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Review

Post-transcriptional gene silencing: Basic concepts and applications

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Post-transcriptional gene silencing (PTGS)-mediated gene silencing exploits the cellular mechanism wherein transcripts having sequence similarity to the double-stranded RNA (dsRNA) molecules present in the cell will be subjected to degradation. PTGS is closely related to natural processes such as RNA-mediated virus resistance and cross-protection in plants. Gene silencing and the cellular machinery for affecting this phenomenon might have evolved as a natural protective measure against viral infection in plants. In PTGS, small interfering RNA (siRNA) molecules of 21–23 nucleotides length act as homology guides for triggering the systemic degradation of transcripts homologous to the siRNA molecules. PTGS phenomenon, first discovered in transgenic petunia plants harbouring chalcone synthase gene and termed co-suppression, has been subsequently exploited to target specific gene transcripts for degradation leading to manifestation of desirable traits in crop plants. Targeted gene silencing has been achieved either through the introduction of DNA constructs encoding dsRNA or antisense RNA or by deploying co-suppression constructs producing siRNAs against the transcript of interest. Understanding the mechanism of gene silencing has led to the development of several alternative strategies for inducing gene silencing in a precise and controlled way. This has paved the way for using PTGS as one of the chief functional genomics tools in plants and has helped in unraveling the mechanism of many cellular processes and identifying the focal points in pathways, besides, opening new vistas in genetic engineering of plants for human benefits. PTGS has shown great potential in silencing the deleterious genes efficiently so that value-added plant products could be obtained. Thus, PTGS has ushered in a new era in the genetic manipulation of plants for both applied and basic studies. In this review, we have outlined the basics of RNAi-mediated gene silencing and summarized the work carried out at our institute using this approach, as case studies. In particular, adopting RNAi-mediated gene silencing (a) as a method to restore fertility in transgenic male sterile lines developed based on *orfH522* gene from sunflower PET1-CMS source, (b) as a tool to suppress the production of toxic proteins, ricin and RCA, in castor, and (c) as an approach to induce bud necrosis virus resistance in sunflower has been discussed. Examples from other plant systems also have been mentioned to exemplify the concept and utility of gene silencing in crop plants.

Keywords. Post-transcriptional gene silencing (PTGS); RNA interference (RNAi); double-stranded RNA (dsRNA); small interfering RNA (siRNA); RNA induced silencing complex (RISC); antisense RNA technology

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1. Introduction

Gene silencing phenomenon was first observed by scientists working with transgenic petunia plants expressing chalcone synthase (*CHS*) gene, the key gene known to be involved in flavonoid anthocyanin pigment production pathway that imparts colour to the flowers (Napoli *et al*. 1990). It was discovered that when the *CHS* gene was introduced into plants for its over-expression to realize dark coloured flowers, it actually induced the opposite phenotype and imparted white colour to the flowers. This phenomenon was termed as co-suppression; the expression of a transgene was suppressed along with the induced suppression of the endogenous gene as well. Later it was discovered that co-suppression could be a result of either suppression of the transcription process itself and/or post-transcriptional degradation of the specific transcripts. Suppression of the transcription, termed as transcriptional gene silencing (TGS), involves changes such as methylation of the promoter sequences and chromatin remodeling at the DNA level, leading to the shut-down of transcription. TGS takes place in the nucleus, exhibits stable inheritance and does not lead to systemic silencing (Mlotshwa *et al.* 2002). On the contrary, post-transcriptional gene silencing (PTGS) occurs in the cytosol, involves sequence-specific mRNA degradation and is reset every generation. PTGS also exhibits systemic silencing (Fagard *et al.* 2000; Chicas *et al.* 2001) with the silencing spreading from one part of the plant to the other indicating that there must be a signal that is carried from one region to the other. However, the exact nature of the movement and extent of spread of the signal have not been fully established. Several studies have established that the major triggers of PTGS are transgenes and viruses, and the process involves production of double-stranded RNA (dsRNA) molecule having homology to the gene(s) silenced.

PTGS in plants shares features of RNA interference (RNAi) reported in animals as well as the quelling observed in fungi with the central commonality being formation of small interfering RNA (siRNA) molecules produced by enzymatic cleavage of long dsRNA molecules by DICER, an RNase III family enzyme. In other words, DICER mediates these three phenomena (Agrawal *et al.* 2003). RNAi was discovered first in *C. elegans* (Fire *et al.* 1998) and subsequently in many trans-kingdom organisms (Romano *et al.* 1992; Cogoni *et al.* 1997). Though originally RNAi was used restrictively to refer to the gene silencing induced by direct introduction/injection of dsRNA designed to target mRNA of a specific gene, later the term has been used as a generic term to denote any gene silencing mediated by siRNA molecules produced from dsRNA molecules. Thus, irrespective of whether the dsRNA is introduced into the cell or it is produced inside the cell, the cognate mRNA is degraded if siRNA molecules with homology are made available from the cleavage of dsRNA molecules. Thus, gene silencing processes, called by different terminologies across kingdoms, are in unison with respect to the trigger that brings about induction as well as the mechanism of induction of gene silencing.

PTGS as a phenomenon, is quite similar to the naturally occurring processes like cross protection and RNA-mediated viral resistance in plants. Therefore, PTGS acts as protective measure against viral infections in plants. PTGS mechanism has been adopted to knock-down specific gene transcripts to induce desirable traits in crop plants. In this review, a concise attempt has been made to succinctly introduce readers to mechanisms of PTGS, strategies for inducing PTGS, and applications of PTGS as adopted in our laboratory for addressing a few issues. Readers are referred to excellent and comprehensive reviews on different aspects of PTGS (Watanabe 2011; de Alba *et al.* 2013; Saikumar and Kumar 2014; Weiberg *et al.* 2015; Zhang *et al.* 2016; Meena *et al.* 2017; Yu *et al.* 2017; Chen *et al.* 2018; Lee and Carroll 2018; Singh *et al.* 2018; Zhang *et al.* 2018; Ramesh *et al.* 2020).

2. Mechanisms of post-transcriptional gene silencing

Several research groups working on gene silencing phenomenon in different systems started observing some common features across kingdoms. A unified concept regarding the mechanism of PTGS was arrived. The common understanding was that the PTGS is triggered by either the homologous genes or dsRNA or siRNAs implying a conserved mechanism operating trans-kingdoms. However, subtle variations have also been observed among different organisms with respect to the mechanism that facilitate PTGS (Vaucheret *et al.* 2001). There are genes reported to be involved in organism-specific fine-tuning of the mechanism of PTGS and it has been demonstrated that some of these are dispensable in some organisms as alternate genes or processes fulfill their task. The central theme observed is that the PTGS exploits the cellular mechanism(s) involved in recognizing the dsRNA and subjecting the corresponding mRNA transcript to a sequence-specific degradation (Wesley *et al.* 2003).

Biochemical studies carried out with *Drosophila* extracts demonstrated an activity capable of processing long dsRNA substrates into small RNAs and this ushered a new insight into understanding the mechanism of PTGS. Further in-depth studies led to the discovery of a candidate protein, an RNase III enzyme, that could cleave longer dsRNA into smaller dsRNA molecules as observed *in vivo* and this protein was rightly called DICER (based on the dicing of longer molecule it does). The resultant smaller dsRNA molecules (siRNAs) were typically 21–25 nt long with characteristic 2 nucleotide 3′ overhangs, and 5′ phosphates. It was also observed that decreased DICER levels *in vivo* correlated with decreased gene silencing, indicating the central role played by DICER enzyme in gene silencing or RNAi. Several studies carried out in various systems and by adopting different techniques, led to stitching up all the pieces of information together to propose the common mechanism involved in all PTGS variants (Qi and Hannon 2005; Lindbo 2012; Meena *et al.* 2017; Singh *et al.* 2018).

The process of PTGS happens as a two step process: the initiator step that involves introduction/formation of dsRNA and the effector step that leads to degradation of target mRNAs (Waterhouse and Heliwell 2003a, b). The initiator step could be achieved by the direct insertion or transfer of dsRNA or siRNA into a cell through direct imbibitions, bombardment, virus-mediated transfer, infiltration (Watson *et al.* 2005) or by using cationic oligo peptides (Narayanan *et al.* 2004). On the other hand, the formation of dsRNA in the cell could be facilitated by transforming the cells with vectors that facilitate production of the dsRNA in the cell. The vectors could be designed to produce stable or transient dsRNA *in vivo*. In the second step of PTGS, degradation of target mRNA begins when the corresponding dsRNA present in the cell is cleaved into siRNAs by the action of Dicer and the so formed siRNAs are incorporated into RNA-Induced Silencing Complex (RISC), initiating the process of cognate mRNA degradation (Agrawal *et al.* 2003; Kuznetsov 2003; Arenz and Schepers 2003). A physical interaction between Dicer and RISC through a common PAZ domain is believed to occur in the cytosol. Upon association with RISC, the double-stranded siRNA unwinds and only one of the strands of RNA (usually guide/antisense strand) will be retained leading to formation of 'activated RISC'. The activated RISC utilizes the bound siRNA strand as a targeting sequence to bind to the complementary mRNA and initiates the cleavage of the targeted mRNA within the region of complementary binding of siRNA and mRNA. The mRNA degradation is executed by the slicing (RNase III enzyme Argonaute) activity of RISC. Different pathways are known to be operating in plants for production of siRNA molecules (de Alba *et al.* 2013; Lee and Carroll 2018).

It has been reported that siRNAs might be involved in the synthesis of long dsRNA mediated by RdRP (RNA-dependent RNA polymerase). This process enhances the effectiveness of RNAi by providing additional substrate for the dicer enzyme and thus resulting in production of secondary siRNAs. These secondary siRNAs actively participate in the degradation of the complementary mRNA. It has also been established in plants that, siRNA molecules of different sizes, produced by dicer-like-proteins (DCLs) are transported systemically either to short or long distance within the plant and thus lead to systemic spread of the silencing (Chen *et al.* 2018).

3. Strategies for inducing PTGS in plants

As stated above, in plants, PTGS has been induced through different strategies- through introduction of DNA construct encoding dsRNA or antisense RNA or the one which produces aberrant RNA molecules with all of them eventually leading to production of siRNAs. Reasonably, the earlier attempts were made to suppress the endogenous gene by transforming plants with the constructs that facilitated production of antisense RNA molecules against the target transcript. One of the earlier classical examples of inactivation of a target transcript through PTGS has been the reduction of polygalacturonase enzyme to delay the onset of ripening in tomatoes (Gray *et al.* 1992), achieved through the antisense RNA technology. In the resultant transgenic plants it was noticed that the protein (polygalacturonase) levels were reduced up to 99%. In similar studies, the fatty acid profiles of rapeseed could be successfully altered by suppressing the key genes encoding Δ9-desaturase (Knutzen *et al.* 1992) and Δ12-desaturase (Kinney 1996) using antisense RNA technology. Later, PTGS has been induced in plants using co-suppression of the endogenous gene by introducing sense copies of the target gene(s). However, antisense RNA and co-suppression strategies resulted in relatively low frequency of PTGS in the transgenic plants and this necessitated the production of a large number of transgenic plants in order to select the ones that exhibited sufficient degree of target gene suppression. This could pose a major hurdle, especially in crops with low transformation and regeneration

frequencies. To counter these limitations, alternative gene silencing strategies with higher efficiencies have been developed. In all these cases, the emphasis was on efficient production of the long dsRNA molecules that could become substrates for Dicer. In a classic experiment, Smith *et al.* (2000) demonstrated that when a functional intron was cloned in between sense and antisense sequences of the target gene, it resulted in a stable (double-stranded) hairpin-RNA after the splicing process and this led to silencing of the target gene(s) in almost 100% of the transgenic plants realized. They also observed that if the functional intron was replaced with any other spacer sequence or non–functional intron or intron in the antisense orientation, it resulted in lower gene silencing efficiencies. These experimental results clearly demonstrated that stable, dsRNA against a target gene results in a highly efficient suppression or down-regulation of the target gene (Smith *et al.* 2000).

In another study, Wesley *et al.* (2001) compared different types of constructs for gene silencing and concluded that intron hairpin RNA strategy abolished the activity of the target gene efficiently. They reported a generic vector, carrying a functional intron, into which fragment of the target gene could be cloned in both sense and antisense orientation in a single step on either side of the intron using gateway cloning technology. The authors also opined that this ihp (intron hairpin) RNAi vector could be used for silencing multiple copies of genes of a gene family by cloning a conserved region (of those genes) in both sense and antisense orientation in the generic vector. Meanwhile, the observation that through RdRP activity, a long dsRNA would be synthesized from the dsRNA structure (formed at the 3′ end of the transcript) due to folding back of the 3′ end of the transcript on itself, a phenomenon termed transitive RNAi, led to the development of a new type of silencing vectors. Brummell *et al.* (2003) utilized this concept and demonstrated that if a fragment of the target gene (in sense version) was cloned between a promoter and an inverted repeat of 3′ un-translated region (3′ UTR) and if this construct is delivered into a plant, a high efficiency gene silencing of the target gene could be achieved due to the *in vivo* formation of dsRNA for the cloned target gene fragment. Thus formed dsRNA could lead to formation of siRNAs due to the activity of DICER and lead to degradation of the target gene transcript through the action of RISC as explained above. This type of gene silencing was termed silencing by heterologous 3′ un-translated regions (SHUTR). Since this approach involves only one cloning step of inserting a fragment of the gene of interest between the promoter and the inverted repeat of the 3′-UTR in a binary vector it is an easy way to prepare the constructs. The utility of this approach for silencing genes has been demonstrated (Brummell *et al.* 2003; Nizampatnam and Kumar 2011).

Another important strategy to silence target genes has been through the use of artificial micro RNAs (amiRNAs). This concept was developed based on the naturally occurring phenomenon of gene regulation through microRNAs (miRNAs). MicroRNAs, a small class of RNA molecules of 19–24 nucleotides length produced naturally from microRNA encoding genes, play an important role in regulating gene expression by binding to the target mRNA with high complementary specificity and effecting its degradation. MicroRNAs are known to play a pivotal role in regulating the plant development as well as in response to different stimuli – both innate to the system as well as external. This feature of target specificity and availability of the entire cellular machinery to effect miRNA-mediated silencing of target genes, has been used to design a new experimental tool termed amiRNA to direct silencing of target genes of choice. Gene constructs of amiRNA are designed by replacing the 21 nt miRNA duplex (miRNA:miRNA*) region within pre-miRNA of a known and well-characterized plant miRNA (e.g., miR319a of *Arabidopsis thaliana*) with the 21 nt long fragment of the target gene and cloning this engineered miRNA under suitable transcription regulators (promoters and terminators) of choice. This gene construct when transferred into the plant, amiRNA is expressed as pre-miRNA and the resultant transcript is subsequently processed in a similar manner as native miRNA *via* small RNA biogenesis. Finally, the resultant active amiRNA molecule triggers silencing of the target gene in a manner similar to miRNAs. The amiRNA-based strategies are considered to be more efficient due to their higher specificity and fewer undesirable off-target effects. Artificial miRNAs have been applied to silence diverse groups of genes in different species (Saikumar and Kumar 2014; Kamthan *et al.* 2015). Similar to miRNAs, amiRNAs might regulate gene expression at the transcriptional (TGS) or post-transcriptional (PTGS) level.

Different strategies adopted for silencing the genes as well as the basic underlying cellular processes leading to targeted silencing of genes are illustrated in figure 1 for the ease of understanding. However, this figure is only indicative of the opportunities and strategies available at present for silencing the genes.

4. Applications of PTGS in plants

RNAi has proven to be an effective tool to impart or enhance molecular, biochemical, physiological, and agronomic traits in plants. RNAi has come a long way since its discovery and there is hardly any sphere of modern biology that has remained un-embraced by this state-of-the-art technology. Of late, it has become a major tool in functional genomics of plants. Detailing the gene silencing studies that have led to the development of various useful and commercial products is beyond the scope of this mini-review and readers are referred to many lucid reviews that illustrate this (Runo 2011; Koch and Kochel 2014; Ibrahim and Aragao 2015; Yu *et al.* 2016; Khalid *et al.* 2017; Lindbo and Falk 2017; Majumdar *et al.* 2017; Meena *et al.* 2017; Islam *et al.* 2018; Yang and Li 2018). Beyond the utility of RNAi for improving traits in plants, RNAi has played a paramount role in functional genomics (Ranjan et al. 2019). Apart from silencing the genes through RNAi in genetically modified (transgenic) plants, the exogenous application of dsRNA (called as environmental RNAi) to bring in transitive and systemic gene silencing in the plants is fast emerging as a technology (Dalakouras *et al.* 2020) for the control of pathogens and pests (Fletcher *et al.* 2020).

There are obvious and distinct advantages of RNA gene silencing approaches over other reverse genetics tools as they

- allow the functional high-throughput analysis of genes through genome-wide screens;
- facilitate local or systemic silencing of the genes in a temporal and or spatial manner using constitutive or tissue-specific promoters respectively;
- enable phenotypes with partial or complete silencing of genes using weak or strong promoters to drive the expression of the target genes;
- are amenable to cultured cells and whole organisms; and
- can potentially target any gene(s) expressed at particular stages of the organism's life cycle.

These perceived advantages have led several plant molecular biologists to use these tools to address queries of both basic and applied interest. Some of the successful applications of RNA gene silencing are in:

- blocking the expression of deleterious (toxic) genes and undesirable substances/phytochemicals;
- improving nutritional quality;
- inducing biotic stress resistance;
- boosting abiotic stress tolerance; and
- altering agronomic or physiological characters

5. Adoption of PTGS approaches for trait improvement at Indian Institute of Oilseeds Research (IIOR), Hyderabad

5.1 *Restoration of fertility in transgenic male sterile tobacco lines expressing orfH522 from sunflower*

In predominately self pollinated crops for the exploitation of heterosis or hybrid vigour, male sterility is a trait that is essential to produce quality F_1 seeds at a reduced cost. However, the fertility must be restored in the F_1 progeny to ensure normal seed production and the crop produce. Induced male sterility using a transgenic approach through barnase-barstar based cell ablation and restoration system has been exploited in several crops (Mariani *et al.* 1990, 1992; Ray *et al.* 2007). In the author's lab, a similar system has been developed using the *orfH522* gene taken from PET1-CMS system of sunflower. Through earlier studies, had indicated that ORFH522, a mitochondrial protein, could be responsible for the induction of male sterility in the PET1-CMS system. But, definitive evidence that ORFH522 is directly responsible for male sterility trait in PET1-CMS of sunflower was established in the author's lab by inducing male sterility in tobacco by ectopically expressing *orf*H522 (Nizampatnam *et al.* 2009). To achieve this, cDNA of *orfH522* was cloned in-frame with yeast *cox*IV pre-sequence (to target the resultant protein ORFH522 into mitochondria), and was expressed under tapetum-specific promoter TA29 (construct designated as TCON). As control vectors, *orfH522* was cloned without the transit peptide under the TA29 promoter (TON) as well as with or without transit peptide under the constitutive CaMV35S promoter (SCON and SON). Among several independent transformants obtained with each of these gene constructs, completely male sterile plants were obtained with only TCON construct while transgenic plants realized with other constructs were fertile. The male-sterile plants were morphologically similar to fertile plants and molecular analysis as well as microscopic studies clearly established that expression of *orfH522* in the tapetal cell layer led to ablation of the nourishing tissue and thus led to male sterility. Stable transmission of induced male sterility trait was confirmed in test cross progeny (Nizampatnam *et al.* 2009) and the sterility trait could be maintained by crossing with fertile untransformed plants. To restore fertility in the F_1 progeny, *orfH522* had to be silenced in the F_1 plants. Two RNAi strategies (ihp and transitive RNAi) were used to silence the *orfH522* transcripts in a post transcriptional manner.

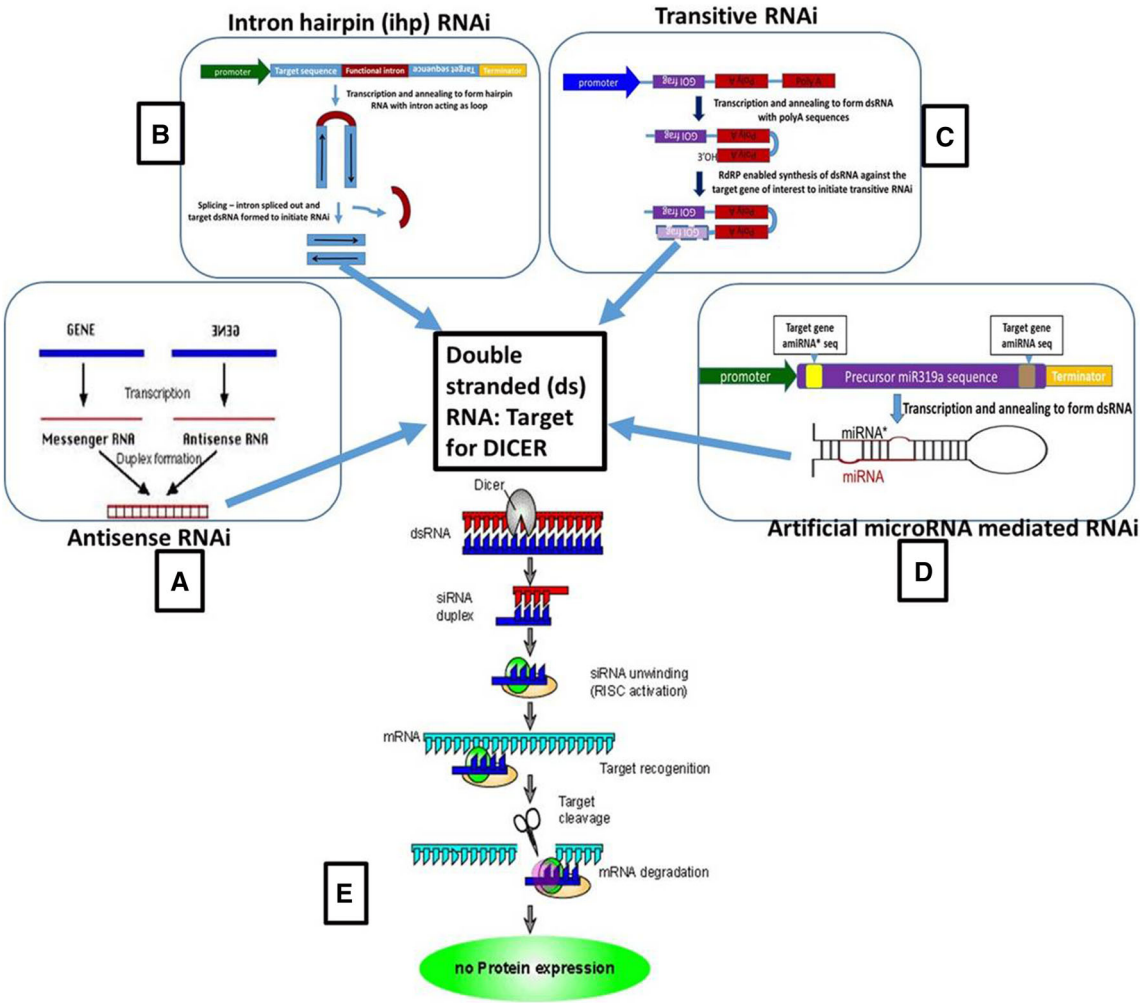

Figure 1. Schematic representation of the major RNAi pathways adopted for silencing the target gene in plants. Double-stranded (ds) RNA, the target for RNAse III enzyme dicer, could be produced through different strategies: antisense mRNA bound to mRNA (antisense RNAi, as depicted in (**A**), intron spliced hairpin RNA (ihpRNAi as depicted in (**B**), RNA produced by RdRP activity (transitive RNAi as depicted in (**C**) and artificial miRNA (as depicted in **D**). This dsRNA will trigger the silencing of target gene(s) through a mechanism as depicted in (**E**). dsRNA will be cleaved by Dicer to produce a population of small dsRNA duplexes called siRNA, siRNA complexes with other proteins to form RNA induced silencing complex (RISC), siRNA duplex will unwind and one of the two strands get associated with RISC to form activated RISC, this active RISC (slicer) will get associated with the target mRNA based on the homology between siRNA* in RISC and the target mRNA, and RISC facilitates target cleavage in the seed region of the siRNA:mRNA binding and thus inactivates the mRNA. Once the target mRNA is cleaved there will be no translation of the transcript and thus there will no protein expression of the targeted gene. In the work presented in the paper, all the four strategies of RNAi have been adopted.

Appropriate constructs that could produce either intron spliced hairpin RNA against the *orfH522* or induce silencing of *orfH522* by heterologous 3′ UTR region were developed by using the selected 316 bp fragment (*orf*316) of *orfH522* gene. These restorer gene constructs (driven by either CaMV35S or TA29 promoters) were independently mobilized into *Agrobacterium* and used for transforming tobacco plants. The T_1 generation plants carrying the restorer gene constructs in homozygous condition were identified and crossed with the male sterile (hemizygous) transgenic tobacco plants expressing *orfH522* to obtain the hybrid seeds. As expected, PCR analysis of the hybrid plants indicated segregation for the sterility inducing cassette (TCON) while all the plants carried the restorer cassette. Hybrid plants produced fertile pollen grains and formed normal capsules upon selfing indicating the restoration of fertility. Further, molecular analyses of these hybrid plants using RT–PCR, Northern blotting and siRNA detection revealed that ihp-RNAi-mediated gene silencing was much more effective compared to silencing by heterologous 3′ UTR (SHUTR) as complete

degradation of *orfH522* transcripts and formation of higher levels of orf316 specific siRNA molecules were indicated in plants carrying ihp-RNA restorer construct. Segregation analyses of F_2 (selfed hybrid) plants confirmed the co-segregation of gene cassettes and the traits in Mendelian di-hybrid ratio (9:3:3:1). Taken together, the results established that intron hairpin and transitive RNAi-mediated silencing of *orfH522* transcripts restored fertility in transgenic male-sterile tobacco plants expressing *orfH522* and the ihpRNAi strategy was more efficient in silencing *orfH522* transcripts than SHUTR approach (Nizampatnam and Kumar 2011).

5.2 *Attempts to reduce ricin and RCA proteins in castor by silencing the ricin gene family members using different PTGS approaches*

Ricin (also known as RCA II or RCA_{60}) and *Ricinus communis* agglutinin (otherwise known as RCA I, RCA_{120}, or simply RCA) are the two highly toxic, endosperm-specific glycoproteins of castor bean. Ricin is a potent cytotoxin but a weak haemagglutinin, whereas RCA is a weak cytotoxin and a powerful haemagglutinin. These two lectin proteins are produced in the developing seeds and stored in the endosperm. Presence of these two toxic proteins, render the castor de-oiled meal unsafe for animal consumption even though it contains about 25% proteins with balanced amino acid composition. Therefore, if the genes encoding these proteins are silenced leading to ricin and RCA free castor lines, the de-oiled meal from such lines could be used as animal feed. Ricin, a type II ribosome-inactivating protein (RIP), is a heterodimeric polypeptide consisting of a 32 KDa ricin toxin A (RTA) chain - a potent inhibitor of 80 S ribosomes - and a 34 KDa carbohydrate binding ricin toxin B (RTB) chain, linked by a single disulfide bond. RCA is a four-chained polypeptide comprising of two A chains and two B chains, slightly less toxic than ricin and causes agglutination of red blood cells (RBCs) in mammals. RCA is a tetramer composed of two ricin-like heterodimers held together by non-covalent forces. The A chain of RCA has a 93% similarity to RTA, while the two B chains are 84% similar to RTB (Roberts *et al.* 1985).

Genome sequencing of castor led to understanding of complex genomics of ricin and RCA gene family. Ricin and RCA proteins are encoded by a family of genes. The draft genome sequence revealed that there are 28 putative genes in the family, including potential pseudogenes or gene fragments, contained in a total of 17 sequence scaffolds. Expression pattern analyses of these identified genes using RT-qPCR at different stages of endosperm development have revealed that in addition to the earlier identified ricin and agglutinin genes, four other RIP I and four RIP II genes are expressed during seed development in castor bean albeit with lower levels of expression (Chan *et al.* 2010). Complex genomics of ricin and RCA gene family indicates that harnessing induced mutations or searching for the mutated alleles from the germplasm collection for ricin family genes could be a daunting task. As genes encoding ricin and RCA share a very high homology, it is logical that the PTGS constructs that target a conserved sequence between the two genes would silence both the proteins. Different PTGS vectors based on four approaches of gene silencing, *viz*., antisense, intron interrupted RNA (ihp RNAi) silencing by 3′ heterologous UTR (SHUTR, transitive RNAi) and amiRNA, targeting either the A chain or the B chain of ricin have been developed at IIOR (Ashfaq *et al.* 2007; Saikumar *et al.* 2009 and personal communication). These vectors have been validated using tobacco as a model system (Reddy *et al.* 2009: 2010). To direct the silencing of these genes only in the endosperm tissue of castor, native promoter of ricin gene has been isolated (Ashfaq *et al.* 2009) and used for driving the silencing gene cassettes. Suitable vectors have also been developed to assess the tissue specificity of the isolated ricin promoter using the *gus* gene. Preliminary results with the transgenic tobacco plants obtained with these constructs have indicated the endosperm-specific expression pattern of the isolated ricin promoter (Ashfaq *et al.* 2010). Efforts are on to generate the transgenic castor using these constructs. Seven putative transgenic plants were realized with ihp-ricinA and ihp-ricinB constructs. Around 25 T_1 plants from each of the seven T_0 castor plants were analyzed using PCR but none showed the presence of the transgene (personal communication). Therefore, further efforts are being made at IIOR, Hyderabad to develop a robust transformation protocol in castor so that the developed constructs for silencing ricin and RCA could be exploited for realizing ricin and RCA free castor lines (Ashfaq *et al.* 2018).

5.3 *Development of transgenic sunflower resistant to SND*

Initial success in incorporating viral resistance by expressing viral coat protein led to serious experimentation to empirically establish the mechanism of

coat protein-mediated resistance (CPMR). Even though it was earlier thought that CPMR was dependent on the level of expression of the introduced coat protein, later it was realized that it was not the case. In fact, the first indications that CPMR could be mediated by RNA came from the experiments wherein it was observed that when non-translatable tobacco etch virus (TEV) coat protein was expressed in tobacco plants, complete immunity was achieved in at least 30 % of the transgenic plants (Lindbo *et al.* 2003; Lindbo and Dougherty 2005). A detailed historical perspective for the present understanding that the CPMR is mediated by RNAi has been provided in a review by Lindbo and Falk (2017).

At IIOR, we have utilized CPMR technique to induce resistance against sunflower necrosis disease (SND). SND incited by tobacco streak virus (TSV) cause significant yield losses in sunflower in the tropics and sub-tropics and we have addressed this burning issue by employing antisense RNA technology. Adopting the *Agrobacterium*-mediated transformation protocol developed at IIOR, split cotyledons from mature seeds of sunflower were transformed with coat protein gene of TSV (*TSV-CP*) cloned under 35S promoter. Putative transgenic plants were selected using kanamycin at a transformation frequency of 3.3%. Putative transgenic plants were confirmed for the transgenicity through PCR and the expression of the transgene was determined through RT-PCR, Northern blot analysis and quantitative Real-time PCR (qRT-PCR). The stable integration of the transgene was ascertained through Southern blot analysis of plants. Inheritance through four generations was confirmed through PCR and Southern analyses. Out of the 102 positive T_0 transgenic plants, 20 plants were carried to T_1 generation from which five lines (*CP*-S-237, *CP*-S-247, *CP*-S-481, *CP*-S-648, and *CP*-S-753) were selected and advanced till T_4 generation. Among these, plants of line No 481 showed resistance to necrosis disease when they were challenge inoculated with TSV. Transgenic lines carrying *TSV-CP* construct grew to maturity while the control plants (untransformed) showed mortality within 1–2 weeks following artificial inoculation. Expression analysis of the *TSV-CP* and *npt*-II genes in different tissues at flowering and seed setting stages revealed constitutive expression of the transgene till seed maturation (Vasavi *et al.* 2018).

Going by the successful application of PTGS to address pollination control system in safflower and SND in sunflower, we at IIOR anticipate furthering the applications of PTGS to improve miscellaneous traits of mandate crops of the institute. Among them, the development of castor with reduced/nil levels of ricin and RCA (on-going) and down-regulation of genes involved in niger-*Cuscuta* interaction assume prior attention.

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