct where the amiRNA and amiRNA* sequences are shown in red. The promoter is shown in blue, and the terminator in textured grey. The predicted amiRNA structure is also shown.

papaya that is known for its edible fruits and as a major source of papain enzyme, virus resistant plants expressing virus coat protein showed significant reduction of coat protein mRNA, revealing RNA silencing-based mechanism [96].

Transgenic potato plants expressing hpRNA construct targeted against *plasma membrane-localized Syntaxin-related 1* (*SYR1*) showed enhanced resistance to the oomycete pathogen *Phytophthora infestans* [97]. In response to infection with *P. infestans*, transgenic potato demonstrated constitutive accumulation of salicylic acid and *PR1* transcripts. Cytological examination revealed the *P. infestans* infection was coincided with aberrant callose deposition and decreased papilla formation, suggesting an involvement of syntaxins in secretory defense responses in potato.

Helicoverpa armigera is an important lepidopteran pest feed on cotton and some other crop plants and cause great yield losses. It has been revealed that a cytochrome P450 gene *CYP6AE14* is responsible for the insect tolerance to a toxic cotton metabolite gossypol at otherwise inhibitory concentrations. Transgenic tobacco plants expressing hpRNA directed against *CYP6AE14* was shown to provide sufficient levels of specific dsRNA to suppress gene expression in the insect midgut [98]. Such a strategy may have laid the basis

for a new strategy of insect control by feeding insects with plant tissues engineered to produce a specific dsRNA that suppresses the vital genes in the pest. Similar strategy was applied to root-knot nematodes (*Meloidogyne* spp.) that infect many plant species and have caused large economic losses worldwide. Transgenic tobacco plants expressing an hpRNA construct targeting a root-knot nematode (*Meloidogyne javanica*) putative zinc finger transcription factor, MjTis11, effectively suppressed the growth of nematodes feeding on the roots of these transgenic plants [99]. Such a host-delivered gene silencing demonstrated that plants can be used as a delivery system to induce dsRNA-mediated gene silencing in parasites such as root-knot nematodes. More recently, hpRNA transgenes against *β*-actin gene of the Colorado potato beetle were designed to be expressed in the chloroplast of potato, which provided strong protection against herbivory by the insect pest [100]. Chloroplasts have no RNA silencing machinery like DCLs allowing the full-length hpRNA to accumulate at high levels, which is likely to account for the high efficacy of chloroplast-expressed hpRNA in directing the silencing of the insect gene. Consistent with this, an hpRNA transgene expressed in the nucleus provided no effective protection against the insect. These results suggest that dsRNA needs to be processed inside the target organism in order to induce the silencing of its gene, and that siRNAs already processed inside plant cells are not effective at inducing cross-kingdom gene silencing in insects.

3.2. Alteration of Plant Architecture and Flowering Time

A number of studies carried out in tomato and petunia on genetic manipulation of plant architecture through RNA silencing have served as platforms for understanding the molecular basis of plant architecture [101]. Such a biotechnological advance could have a wide utility in flowering, ornamental, and horticultural crops, as exemplified in the area of machinery harvest of fruits or seeds from tall trees, leaf plucking in tea or mulberry plants. Interestingly, carotenoid cleavage dioxygenase (*CCD*) genes have been demonstrated to play an integral role in the control of branch development in model plants [102]. The reduction of *AcCCD8* expression in transgenic kiwi fruit (*Actinidia chinensis* L.) plants through hpRNA-induced silencing was found to be concomitant to an increase in the total number of branches and delayed leaf senescence over two growing seasons [103]. Such an improvement in plant architecture is anticipated to increase the number of flowers produced on kiwifruit vines by increasing the proportion of nodes with the potential to set fruits.

European pear (*Pyrus communis* L.) normally requires a long juvenile phase before they can flower and set fruits. A transgenic line termed as Early Flowering-Spadona (EF-Spa) was produced expressing an hpRNA cassette targeting the *Terminal flower 1* (*TFL1*) that is a key gene involved in repressing flowering and maintaining the inflorescence meristem by preventing the expression of *Apetala 1* (*AP1*) and *Leafy* (*LFY)* genes [104], providing an interesting tool to accelerate pear breeding.

3.3. Development of Seedless Fruits

Absence of seeds in fruits is appreciated by consumers for fresh consumption as well as in conserved or processed fruit products. It is also a desirable agronomic trait that enhances fruits' marketing values as high yield can be achieved even under environmental conditions unfavourable for pollination and fertilization. Seedless fruit development (parthenocarpy) is a process of fruit development from the ovary without pollination and fertilization. Seedless fruits were observed in tomato plants when auxin response factor 7 (ARF7) function was suppressed using RNA silencing [105]. Transgenic tomato plants expressing the *AUCSIA* genes coding for a short peptide specifically expressed in the ovary were functionally suppressed by hpRNA-induced silencing and produced seedless fruits after flower emasculation [106].

Seedless tomato has been obtained by down-regulating *CHALCONE SYNTHASE (CHS)*, the first gene in the flavonoid biosynthetic pathway [107]. Although the mechanism is not entirely clear, it is likely that in *CHS*-silenced tomato, parthenocarpy results from an altered distribution of auxin caused by the reduced level of flavonoids. Phytohormones such as auxin and gibberellins are closely associated with the trait of parthenocarpy [108] which in turn are regulated by many miRNAs. For example, expression of an aberrant form of ARF8, a target of miR167 [109-111], gave rise to parthenocarpic fruit in tomato [112].

3.4. Modification of Flower Color and Scent

A change in color or pattern of ornamental flowers could enhance value in the market. Flower color is mainly produced by the synthesis of flavonoid pigments and anthocyanins that could be genetically altered more efficiently than the conventional breeding. The introduction of an hpRNA construct targeting the gene into the garden plant *Torenia* hybrid was able to modulate its original blue flower color into white and pale colours [113]. Effective suppression of the genes coding for chalcone isomerase (CHI) by RNA silencing had reduced pigmentation and change of flavonoid components in flower petals in tobacco [114]. Other approaches such as altering the polyacylation of anthocyanins could also change flower colour. For example, simultaneous silencing of anthocyanin 5,3'-aromatic acyltransferase (5/3' AT) and flavonoid 3',5'-hydroxylase (F3'5'H), two key enzymes for gentiodelphin biosynthesis gave rise to transgenic lines with reduced flower color, such as lilac or pale-blue flowers [115]. The solubility of anthocyanins in plant cells was found to be enhanced by the expression of UDP glucose: flavonoid 3-O-glucosyltransferase (UFGT) through transfer of the glucosyl moiety from UDP-glucose to 3-hydroxyl group. In the red colored moth orchid Phalaenopsis, *PeUFGT3* was highly expressed in petals, and hpRNA-*PeUFGT3* Phalaenopsis plants displayed various levels of flower color fading that was well correlated with the extent of reduced level of *PeUFGT3* transcriptional activity and significant decrease in anthocyanin content [116].

In addition to color alteration, the scents of a flower could also be manipulated through RNA silencing application. The floral scent is a mixture of volatile phenylpropanoid/benzenoid compounds, which can be altered by RNA silencing technology as demonstrated in petunia by the elimination of some volatile compounds from the scent bouquet [117]. A number of genes including benzyalcohol/phenyl-ethanol benzoyl-transferase (*PhBSMT*), phenylacetaldehyde synthase gene (*PhPAAS*), benzyl-alcohol/

phenyl-ethanol benzoyl-transferase (*PhBPBT*) and coniferyl alcohol acyl-transferase (*CFAT*) have been individually down-regulated by RNA silencing and selectively altered scent component, with minimal changes in the emission of other volatiles in petunia [118, 119]. In the latter case, CFAT catalyzes the formation of coniferyl acetate in petunia flower, and its RNA silencing down-regulation led to almost complete elimination of isoeugenol emission without affecting other phenyl-propanoid/benzenoid volatiles [119].

3.5. Secondary Metabolites for Neutraceutical and Pharmaceutical Applications

RNA silencing technology has been used to enhance nutritional value by altering the accumulation of specific metabolites in fruits, as exemplified by the carotenoid and flavonoid content in tomato [120]. In tomato, a photomorphogenesis regulatory gene involved in repression of several light controlled signalling pathways, *DET1*, was found to be a negative factor for accumulation of carotenoids and flavonoids which are highly beneficial for human health [120]. Transgenic tomatoes expressing an hpRNA configuration of *DET1* showed gene-specific mRNA degradation accompanied by significant increase in the level of both flavonoid and carotenoid, whereas other parameters of fruit quality were largely unaffected [120]. This work is particularly interesting as it demonstrates that manipulation of a plant regulatory gene can simultaneously alter multiple independent phytonutrients biosynthetic pathways, leading to genetic improvement of the nutritional value of plant-derived products.

9-cis-epoxycarotenoid dioxygenase (NCED) is known to play a key role in abscisic acid (ABA) biosynthesis. Interestingly, its down-regulation by RNA silencing led to enhanced accumulation of upstream compounds in the ABA pathway, mainly lycopene and β-carotene. RNAi technology has also been used to enhance β-carotene and lutein contents in potato by down-regulating the expression of β-carotene hydroxylase (BCH) that converts β-carotene to zeaxanthin [121]. The transgenic potato lines generated by transformation controlled by a tuber-specific granule bound starch synthase (GBSS) promoter contained more β-carotene than those lines controlled by Cauliflower mosaic virus (CaMV) 35S promoter.

Squalene epoxidase catalyzes a rate-limiting step in the biosynthesis of phytosterol and triterpenoid saponin pathway. Two squalene epoxidase genes (*pgSQE1* and *pgSQE2*) derived from *Panax ginseng* were examined by RNA silencing approach, and only the down-regulation of *PgSQE1* resulted in reduction of ginsenoside production [122]. *pgSQE2* was found to be mainly involved in the formation of phytosterols, while exhibited some minor compensational activity when *PgSQE1* was silenced. It was envisaged that, based on this work, the overexpression of *PgSQE1* might lead to enhanced production of pharmacologically important ginsenosides in *P. ginseng*. Artemisinin is an effective antimalarial drug isolated from *Artemisia annua*. Transgenic *A. annua* expressing an hpRNA construct targeting squalene synthase (SQS) that is a key enzyme in the sterol biosynthesis pathway produced significantly increased artemisinin by 3.14-fold as compared to untransformed control plants [123].

In transgenic opium poppy (*Papaver somniferum* L.), RNA silencing down-regulation of codeinone reductase (COD) gave rise to yield increase of the non-narcotic alkaloid (s)-reticuline that is a key compound in the biosynthetic pathway for isoquinoline alkaloids at the expense of downstream products, including morphine, codeine, oripavine and thebaine [124]. In another study, the RNA silencing down-regulation of salutaridinol 7-O-acetyltransferase (salAT) in opium poppy significantly increased the pharmaceutically potent intermediate compounds, salutarydine and salutaridinol [125].

3.6. Applications in Developing High Value Industrial Products

Starch are made of amylose and amylopectin consisting of D-glucose residues linked by α-1,4 glucosidic bonds. Amylopectin differs from amylose by having a highly branched structure catalyzed by starch branching enzymes (SBE1 and SBE2). High-amylose starch has desirable physicochemical properties in film-forming and gelling properties, hence having broad applications in manufacturing candy, gums and packaging materials [126]. It has been shown that high-amylose potatoes can be produced by simultaneous down-regulation of two SBE coding genes [127]. The use of antisense technology for gene inhibition has yielded a low frequency of high-amylose lines that mostly harboured multiple T-DNA copies. In contrast, hpRNA expressed by a potato GBSS promoter was demonstrated to be very efficient, with more than 50% of the transgenic lines showing the desirable high-amylose trait [128].

When some valuable recombinant protein, such as human serum albumin, was expressed in transgenic potato, it was discovered that the steps involved in the purification of target proteins are major cost factors due to the contamination of patatin that is a family of glycoproteins representing up to 40% of total soluble proteins in potato tuber. Patatin content was effectively reduced by approximately 99% at both the protein and mRNA levels in transgenic potato specifically targeting patatin gene using the hpRNA approach, allowing rapid purification of other potato glycoprotein or transgenically produced glycoproteins with less contamination [129].

3.7. Prolongation of Shelf-life

The increase in shelf life of vegetables and fruits by delayed ripening process is highly desirable as the post-harvest deterioration and spoilage is one of the major causes of economic loss in horticultural plants. Initiation of ripening in climacteric fruits like tomato is largely controlled by ethylene that regulates a suite of ripening-specific genes [130]. RNA silencing down-regulation of *1-aminocyclopropane-1-carboxylate oxidase* (*ACCO*), a gene of ethylene biosynthesis pathway in tomato significantly reduced the rate of ethylene production in the ripening tomato fruits of transgenic plants leading to extended shelf life of more than 120 days with similar levels of total soluble sugar, titratable acid, amino acids, and total soluble solids as the control plants [131]. Recently simultaneous targeting of multiple genes in the same pathway has proven to be more effective. Chimeric hpRNA technology concomitantly targeting three homologs of ACC synthase gene during the course of ripening was able

to extend shelf life of transgenic tomatoes for further 45 days [132]. RNAi-*ACCO* knockdown kiwifruit lines were produced in which fruit softening was arrested at the desirable stage with 'eating-ripe' firmness and thereby significantly extending shelf life [133]. Strawberry (*Fragaria* × *ananassa*, Duch. cv Chandler) is a soft fruit with a short shelf life, mainly due to a rapid loss of firm texture. To control the strawberry fruit softening, the expression of strawberry pectate lyase gene was targeted by antisense under the control of the CaMv 35S promoter. Coinciding with the significantly reduced pectate lyase gene expression level, cell walls isolated from ripened transgenic strawberry fruits showed a reduced *in vitro* swelling and lower level of pectins compared to the untransformed fruits, indicating increased firmness [134].

A number of key genes that play an important role in regulating both fleshy fruit expansion and the ripening process have been identified through RNA silencing repression, such as *TOMATO AGAMOUS-LIKE1* (*TAGL1*) and *α-MANNOSIDASE* (*α-MAN*) and *α-D-N-ACETYL HEXOSAMINIDASE* (*α-HEX*) [135]. Transgenic tomato and capsicum fruits with significantly extended shelf life and enhanced firmness according to texture analysis as rate of fruits softening were significantly reduced as a result of RNA silencing down-regulation of the N-glycoprotein modifying enzymes [136, 137]. Further, microRNAs that are involved in tomato fruit development and ripening have also been identified [111, 138]. miR156 and miR172 have been found to be involved in the regulation of fruit development and ripening through an important gene in fruit ripening *COLORLESS NEVER RIPE* (*CNR*) [111, 138].

Polyphenol oxidase (PPO) catalyzes the oxidation of phenolics to quinones that are subsequently polymerized to form brown pigments. Several reports have described reduced browning in potato and apples by suppression of *PPO* gene expression using either antisense or hpRNA [139-141]. More recently, an amiRNA strategy was used to suppress four members of the *PPO* gene family either individually or in combination, generating low-browning potato with small DNA inserts [142]. The commercial development of potato and apples that will not brown during food processing and consumption is appealing to consumers, in addition to reduction of waste due to browning.

3.8. Removal of Toxic Compounds and Allergens

Plants are known to contain nutritionally undesirable compounds or toxins of various types, removal of which from plants is often a cumbersome and a costly process. Numerous studies have proven RNA silencing as a powerful technology to make the plants toxin-free. Simultaneous down-regulation of three distinct methylation steps of the caffeine biosynthetic pathway, by suppressing the expression of *7-Nmethylxanthine methyl-transferase* gene (*CaMXMT1*) was shown to reduce caffeine content of transgenic plants by up to 70%, indicating the feasibility of producing decaffeinated coffee beans [143]. Cassava (*Manihot esculenta*) is a major staple food in tropical countries but it contains toxic cyanogenic glucosides in its tuber. Antisense down-regulation of cytochrome P450 enzymes CYP79D1 and CYP79D2 that catalyze the first committed step in the biosynthesis of linamarin and lotaustralin generated transgenic cassava plants with more than 90% reduction of cyanogenic glucoside amounts in tubers [144]. A similar strategy was used to generate commercial potatoes with significantly reduced asparagine, one of the major precursors of neural toxin acrylamide, by simultaneously silencing the *ASPARAGINE SYNTHETASE* genes (*StAs1* and *StAs2*) [145]. Also in potato, sprouts and green tubers will normally lead to high level accumulation of α-solanine and α-chaconine, two toxic steroidal glycoalkaloids (SGAs). A recent study identified sterol side chain reductase 2 (SSR2) as a key enzyme in the biosynthesis of cholesterol and related SGAs. The subsequent genetic manipulation by RNA silencing down-regulating *SSR2* has generated potato lines showing the significant reduction in the levels of predominant SGAs by at least 10% compared with the non-transformed control plants, without affecting plant growth [146].

Food allergy is a hypersensitive response to normally harmless protein components in food mainly through immunoglobulin (IgE) mechanism [147]. RNA silencing technology was successfully used to down-regulate the expression of a prominent apple allergen Mal d1 that displays IgE antibodies [148]. Transgenic apple plantlets, transformed with a construct coding for an intron-spliced hpRNA construct containing a *Mal d 1*-specific inverted repeat sequence showed an approximate 10 fold reduction in *Mal d 1* leaf expression without compromising normal plant growth [148]. Hypoallergenic tomatoes were developed through RNA silencing down-regulation of Lyc e 3, a nonspecific lipid transfer protein of tomato [149]. The transgenic lines showed up to 10-fold reduction in the targeted protein accumulation in transgenic fruits and skin prick tests revealed highly reduced allergenicity. Tear-less onion was developed through RNA silencing down-regulation of the conversion of 1-propenylsulfenic acid to propanthial S-oxide that is a tear-inducing, lachrymatory factor [150]. RNA silencing down-regulation of carrot allergen Dau c 1 that belongs to pathogenesis-related 10 (PR 10) family of plant protein resulted in transgenic carrot lines showing drastically reduced specific allergenic reactivity in patients in skin prick tests [151]. Many of such allergenic proteins are ubiquitous small plant proteins existing in various fruits, vegetables and tree nuts. The clinical results from above exemplified studies combating plant allergy have provided support for the feasibility of creating low-allergenic foods using RNA silencing-mediated biotechnology.

4. CONCLUDING REMARKS

Every technology has its limitations. Post-transcriptional RNA silencing usually does not result in complete gene silencing. In addition, it remains unclear if RNA silencing technologies can be used to consistently switch off endogenous gene promoters in plants, which, unlike transgene promoters, appear to be resistant to siRNA-directed transcriptional silencing. These would limit the applications of RNA silencing technologies, particularly in cases where complete and stable gene knock-out is required. Combinations of RNA silencing technologies based on the different RNA silencing pathways could enhance the efficiency of silencing. For instance, combining PTGS with TGS-based technologies, siRNA with miRNA-based technologies, or technologies

based on all the different RNA silencing pathways, could potentially result in more potent gene silencing than using technologies based on a single pathway.

Recent years have seen great advances in developing technologies for targeted mutagenesis in plants. The CRISPR/Cas9 technology can now be used to mutagenize or edit nucleotide sequences of selected genes or genomic loci in plants [152-154]. Such technologies would be particularly useful for generating genetically stable gene knock-out lines. However, RNA silencing technologies will continue to serve as a useful tool in gene function analysis and crop improvement for several reasons. i) They are now well established technologies and easy to use. ii) Complete knock-out of essential genes is lethal to plants and therefore such mutants cannot be recovered by CRISPR/Cas9-like mutagenesis technologies. However, mutants of such genes could be recovered for gene function analysis by incomplete gene knockdown with RNA silencing technologies. This is demonstrated in rice where transformation with hpRNA libraries results in the recovery of essential gene mutants [77]. iii) RNA silencing technologies allow for tissue-specific silencing of a gene using a tissue-specifically expressed transgene, whereas genetic mutation result in gene knock-out in all tissues. iv) RNA silencing technologies can be used to simultaneously silence multiple genes using transgenes containing either a conserved sequence or a composite sequence from multiple genes, whereas this would be difficult to achieve using CRISPR/Cas9-like mutagenesis methods. With continuing efforts in further understanding the RNA silencing mechanisms in plants, it can be anticipated that RNA silencing technologies will be further improved to overcome potential limitations allowing for wider applications in agriculture.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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