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Small RNAs landscape (sRNAome) of Soybean [*Glycine max* (L.)]: Biogenesis, vital functions and potential applications

S. Veluchamy Ramesh [1,*], Nikita Admane[2], Syed Masroor Husain[1, 3]

[1]Directorate of Soybean Research, Indian Council of Agricultural Research, Indore -452001, Madhya Pradesh, India
[2]Pt. Ravishankar Shukla University, Raipur - Chhattisgarh, India
[3]Senior author

***Corresponding author: rameshsvbio@gmail.com**

Abstract

Small non coding RNAs (ncRNAs) are characterized by 20-30 nucleotides (nt) long RNA molecules that have emerged as negative regulators of gene expression both at transcriptional and post-transcriptional stages in eukaryotic organisms. Small RNA (sRNA) repertoire of soybean arises from endogenous or exogenous, duplex RNA or single-stranded RNA and is characterized with perfect or imperfect hairpin (stem-loop) structures. The diversity of soybean small RNA (sRNA) population is enormous encompassing microRNAs (miRNAs) and diverse class of small interfering RNAs (siRNAs). sRNAs entail dedicated cellular machinery for their biogenesis, mode of action and are expressed in response to various environmental or developmental circumstances. The scientific understanding on the biological role of plant sRNA metabolism has indeed resulted in the exploitation of sRNA mediated gene suppression. In recent years, sRNA induced gene suppression has been shown to be an effective tool for modulating the expression levels of several genes to develop desired soybean phenotype and also in targeted down regulation of genes in functional genomics studies.

Keywords: microRNA (miRNA); non-coding RNA (ncRNA); RNA interference (RNAi); small interfering RNA (siRNA); Virus Induced Gene Silencing (VIGS).
Abbreviations: amiRNA_artificial miRNA, DCL1_Dicer-Like 1, hpRNA_hairpin RNA, lsiRNA_long siRNA, miRNA_ microRNA, MPSS_Massively Parallel Signature Sequencing, natsiRNA_natural antisense transcripts siRNA, ncRNA_non- coding RNA, Pre-miRNA_Precursor miRNA, Pri-miRNA_Primary miRNA, PTGS_Post Transcriptional Gene Silencing, rasiRNA or hcsiRNA_repeat associated (or) heterochromatic siRNA, RISC_RNA-Induced Silencing Complex, RITS_RNA-Induced Transcriptional Silencing Complex, RNAi_RNA interference, siRNA_small interfering RNA, tasiRNA_*Trans*-acting siRNA, VIGS_Virus Induced Gene Silencing, VRTP_Virus Resistance Transgenic Plants.

Introduction

Small non coding RNAs (ncRNAs) are indispensable component of cellular regulatory mechanisms because they play diverse roles in mediating many biological processes like gene expression, chromatin formation, defence of genome against invading nucleic acids like viruses and transposons in eukaryotes. Ever since the ground breaking discovery of small ncRNA, *lin-4* in *C. elegans* and its role in sequence dependent translational repression (Lee et al., 1993) an exciting small RNA (sRNA) world has been unfastened. The inadvertent discovery of double-stranded RNA (dsRNA) triggered RNA interference (RNAi) phenomenon in *C. elegans* (Fire et al., 1998) has significantly expanded our understanding on molecular mechanism underlying antisense and sense RNA (co-suppression) mediated gene silencing known earlier in plants. RNA interference identified in animals is termed as Post Transcriptional Gene Silencing (PTGS) in plants (Napoli et al., 1990). Later on role of small ncRNAs as specificity determinants in transcriptional or post transcriptional gene regulations were well elucidated with the discovery of microRNAs (miRNAs), a class of ncRNAs, in plants (Reinhart et al., 2002; Bartel, 2004). The insights gained into the regulatory roles of small ncRNAs in general and miRNAs in particular strengthened the view that sRNAs are the ultimate regulators of gene expression in plants and animals. The RNA interference (RNAi) phenomenon along with large scale genome sequencing of economically important crops have opened up the floodgates in unearthing and characterization of small ncRNAs, ultimately to decipher their regulatory role in multitude of cellular processes. Soybean is a nutritionally important grain legume as more than half its seed (~60%) is endowed with oil and protein. Information on molecular markers, complete genome sequence, transcriptome records, online genomic repositories and tools made exclusively available on soybean makes it one of the most comprehensively investigated crops. The small ncRNAs documentation in soybean is no exception as is evident from microRNAs registry, miRBase version 18 (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2011). The database went from 218 entries in 2002 to 18226 entries currently accommodating hairpin precursor miRNAs and 21643 mature miRNA products representing 168 species. Thus far 362 precursor miRNAs and 395 mature miRNA sequence entries were reported in soybean and the inventory is growing rapidly. This review attempts to elucidate the various kinds of non-coding RNAs explored, their biogenesis, functions, successful and potential applications

for crop improvement in the context of soybean a representative grain legume.

Classes of small non coding RNAs (ncRNAs)

Endogenous small RNAs

Endogenous small non coding RNAs (ncRNAs) are class of innate RNAs that forms an inalienable component of gene regulatory mechanisms as they influence the spatial and temporal gene expression in transcriptional and post transcriptional stages.

microRNAs (miRNAs)

microRNAs are a class of endogenous, 18 to 24 nucleotide (nt) long, well characterized small RNAs which enhanced our understanding on RNA mediated gene regulatory mechanisms in plants. Biogenesis of miRNAs encompasses co-ordinated interplay of quite a few cellular proteins in and outside the nucleus (Fig 1). Mature miRNAs are produced from a pathway starting with primary miRNA transcripts (pri-miRNAs) transcribed from miRNA genes by RNA polymerase II (Mallory et al., 2008). The imperfect hairpin like primary miRNA (pri-miRNA) transcript undergoes cleavage to form a perfect hairpin precursor called precursor miRNA (pre-miRNA) with the aid of Dicer-Like enzyme (DCL1), a plant counterpart of animal Dicer enzyme. In the dicing process DCL1 interacts with pri-miRNA with the abetment of protein DAWDLE (DDL), which plays a significant role in recruiting DCL1 to pri-miRNA (Yu et al., 2008). Pre-miRNAs of animal origin are characteristically 60-70 bp in long whereas fairly long (~ 90-140 bp) pre-miRNAs are observed in plants. The pre-miRNA is further cleaved in to smaller, double stranded mature miRNA (miRNA:miRNA*) inside the nucleus and transported to cytoplasm by EXPORTIN-5 (Kurihara and Watanabe, 2004). In addition cellular enzymes like HYL1 (HYPONASTIC LEAVES 1), HEN 1 (HUA ENHANCER 1) and HST 1 (HASTY 1) are obligatory for the maturation of miRNA. DCL 1 interacts with its cohort HYL 1 (HYPONASTIC LEAVES 1) which possibly interacts with a zinc finger protein SERRATE (SE) to produce mature miRNA (Han et al., 2004). The processed miRNA is methylated and polyuridylated by HEN 1 (HUA Enhancer 1) to protect it from degradation (Park et al., 2002). The mature miRNA is then exported out of the nucleus by HST 1 (HASTY 1) an EXPORTIN orthologue in plants (Bollman et al., 2003; Yi et al., 2003). Out of two strands of the mature miRNA duplex (miRNA:miRNA*) one with the least 5' end thermodynamic stability will function as a mature miRNA, whereas the other strand (miRNA*) termed as passenger strand is specifically degraded (Khvorova et al., 2003). However, miRNA* passenger strands have also been implicated in gene regulatory mechanisms with the increasing evidence suggesting this aspect of sRNA mediated silencing (Pant et al., 2009). The mature miRNA strand in the cytoplasm forms an RNA-protein complex with ARGONAUTE (AGO 1) a component of gene silencing protein co-ordination called RNA-Induced Silencing Complex (RISC). The RISC complex cleaves specifically those transcripts that share sequence complementarity with the recruited miRNA on Watson-Crick base pairing principle (Llave et al., 2002). Alternatively the complex represses the target mRNA translation, either way it ultimately effects the down regulation of specific gene expression (Chen, 2004). The widely held view that the translational repression mechanism is prevalent only in animals requires a reconsideration as evidences for the presence of such apparatus in plants are also emerging (Aukerman et al., 2003; Brodersen et al., 2008). Computational analysis of soybean genome for miRNAs, based on the homology search predicted 22 miRNAs (Dezulian et al., 2005). Subsequently, intensive efforts by researchers involved in legume transcriptomics, following various approaches, have uncovered countless miRNAs regulating various stages of soybean growth and development (Table 1). Unlike the animal sRNAome, clustered miRNA genes are uncommon in plants (Jones-Rhoades and Bartel, 2004; Talmor-Neiman et al., 2006; Zhang et al., 2006b); however, computational analysis on soybean genome revealed the presence of 5 miRNA gene clusters (Zhang et al., 2008). The importance of clustered miRNAs is unclear nevertheless the clustered miRNAs in animal system are under a control of a unified gene regulatory mechanisms (Tanzer and Stadler, 2004; Altuvia et al., 2005) hence it is tempting to speculate that soybean miRNAs tend to evolve in clusters to maintain the single line of command in gene expression networks. Furthermore absence of such clusters in Rice and *Arabidopsis* implies that the evolution of miRNAs tends to be lineage specific. Soybean genome wide scrutiny for miRNA genes uncovers that the miRNA encoding regions are intergenic and few of those are intragenic or near the coding sequences thus restricted co-regulation and in turn co-evolution of miRNA genes with the target genes (Turner et al., 2012).

miRNA discovery

The field of plant miRNA discovery was initially dominated by the identification of conserved miRNAs expressed across the diverse plant species (Axtell and Bowman, 2008). MicroRNAs identification and characterization greatly relied on two approaches *viz*., computational approach and experimental validation. The former relies on the prospects of bio-informatics tools' prediction of evolutionarily conserved miRNAs based on sequence homology and secondary structural features (Wen et al., 2008). Computational programs like MIRscan (Lim et al., 2003) and MiRAlign among others yielded immense number of conserved miRNAs. *Arabidopsis* genome identified miRNAs like miR166, miR159 were found to be conserved across many of the unrelated plant species as well. Needless to mention that the approach is suitable for the plant species whose genome sequence is made available public. To vindicate this methodology nineteen miRNA families have been identified and reported in legumes *viz*., *Medicago truncatula*, *Lotus japonicus* and *Glycine max*. Nonetheless slack standards for stem-loop structures and miRNA prediction algorithms are the demerits of the approach that resulted in numerous false positives. Moreover the scheme generally identifies only conserved miRNAs that are necessitated to be experimentally validated. The alternative experimental approach (Fig 3) involving conventional cloning and deep sequencing of small RNA libraries has disclosed variety of low abundant, non-conserved, tissue and environment specific miRNAs (Lu et al., 2005; Sunkar and Zhu, 2004**;** Sunkar et al., 2005**;** Yao et al., 2007; Subramanian et al., 2008**).** Besides the traditional method of sequencing, MPSS (Massively Parallel Signature Sequencing) approach is also generally employed. It involves the identification of ~17 nucleotides long signature sequences of the template with the aid of restriction enzyme digestion, ligation and hybridization (Brenner et al., 2000). However,

Table 1. Salient miRNAs unearthed in soybean.

S.No	Trait responsive /organs specific miRNAs	Reference
1.	*B. japonicum* responsive • miR168 and miR172 (up regulated) miR160 and miR169 (Down regulated) and 35 novel miRNA families • miR482, miR1512 and miR1515	Subramanian et al., 2008 Li et al., 2010
2	EST, genome database screening and qRT-PCR validated 69 miRNAs involving 33 families	Zhang et al., 2008
3	Root nodules 32 miRNAs involving 11 families	Wang et al., 2009
4	Wild type *Glycine sojae* 9 novel miRNAs	Chen et al., 2009
5	Seed 26 novel miRNAs	Song et al., 2011
6	Shoot apical meristem 32 conserved miRNA families and 8 putative novel miRNAs Nuclear miRNA- miR4422	Wong et al., 2011
7	Drought and rust responsive 256 miRNAs including 24 families of novel miRNAs	Kulcheski et al., 2011
8	Sequencing of sRNAs and computational prediction 129 miRNAs inclusive of 87 novel miRNAs	Joshi et al., 2011
9	Drought, salinity and alkalinity stress 133 miRNAs belonging to 95 miRNA families	Li et al., 2011
10	Differential / spatial regulation of miRNA target transcripts expression in root and nodules 120 unknown miRNA genes including 5 novel miRNA families	Turner et al., 2012

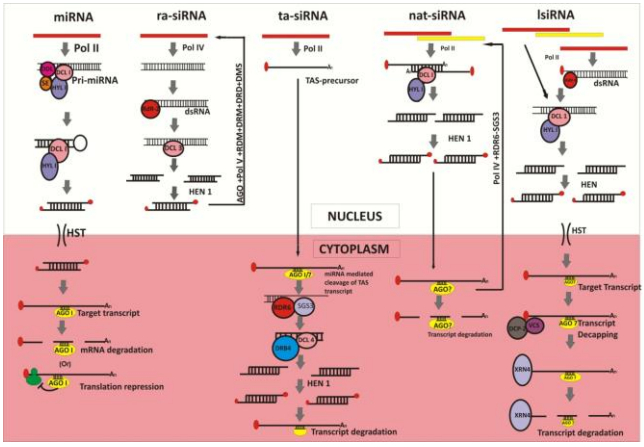

Fig 1. Endogenous small RNA biogenesis and mode of action (Adapted from Katiyar-Agarwal and Jin 2010).
Different kinds of endogenous small RNAs (sRNAs) *viz*., microRNAs (miRNAs), short interfering RNAs (siRNAs) comprising repeat associated siRNAs (rasiRNAs), *trans*-acting siRNAs (tasiRNAs), natural antisense transcript siRNAs (natsiRNAs), long siRNAs (lsiRNAs)- biogenesis along with the protein machinery involved and the mode of action

Protein machinery and functions

Pol:RNA POLYMERASE, Pri-miRNA:primary miRNA transcript, dsRNA:double-stranded RNA, DCL: DICER-LIKE, DDL:DAWDLE (involved in recruiting DCL 1 to pri-miRNA for downstream processing), SR:SERRATE, HYL:HYPONASTIC LEAVES (SR,HYL in cohort with DCL 1 is involved in generation of sRNA duplex from pre-miRNA), HEN: HUA ENHANCER (methylates sRNAs at 3' end), HST:HASTY (exportin homologue of plants involved in export of sRNAs from nucleus to cytoplam), AGO:ARGONAUTE (forms a component of RISC: RNA-Induced Silencing Complex- thus involves in mRNA degradation or translational repression, RdR: RNA DEPENDENT RNA POLYMERASE, RDM: RNA DIRECTED DNA METHYLASE, DRM: DOMAIN REARRANGED METHYLTRANSFERASE(catalyzes *de novo* DNA methylation), DRD: DEFECTIVE IN RNA-DIRECTED DNA METHYLATION(a chromatin-remodeling protein), DMS: DEFECTIVE IN MERISTEM SILENCING (A transcription factor required for Pol mediated transcription), SGS: SUPPRESSOR OF GENE SILENCING (A protein with zinc finger domain involved in binding of 5'overhangs of dsRNA), DCP: DECAPPING, VCS :VARICOSE (Both DCP and VCS are involved in mRNA decapping), XRNA :5′-3′ RNA exoribonuclease.

with the advent of high throughput sequencing facilities like Illumina Genome Analyzer (GA), Roche/454 FLX and the ABI SOLiD system coupled with computational predictions and experimental validation tools available the discovery of miRNAs have witnessed quantum improvement across various plant species *viz*., *Arabidopsis* (Xie et al., 2005; Lu et al., 2006; Rajagopalan et al., 2006), *Oryza sativa* (Sunkar et al., 2005), *Triticum aestivum* (Yao et al., 2007), *Medicago truncatula* (Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009) and *Glycine max* (Subramanian et al., 2008; Joshi et al., 2010 ; Kulcheski et al., 2011).

Comparative microRNA (miRNA) transcriptomics

In the early stages of miRNA discovery comparative transcriptomics predominated as previously identified,

Table 2. miRNA gene families, precursors and mature miRNA sequences identified in various legumes as in miRBase (v18.0) (Kozomara and Griffiths-Jones, 2011)

S.No	Legume species	miRNA gene family	miRNA precursors	Mature miRNAs
1	*Acacia auriculiformis*	7	7	7
2	*Acacia mangium*	3	3	3
3	*Arachis hypogaea*	21	23	32
4	*Glycine max*	187	362	395
5	*Glycine soja*	6	13	13
6	*Lotus japonicus*	3	3	4
7	*Medicago truncatula*	233	635	674
8	*Phaseolous vulgaris*	8	8	10
9	*Vigna unguiculata*	14	18	18

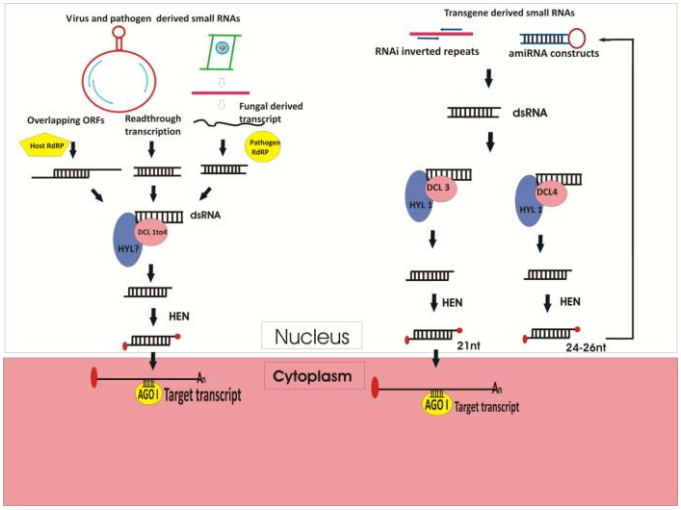

Fig 2. Exogenous small RNA biogenesis and mode of action (Adapted from Praveen and Ramesh 2007).
Various kinds of exogenous small RNAs (sRNAs) comprising virus, pathogen and transgene derived siRNAs their biogenesis and the mode of action
Acronyms
dsRNA: double-stranded RNA, RNAi: RNA interference, amiRNA: artificial miRNA, DCL: Dicer-Like, HYL: HYPONASTIC LEAVES, HEN: HUA ENHANCER, AGO:ARGONAUTE, RdRP: RNA dependent RNA polymerase (host/pathogen derived)

conserved miRNA families in model plants form the foundation of search for miRNAs in a particular species. The conserved miRNAs in model legumes revealed that their biological function is also conserved thus provides an opportunity for rapid *in silico* prediction and functional characterization of miRNAs in new species. For instance *P. vulgaris* derived miR399 had been implicated to play a role in modulation of ubiquitin conjugase enzyme under the condition of phosphorus starvation which is consistent with role previously characterized for the similar miRNA in *Arabidopsis*. EST based computational survey of miRNAs forms an important module of comparative transcriptomics as it precludes data on whole genome sequence. Moreover EST being expressed transcripts provides direct evidence for miRNA expression (Matukumalli et al., 2004). Purging the need for special software in miRNA exploration, openly available search algorithm like BLASTn could well be utilized for the purpose. On the above premise an expressed sequence tag (EST) and a genome survey sequence (GSS) approach was developed to identify miRNAs (Zhang et al., 2005). The EST- GSS approach identified 69 miRNAs belonging to 33 families in soybean and five miRNAs in *Glycine soja* and *Glycine clandestine* (Zhang et al., 2008). Thus the miRNA discovery and functional validation were based generally on the investigations of hardly any conserved miRNA families (Bartel, 2009). In legumes besides conserved miRNAs, species specific novel miRNAs have also been discovered. Investigations on soybean by various workers have yielded 87 novel in addition to 42 conserved miRNAs (Joshi et al., 2010; Subramanian et al., 2008; Wang et al., 2009). In the model legume *M. truncatula*, besides the conserved miRNAs families not less than 100 novel miRNAs were identified (Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009; Szittya et al., 2008). In case of tropical legume *P. vulgaris* six stress responsive miRNAs and 16 evolutionarily conserved miRNA families have been identified (Arenas-Huertero et al., 2009). Plethora of miRNA gene families (482), miRNA precursors (1039) and mature miRNA (1114) sequences from *Glycine max* and related legume species have been identified based on computational predictions and sequencing approaches (Table 2).

miRNAs and soybean nodulation

Legume-*Rhizobium* symbiotic nitrogen fixation is a multifaceted process that necessitates interplay of gene regulatory networks between two diverse organisms *i.e*., plant legume and bacterial *Rhizobium*. Host-derived miRNAs in the early stages of soybean-*Rhizobium* nodule development are implicated in the phytohormones homeostasis and systemic signaling. Expression analysis of *Bradyrhizobium japonicum* responsive miRNAs divulges the up-regulation of miR168 and miR172 whereas miR169 showed down regulation in expression. The changes in the expression levels of these miRNAs thus concertedly amend the concentration of Auxin Response Factors (ARFs) necessary for phytohormones homeostasis (Subramanian et al., 2008). Similarly 32 different miRNAs belonging to 11

Table 3. Online small RNA (sRNA) data analysis and repository resources.

S.No	Host	Datasets and Computational tools
1.	miRBase http://www.mirbase.org/ Faculty of Life Sciences, University of Manchester (Kozomara and Griffiths-Jones, 2011)	• hairpin, mature miRNA sequences and family classification • miRNA search by name, keyword, accession and tissue specific expression • miRNA targets (microcosm database)
2.	PMRD: Plant microRNA database http://bioinformatics.cau.edu.cn/PMRD China Agricultural University (Zhang et al., 2010)	• miRNA search by name, location, target gene ID or stem-loop sequence • microRNA prediction, secondary structure and expression profile • Genome browser and promoter sequences
3	Rfam http://rfam.sanger.ac.uk/ Wellcome Trust Sanger Institute (Griffith-Jones et al., 2003)	• Search for Rfam matches • Rfam family annotation and alignments, Rfam clan search • RNA families-multiple alignments, secondary structures • RNA gene annotations
4.	miRU: psRNATarget A Plant Small RNA Target Analysis Server http://www.plantgrn.org/psRNATarget/ (Dai and Zhao, 2011)	• miRNA target prediction with the pre-loaded *Glycine max* transcripts • target site accessibility evaluation
5.	Deepbase http://deepbase.sysu.edu.cn/index.php	• Small and long ncRNAs • miRNA target prediction • Deepview genome browser: large scale analysis of deep sequencing reads • libView Library Browser: comparisons of multiple small RNA libraries • nasRNAs(ncRNA-associated small RNAs) browser • nasView Graphic Browser: comparisons of multiple small RNA libraries in ncRNAs • Heatmap Browser: expression pattern of ncRNAs • snoSeeker: orphan and guide snoRNAs
6.	UEA sRNA toolkit http://srna-tools.cmp.uea.ac.uk/ University of East Anglia	• miRProf: Expression profiles of miRNAs • miRcat: identification of new miRNAs • miRNA and ta-siRNA target prediction • SiLoCo: identification and prediction of siRNA producing loci • RNA folding and annotation
7	NCBI http://www.ncbi.nlm.nih.gov/ The National Center for Biotechnology Information, National Institutes of Health (NIH)	• Genome sequences • Gene annotation • Transcriptome • Gene expression • BLAST • Conserved Domain Database (CDD)
8	Phytozome http://www.phytozome.net/ Joint Genome Institute and Center for Integrative Genomics, University of California (Goodstein et al., 2012)	• Assembled sequences and sequence annotation • Genome browser • BLAST • Cross species homology search
9.	SoyBase http://soybase.org/ USDA and Iowa State University (Grant et al., 2010; Du et al., 2010	• Genome sequence and Gene annotation • BLAST • EST library • Tissue based expression profile • Williams 82 Transposable element (TE) database
10	SoyKB: Soybean Knowledge Base http://soykb.org/ University of Missouri (Joshi et al., 2012)	• GBrowse for annotated genes and miRNA • BLAST • Transcriptomics data
11	Soy-TFKB : Soybean Transcription Factor Knowledge Base http://www.igece.org/Soybean_TF/ Institute for Green Energy and Clean Environment	• Transcription Factors (TFs) • Transcripts sequence • TF families browser

miRNA families (for instances miR167, miR172, miR396, and miR399 families) have been incriminated in the later stages of soybean-*Rhizobium* nodulation process (Wang et al., 2009). In a quest to delineate the role of miRNAs in soybean-*Bradyrhizobium* interactions transgenic expression of novel miR482, miR1512 and miR1515 recognized them to substantially increase the number of nodules. Moreover differential expression pattern of these miRNAs have been associated with non-nodulating and super nodulating genotypes of soybean (Li et al., 2010). The unraveling of the role of miR482 in repression of disease resistance R genes is perceivable as symbiotic nitrogen fixation entails the establishment of non-native alive entity, *Bradyrhizobium*, in the roots of legumes. In addition, the target predictions of *Bradyrhizobium* responsive miRNAs in *M.truncatula* revealed mRNAs encoding for transcription factors (TFs), proteins involved in hormonal signaling pathways and cell cycle (El-Yahyaoui et al., 2004). Undoubtedly it can be inferred that soybean-*Rhizobium* nodulation is a multifarious process mandated by the complicated gene regulatory cascades involving phytohormones mediated cell division for the symbiotic benefit of both the organisms.

Endogenous siRNAs

Notwithstanding similarity in size, miRNAs and small interfering RNAs (siRNAs) differ greatly in their biogenesis and their functions are considerably diverse. While the miRNAs are elucidated to originate from hairpin (stem-loop) precursors derived from single strand RNA, siRNAs are processed from longer double-stranded RNA (dsRNA) molecules with near perfect complementarity. Furthermore mature siRNAs embodies both the strands of RNA whereas active miRNAs are characterized by single strand of RNA the inactive strand called passenger strand is degraded eventually. The different kinds of endogenous siRNAs identified in the plant RNA silencing mechanism are: repeat associated siRNAs (rasiRNAs) or heterochromatic siRNAs (hcsiRNAs), *trans*-acting siRNAs (tasiRNAs) and natural antisense transcript siRNAs (natsiRNAs) and long siRNAs (lsiRNAs) (Fig 1). The mode of siRNA biogenesis differs with the kind of siRNAs produced as the protein machinery involved varies.

Repeat associated siRNAs (rasiRNAs) or heterochromatic siRNAs (hcsiRNAs)

repeat associated siRNAs (rasiRNAs) or heterochromatic siRNAs (hcsiRNAs) are 24-26 nucleotides (nt) long endogenous siRNAs uncovered in protists, *Drosophila* and plants. rasiRNAs are the cleaved products of transcripts derived from recurring genome sequences present in centromere or transposons. The double stranded repeat associated primary transcripts (Pri-rasiRNA) are initially transcribed by RNA polymerases Pol IV and RNA dependent RNA Polymerase 2 (RdRP 2) inside the nucleus. The pri-rasiRNA transcripts are further processed by DCL 3 enzyme to generate rasiRNAs that are further methylated by the action of HEN 1 to produce mature rasiRNA. The mature rasiRNAs effect the process of transcriptional gene silencing (TGS) with the involvement of multi-protein complex called RNA induced transcriptional gene silencing complex (RITS) in the target genomic region. RITS executes the process of TGS with the aid of Pol V which is consecutively involved in recruiting DNA methyl transferases and histone modifying enzymes. Further RITS is known to comprise ARGONAUTE 4 (AGO 4), RNA binding protein known as KTF 1 (He et al.,

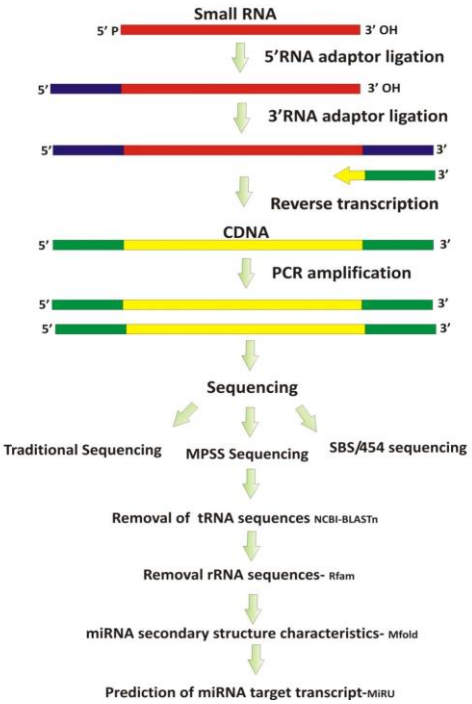

Fig 3. The workflow diagram for small RNA cloning and characterization by experimental approach (Adapted from Meyers et al., 2006).

The figure explains the steps involved in sRNA characterization which entails PAGE-size fractionation of sRNA species followed by reverse transcription-PCR to obtain the dsDNA complement, then sequencing of amplicons by traditional or MPSS (Massively Parallel Signature Sequencing) or NGS (Next Generation Sequencing) methodologies. It is followed by *in silico* analysis to trim down the undesirable sequences and to predict secondary structural features and the target transcripts.

2009; Wierzbicki et al., 2009) in realizing the rasiRNAs mediated sequence dependent chromatin modification through RNA-dependent DNA methylase (RdDM). It is comprehensible that the tandem repeat elements and transposons are strongly regulated by rasiRNAs because the liberal movement of such elements across the genome may cause a colossal damage to the genome integrity of the plants. Nevertheless it is noteworthy to learn that chromatin regulation by rasiRNAs is wobbly controlled all through the moment of external abiotic or biotic stress. Hence it is safe to speculate that the condition of adversity provides an opportunity for gene regulatory mechanism to exercise the transposon mediated genome rearrangements as a last resort to increase the genetic diversity.

Trans-acting siRNAs (tasiRNAs)

Transacting small RNAs which target mRNAs that show little semblance to the gene from which they originate, contrary to the widely held principle that siRNAs silence the transcript from which they originate have led to the advent of *trans*-acting siRNAs (ta-siRNAs) in plants and also in *C.elegans* . The biogenesis of tasiRNAs comprises DCL 1, HYL 1, HEN 1 and AGO 1/7 along with involvement of miRNA in processing a long primary transcript derived from *trans*-acting siRNA genes (TAS genes). The resultant transcript is further processed by RDR 6 (RNA Dependent RNA Polymerase 6) and SGS3 (Suppressor of Gene Silencing 3) to generate dsRNA, with an eventual processing

Table 4. Applications of sRNA mediated downregulation to obtain desired soybean phenotypes and in functional genomics studies.

S.No	Target gene and its silencing effect(s)	Reference
1	Fatty acid desaturase (*GmFAD2-1*): High oleic acids	Kinney, 1996; Wang and Xu, 2008; Wagner et al., 2011
2	â-conglycinin á and á′ subunit : Decreased conglycinin, increased glycinin and protein body accumulation in ER derived vesicles	Kinney et al., 2001
3	P34/ Bd 30 K gene: Reduced Bd 30 K	Herman et al., 2003
4	Flavanone 3-hydroxylase: Increased isoflavone	Yu et al., 2003
5	â-glucuronidase (*GUS*): No *GUS* expresssion	Reddy et al., 2003
6	Isoflavone synthase (*IFS1* and *IFS2*): Reduced isoflavone and increased susceptibility to *P. sojae* and root nodules	Subramanian et al., 2005 ; Subramanian et al., 2006
7	Thioredoxin gene: Reduced root nodules	Lee et al., 2005
8	Delta 15 desaturase: Production of arachidonic acid	Chen et al., 2006
9	Myo-inositol-1-phosphate synthase (*GmMIPS1*): Reduced phytate and impaired seed development	Nunes et al., 2006
10	ATP binding cassette (ABC) embryo specific transporter: Reduced phytate	Shi et al., 2007
11	Chalcone synthase (*CHS6*) and isoflavone synthase (*IFS2*): Reduced isoflavone, coumesterol and increased growth of *F. solani*	Lozovaya et al., 2007
12	Chalcone reductase and isoflavone synthase: breakdown of resistance to *P. sojae* and cell death	Graham et al., 2007
13	*GmFAD-3*: Low á-linolenic acid	Flores et al., 2008
14	24-kDa oleosin gene: Changes in seed oil body size and slow plant growth	Schmidt and Herman, 2008
15	Lipoxygenases (*LOX9* and *LOX10*): No effect on root nodule development	Hayashi et al., 2008
16	Glutathione S-transferase (*GST9*): Reduced nitrogenase activity and increased oxidatively damaged proteins	Dalton et al., 2009
17	Ecto-apyrase gene (*GS52*): Suppression of root nodule development	Govindarajulu et al., 2009
18	FW2.2-like gene (*GmFWL1*): Suppression of root nodule development	Libault et al., 2010
19	MYB transcription factor (*GmMYB176*): Reduced isoflavanoids	Yi et al., 2010
20	Phospholipase D gene (*SPLDá*): Changes in phospholipid and triacylglycerol (TAG) Composition. Reiterated the positive role of SPLDá in Phosphatidyl choline (PC) to TAG conversion	Lee et al., 2011
21	Glycinin A1bB2 subunit and *FAD-2* genes: Rebalance of seed protein content	Schmidt et al., 2011
22	â-amyrin synthase (*GmBAS1* and *GmBAS2*): Reduced saponins	Takagi et al., 2011
23	Amino aldehyde dehydrogenase: Biosynthesis of 2-acytyl-1-pyrroline hence the associated aroma	Arikit et al., 2011

by DCL 4 and DRB 4 to generate mature 21nt long tasiRNAs. The primary function of these siRNAs is in gene silencing through perfect complementarity and they act in *trans* (Allen et al., 2005; Gasciolli et al., 2005)

Natural antisense transcripts siRNAs (natsiRNAs)

Natural antisense transcripts siRNAs (natsiRNAs) are a class of endogenous siRNAs 21 to 24 nucleotides (nt) long and are derived from overlapping convergent genes hence the name natural antisense transcript (Borsani et al., 2005; Katiyar-Agarwal et al., 2007). It is a naturally present mechanism in plants to tide over any adverse environmental conditions. The concomitant presence of constitutively expressed sense transcripts and the natural antisense transcripts triggers the generation of natsiRNAs by the processing of DCL 2 and RdRP 6, SGS 3 and Pol IV. The mature natsiRNAs thus effect the sequence dependent silencing of constitutively expressing sense transcripts under the conditions of extreme stress.

Long siRNAs (lsiRNAs)

Another class of endogenous siRNAs is characterized by 30 to 40 nucleotides (nt) long effectors called as long siRNAs (Katiyar-Agarwal, 2007). The long siRNAs are produced in the wake of pathogen infection as a defence mechanism to combat the infection. *Arabidopsis thaliana* derived AtlsiRNA-1 is the first characterized lsiRNA, induced by bacterial pathogen *Pseudomonas syringae* and is involved in the target mRNA decapping and mRNA degradation. The protein machinery involved in the biogenesis is DCL 1, HYL 1, HEN 1 and AGO 7 besides the biogenesis partially depends on RDR6 and Pol IV (Katiyar-Agarwal, 2007). Microarray profiling of soybean miRNAs under the *Phytophthora sojae* infection, revealed altered expression levels of specific miRNAs across different cultivars like Williams (susceptible), Conrad (quantitative resistance) and Williams 82 (qualitative resistance) suggesting the existence of lsiRNAs kind feedback circuit between the miRNAs and target coding genes (Guo et al., 2011)

Exogenous Small RNAs

Virus and other pathogen Derived Small RNAs

Virus derived small RNAs form major component of exogenous ncRNAs in plants (Fig 2). Soybean infecting viruses are known to produce two different kinds of small RNAs *viz*., viral siRNAs and miRNAs with a perceivable difference in their biogenesis and mode of action. Viral derived siRNAs have been characterized from both RNA and DNA viruses infecting soybean. The trigger for their biogenesis is host RNA dependent RNA polymerase (RdRP)-

Table 5. Virus induced gene silencing (VIGS)-based functional genomics studies on soybean.

VIGS vector	Target gene and its silencing effect(s)	Reference
1. BPMV (*Bean Pod Mottle Virus*)	Phytoene desaturase (*PDS*): Photobleaching	Zhang and Ghabrial, 2006; Zhang et al., 2010
	Stearoyl-acyl carrier protein-desaturase: Reduced oleic acid, increased stearic acid, resistance to pathogens due to Salicylic acid	Kachroo et al., 2008
	GmRAR1(for *Mla12*-mediated resistance) and *GmSGT1* (suppressor of the G2 allele of *skp1*): compromised resistance to *Soybean mosaic virus* and *Pseudomonas syringae*	Fu et al., 2009
	Actin:Resistance to *Soybean mosaic virus* infection	Zhang et al., 2009
	Mitogen activated protein kinase (*Mpk4A* and *Mpk4B*): Stunted growth	Zhang et al., 2009
	GMSgt1A and *GMSgt1B:* Mild symptoms	Zhang et al., 2009
	Ribosomal protein genes (*Rps6* and *Rps13*): Foliar symptoms and stunted roots	Zhang et al., 2009
	Candidate rust resistance genes: Altered resistance against *Phytophthora pachyrhizi*	Meyer et al., 2009; Pandey et al., 2011
	Fatty acid desaturase (*FAD3*): Changes in seed size, susceptibility to *Pseudomonas syringae*	Singh et al., 2011
2. CMV (*Cucumber Mosaic Virus*)	Chalcone synthase (*CHS*) : No pigmentation in seed coat, reduced isoflavone	Nagamatsu et al., 2007
	Flavonoid 3′-hydroxylase : Reduced quercetin	Nagamatsu et al., 2007
	Terminal flower-1b (*GmTFL1b*): Reduction in node number	Liu et al., 2010
3. ALSV (*Apple Latent Spherical Virus*)	Isoflavone synthase (*IFS-2*): Low isoflavone	Yamagishi and Yoshikawa, 2009
	Phytoene desaturase (*PDS*): Photobleaching	Yamagishi and Yoshikawa, 2009

induced perfect stem-loop structure produced from the aberrant, read through transcripts or overlapping ORFs of virus genome. The resultant viral derived hairpin RNAs are processed by different types of DCLs (DCL 1 through DCL 4) to generate three kinds of siRNAs of varying length (21, 22 and 24 nucleotides long) that are involved in plant's defence mechanism. Whereas the RNA virus derived hairpin RNAs (hpRNAs) are processed only by DCL 4 and the consequential siRNAs also acts as systemic signal. Resistant cultivar of soybean when agro-infected with *Mungbean yellow mosaic Indian virus* (MYMIV) exhibited the generation of 24 nucleotides long siRNAs targeting intergenic regions (IR) of viral genome. The siRNAs accomplish the methylation of target viral genome with the assistance of host protein machinery consequently repressing the viral gene expression. The siRNAs characterized in susceptible cultivars were found to be targeting coding regions of the viral genome and are futile in conferring resistance against *Yellow mosaic virus* infection (Yadav and Chattopadhyay, 2011). In consequence the resistance reaction in YMV infection could conceivably be due to siRNA mediated Transcriptional Gene Silencing (TGS) whereas siRNA mediated PTGS is unsuccessful. Thus, siRNA generating loci of virus genome consequently decides the mode of siRNA action and determines the outcome of disease reaction. Unlike the viral pathogens which are dependent on the host molecular machinery to gain entry and establishment within the host cell, fungal pathogens own their cellular machinery to initiate and sustain the process of infection. As a model of host-pathogen interaction a study on soybean-*Phytophthora sojae* small RNAs interface revealed the presence of pathogen derived siRNAs and miRNAs inside the host system. Small RNA libraries derived from *Phytophthora sojae, Phytophthora infestans and Phytophthora ramorum* disclosed the existence of two classes of siRNAs (21 and 25 nucleotides long) that help the pathogen to overcome host defence mechanism. In addition eight candidate miRNA genes have also been identified in soybean in response to the infection. The protein complement of the fungal gene silencing machinery has been recognized to comprise DCL 1, DCL 2 and RdRP among others (http://www.ars.usda.gov/research/projects/).

Transgene derived siRNAs

Transgene derived siRNAs are the result of concerted and directed efforts to definitely alter the plant genetic makeup in order to obtain desirable plant phenotype. Transgenes are designed to produce perfect hairpin or inverted repeat RNAs. The perfect stem-loop duplex RNAs are processed by DCL 3 and DCL 4 to produce two varieties of siRNAs differing in length *i.e*., 24 -26 and 21-22 nucleotides long respectively. A shorter class of siRNA (21–22 nt long) is implicated in mRNA degradation while a longer one (24–26 nt long heterochromatic siRNAs) is involved in directing DNA methylation and additionally causing systemic spread of silencing message (Tang et al., 2003).

Small RNA database

The plethora of small RNA (sRNA) data made possible by the high-throughput sequencing technologies requires a thorough *in silico* analysis for validation of small RNAs, secondary structure characterization, identification of novel small RNAs, target transcripts prediction and development/ maintenance of repository of small RNAs in a retrievable configuration. The concern is addressed by many publicly available bio-informatics software packages which help in converting the raw sequence data in to accessible and manageable formats. Multitude of small RNA databases have been created with an outlook to compile species specific information, target transcripts in the genome, sRNA data analysis, repository resource in order to make the sRNAome record complete (Table 3).

Potential applications of small RNA (sRNA) metabolism

Our understanding on the phenomenon of RNAi (RNA interference) in plants, hence on sRNA metabolism, came predominantly from the generation of virus resistance

transgenic plants (VRTPs). While the phenomenon of dsRNA mediated gene silencing was discovered in *C. elegans* (Fire et al., 1998) it was also deduced that simultaneous expression of sense and antisense RNA strands cognate to virus genome delivers resistance against the pathogen (Waterhouse et al., 1998). Subsequently the ultimate effector molecules of RNA mediated silencing are identified as small non coding RNAs. sRNA mediated modulations in soybean disclose that the qualitative traits, governed by single or few genes, are the preferred targets for manipulation (Table 4). The intricacy of the plant response to biotic and abiotic stresses involves many genes and cellular mechanisms and hence adjustment to these stresses is achieved through regulating multiple gene expression. The strategic position of sRNAs, particularly the cellular functions of rasiRNAs or lsiRNAs, in plant molecular pathways indicates that multiple genes involved in quantitative trait like drought tolerance are the prospective targets for modulation.

Small RNAs as Functional genomics tool in soybean

Small RNAs (miRNAs and siRNAs) mediated gene silencing is a potential technique for silencing of genes in a specific and efficient manner in soybean. The mutant resources of soybean have been developed using transgene induced silencing and Virus Induced Gene Silencing (VIGS) modalities (Table 4 and Table 5). RNAi based negative regulation of isoflavone synthase genes (*IFS1* and *IFS2*) uncovered its role in conferring race specific resistance against *Phytophthora sojae* infection (Subramanian et al., 2005; Graham et al., 2007). Similarly the perturbations of total glycinin and conglycinin seed storage proteins through siRNA mediated silencing unearthed the rebalance of the total protein content in soybean seeds without any compromise on the metabolome (Schmidt et al., 2011). RNAi mediated silencing of *GmFWL*1 resulted in reduced number of soybean nodules with the simultaneous reduction in nuclear size thus implicating its role in nodule organogenesis (Libault et al., 2010). siRNA mediated silencing of saponin biosynthesis gene β amyrin synthase besides reducing the level of saponins it also demonstrated that soybean seeds can sustain the normal development process and survive with no remarkable reduction in antioxidant activity in the absence of saponins (Takagi et al., 2011). On the high throughput studies RNAi knock-out soybean lines have been generated targeting the transcripts involved in soybean-*Bradyrhizobium* nodulation process and seed specific transcriptional factors (http://seedgenenetwork.net/) to ascertain their role in the normal developmental processes. Nevertheless the approach greatly relies on the transformation efficiency of soybean that is low and genotypic specific (Ko et al., 2006; Olhoft et al., 2006). Hence an alternative transient methodology for small RNA mediated gene silencing would accelerate the process. Virus induced gene silencing (VIGS) which exploits the small RNA mediated antiviral defence mechanism to induce knock-down of specific gene expression employing a virus vector carrying a fragment of target gene. It is a valuable tool for all-encompassing forward genetics studies in soybean. In the arena of soybean functional genomics studies, *Bean pod mottle virus* (BPMV), *Cucumber mosaic virus* (CMV) and *Apple latent spherical virus* (ALSV) based virus induced gene knock-out vectors have been successfully developed and employed extensively (Table 5). Among these BPMV is a DNA virus based VIGS vector that offers an advantage of siRNA mediated silencing and concomitant expression of foreign genes (Yamagishi and Yoshikawa, 2009; Zhang et al., 2010). VIGS methodology had facilitated significantly in the functional genomics studies on many disease resistant genes for instance it uncovered the role of actin gene in *Soybean mosaic virus* infection (Zhang et al., 2010). VIGS offers advantage over the insertion mutagenesis as the former allows for targeted negative regulation of transcript of interest. On the other hand silencing a conserved region of a gene would generate knock-outs for the entire family of genes thus accelerates soybean functional genomics research. Moreover with the incorporation of inducible promoters the time and degree of gene suppression could be achieved consequently providing greater flexibility to the process. Thus far the potential of RNAi mediated functional genomics tool can be exploited where transformation efficiency of the crop is relatively high, where not available, VIGS offers hope but the compatibility of the virus vectors for VIGS with plants required to be studied elaborately. The outcome of the high-throughput knock-out studies based on sRNA modulation possibly will help us in understanding the molecular processes involved in complex traits like drought tolerance, yield levels of economic parts and so on. It will facilitate in shifting the sRNA based trait engineering in favour of quantitative characters which are relatively complicated to manipulate through conventional approaches from currently predominating qualitative traits.

Conclusions

The small RNA mediated gene regulatory system is the master controller of physiological and developmental processes in plants. Whereas the discovery and expression analysis of sRNA in soybean is predominated by miRNA profiling, siRNA mediated gene silencing dominates on the aspect of sRNA applications. While the sRNA based applications are embarked upon a finding that transgene triggered sRNA mediated gene silencing occurs even inside the nucleus unlocks another feature of sRNAome (Hoffer et al., 2011). It establishes that the protein machinery obligatory for RNA silencing is available inside the nucleus as well. Hence the consensus model for sRNA mediated gene regulatory networks in plants, generated over the years, requires to be re-examined and essentially for the opportunities in exploration and exploitation of plant sRNAome to further the crop improvement programmes. Of late sRNAs are gaining attention for its cross-kingdom presence and its active role in metabolic regulation of mRNAs in biologically unrelated organisms. The surprising finding that mammalian mRNAs are under modulation of plant derived sRNAs, obtained principally through diet, appends to yet another facet of sRNA metabolism (Zhang et al., 2011). Nonetheless, studies have revealed on safe consumption of food derived sRNAs despite sRNAs sharing perfect sequence complementarity with the human and mammalian mRNAs (Ivashuta et al., 2009). Albeit the harmless effects of plant derived sRNAs on human transcriptome analogous consequence cannot be envisaged with sRNAs generated from RNAi based genetically modified crops. The credible ecological risk assessment (ERA) criteria including sRNAs persistence, their off-target or unintended effects on the transcriptome of related species is inevitable in order to evaluate transgene derived sRNAs for safe human consumption. The discovery of non-coding RNA molecules called miRNA decoys or miRNA mimics in *Arabidopsis* (miRNA sponges in animals) characterized by non-cleavable miRNA target sites and their deployment in manipulating the miRNA activity arouses much interest (Axtell et al., 2006; Franco-Zorrilla et al., 2007; Ebert et al.,

2007). The successful engineering and manipulation of endogenous RNA regulatory network by altering the miRNA target decoys a range of plant phenotypes are possible (Ivashuta et al., 2011) hence miRNA decoys offer a feasible opportunity for employing the modus operandi to engineer quantitative traits like drought tolerance in soybean. sRNA repertoire demonstrates that targeted gene silencing approach could be employed to achieve multitude of gene modulations which are impossible with traditional plant breeding approaches. The improved sRNA based silencing platforms offers a new hope for obtaining improved crop plants to enhance the global food supply with greater food safety standards. Different kinds of endogenous small RNAs (sRNAs) *viz*., microRNAs (miRNAs), short interfering RNAs (siRNAs) comprising repeat associated siRNAs (rasiRNAs), *trans*-acting siRNAs (tasiRNAs), natural antisense transcript siRNAs (natsiRNAs), long siRNAs (lsiRNAs)- biogenesis along with the protein machinery involved and the mode of action

Protein machinery and functions

Pol:RNA POLYMERASE, Pri-miRNA:primary miRNA transcript, dsRNA:double-stranded RNA, DCL: DICER-LIKE, DDL:DAWDLE (involved in recruiting DCL 1 to pri-miRNA for downstream processing), SR:SERRATE, HYL: HYPONASTIC LEAVES (SR,HYL in cohort with DCL 1 is involved in generation of sRNA duplex from pre-miRNA), HEN: HUA ENHANCER (methylates sRNAs at 3' end), HST:HASTY (exportin homologue of plants involved in export of sRNAs from nucleus to cytoplam), AGO:ARGONAUTE (forms a component of RISC: RNA-Induced Silencing Complex- thus involves in mRNA degradation or translational repression, RdR: RNA DEPENDENT RNA POLYMERASE, RDM: RNA DIRECTED DNA METHYLASE, DRM: DOMAIN REARRANGED METHYLTRANSFERASE(catalyzes *de novo* DNA methylation), DRD: DEFECTIVE IN RNA-DIRECTED DNA METHYLATION(a chromatin-remodeling protein), DMS: DEFECTIVE IN MERISTEM SILENCING (A transcription factor required for Pol mediated transcription), SGS: SUPPRESSOR OF GENE SILENCING (A protein with zinc finger domain involved in binding of 5'overhangs of dsRNA), DCP: DECAPPING, VCS :VARICOSE (Both DCP and VCS are involved in mRNA decapping), XRNA :5′-3′ RNA exoribonuclease. Various kinds of exogenous small RNAs (sRNAs) comprising virus, pathogen and transgene derived siRNAs their biogenesis and the mode of action

Acronyms

dsRNA: double-stranded RNA, RNAi: RNA interference, amiRNA: artificial miRNA, DCL: Dicer-Like, HYL: HYPONASTIC LEAVES, HEN: HUA ENHANCER, AGO:ARGONAUTE, RdRP: RNA dependent RNA polymerase (host/pathogen derived) The figure explains the steps involved in sRNA characterization which entails PAGE-size fractionation of sRNA species followed by reverse transcription-PCR to obtain the dsDNA complement, then sequencing of amplicons by traditional or MPSS (Massively Parallel Signature Sequencing) or NGS (Next Generation Sequencing) methodologies. It is followed by *in silico* analysis to trim down the undesirable sequences and to predict secondary structural features and the target transcripts

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