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REVIEWS

Non-coding RNAs in Crop Genetic Modification: Considerations and Predictable Environmental Risk Assessments (ERA)

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Abstract Of late non-coding RNAs (ncRNAs)-mediated gene silencing is an influential tool deliberately deployed to negatively regulate the expression of targeted genes. In addition to the widely employed small interfering RNA (siRNA)-mediated gene silencing approach, other variants like artificial miRNA (amiRNA), miRNA mimics, and artificial transacting siRNAs (tasiRNAs) are being explored and successfully deployed in developing non-coding RNA-based genetically modified plants. The ncRNA-based gene manipulations are typified with mobile nature of silencing signals, interference from viral genome-derived suppressor proteins, and an obligation for meticulous computational analysis to prevaricate any inadvertent effects. In a broad sense, risk assessment inquiries for genetically modified plants based on the expression of ncRNAs are competently addressed by the environmental risk assessment (ERA) models, currently in vogue, designed for the first generation transgenic plants which are based on the expression of heterologous proteins. Nevertheless, transgenic plants functioning on the foundation of ncRNAs warrant due attention with respect to their unique attributes like off-target or non-target gene silencing effects, small RNAs (sRNAs) persistence, food and feed safety assessments, problems in detection and tracking of sRNAs in food, impact of ncRNAs in plant protection measures, effect of mutations etc. The role of recent developments in sequencing techniques like next generation sequencing (NGS) and the ERA paradigm of the different countries in vogue are also discussed in the context of ncRNA-based gene manipulations.

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Introduction

Enhanced tolerance to biotic and abiotic stresses, resistance to herbicides, increased yield, and crops with superior nutritional value represent major crop improvement goals. Genetic engineering approach contributes to realizing these goals in an unprecedented manner by altering the genetic makeup of the crop. Genetic manipulation in crops toward the successful incorporation of desired trait, till recently, involves purposeful introduction and expression of foreign genetic elements into the plant genome's background. With the arrival of RNA interference (RNAi) phenomenon—a natural defense mechanism against invading viruses, nucleic acids, and transposons [1]–non-coding RNAs (ncRNAs) are identified as effector molecules of RNA-mediated gene silencing and are being exploited in genetic alteration of the crops [2]. Heterologous protein expression-based transgenic plants differ from ncRNAs-mediated genetic modulation, as in the latter, desired trait is attained by negatively regulating the expression of endogenous or exogenous gene(s) deploying small non-coding RNAs in nucleic acid sequence-dependent manner. The better understanding on the small RNA landscape (sRNAome) of plants, greater appreciation of versatile small RNA-based gene silencing systems with diverse forms of ncRNAs have unopened an era of non-coding RNAs-based genetic trait manipulation (Fig. 1).

One of the most extensively employed techniques for selective down regulation of target genes is small interfering RNA (siRNA)-based gene silencing, chiefly to incorporate virus resistance trait [3], secondary metabolite

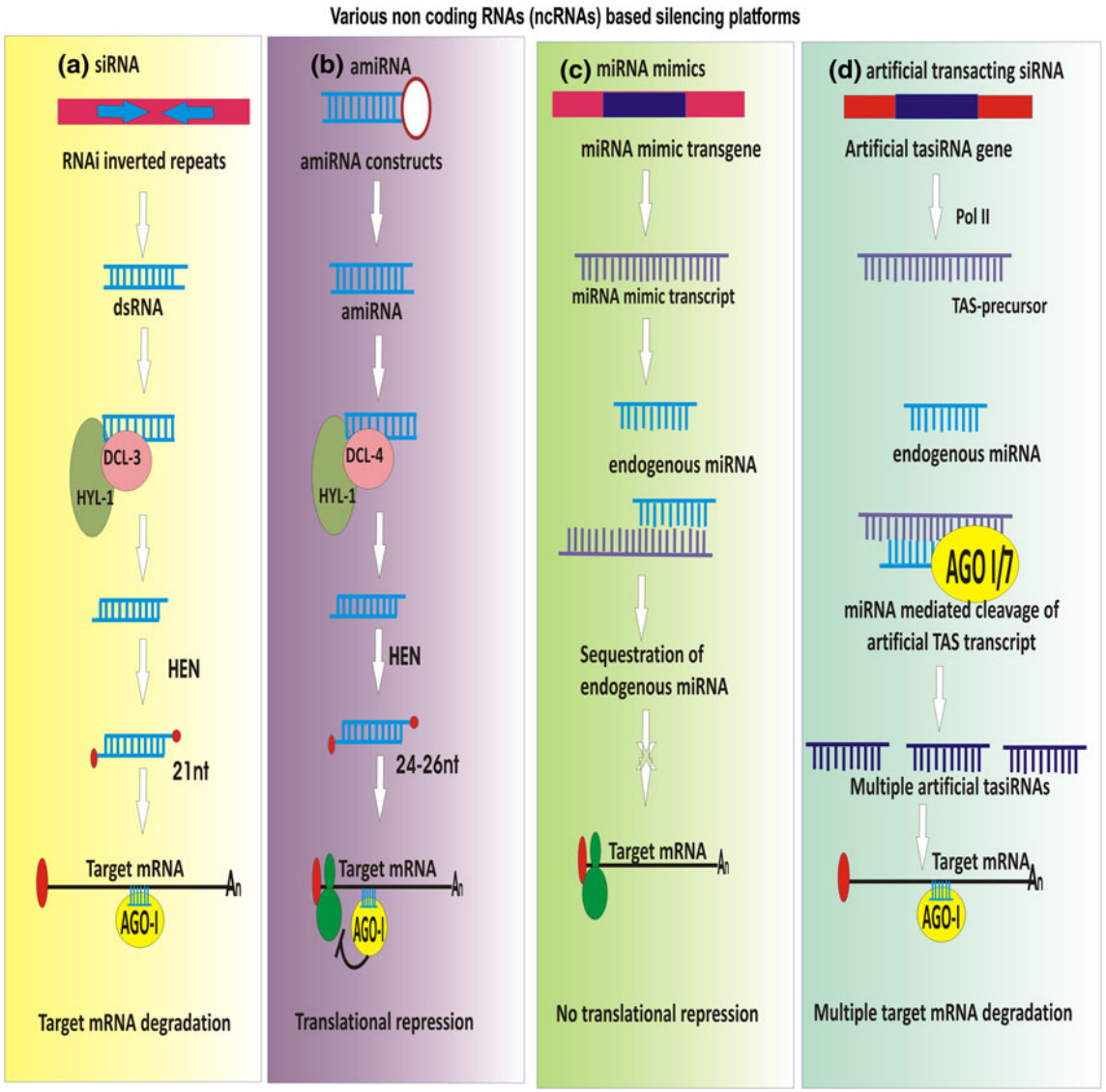

Fig. 1 Various non-coding RNAs-based silencing platforms. **a** siRNA-mediated targeted gene regulation involves the generation of inverted repeats or hairpin RNA expressing gene constructs, and the resultant dsRNA is processed by protein machinery comprising Dicer-Like (DCL-3), Hyponastic leaves (HYL-1), and HUA Enhancer (HEN-1) to produce methylated, functional small interfering RNA (siRNA) which in turn causes sequence-dependent target mRNA degradation with the aid of RNA-Induced Silencing Complex (RISC) containing Argonaute (AGO-1). **b** In artificial miRNA (amiRNA)-mediated gene silencing, the 21-nt long miRNA segment of endogenous miRNA is replaced with similar length transgene complementary for target gene resulting in the translational repression of the target gene. **c** miRNA mimics platform utilizes the uncleavable miRNA target transcript as transgene, thereby resulting in sequestration of endogenous miRNA, thus precluding it from functioning in translational repression. **d** Artificial transacting siRNAs (tasiRNAs) involve the expression of artificial transacting siRNA (TAS) gene, then endogenous miRNA-mediated regulation results in the production of multiple artificial tasiRNAs, each targeting a separate mRNA, a strategy thus applicable for realizing metabolic engineering in plants

engineering [4, 5], removal of toxins or undesirable substances [6], functional genomics studies [7], improving nutritional value of the crop [8], pest and pathogen tolerance [9, 10]. Small interfering RNA (siRNA) is a class of 21–24 nucleotides (nt) long non-coding, effector RNA molecule derived from dsRNA or fold back of transcribed ssRNA. Transgenes are designed in order that the transcribed mRNA folds back to produce perfect hairpin or inverted repeat RNAs [11, 12]. The perfect stem-loop duplex RNAs are processed by cellular enzymes called DCL 3 (Dicer-Like 3) and DCL 4 (Dicer-Like 4) to produce two varieties of siRNAs differing in length i.e., 24–26 and 21–22 nucleotides long, respectively. The 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 [13].

In addition to siRNA-based gene silencing mechanism, endogenous microRNAs (miRNAs)-based gene repression phenomenon is in vogue recently [14, 15]. miRNAs are a class of small ncRNAs transcribed from longer miRNA genes that are involved in the endogenous gene regulation. In this methodology, the endogenous miRNA gene is isolated and characterized for the 21–24 nucleotides long miRNA segment which is replaced with similar length transgene complementary for target gene intended to be silenced. This way the miRNA silencing machinery originally intended for endogenous gene regulatory network is modified to downregulate any gene of interest [16, 17]. The greatest advantage of methodology over siRNA-mediated gene silencing is immobile silencing signal; hence tissue-specific silencing is possible [18].

miRNA mimics or target mimicry approach is a recent, potent addition to the existing arsenal of ncRNA-mediated gene silencing. It involves the investigational approaches that intend to deregulate either the miRNA activity or its biosynthesis. The former involves the expression of short ncRNA molecule called miRNA mimics, characterized with non-cleavable miRNA target transcripts [19, 20]. Constitutive expression of miRNA mimics leads to its overwhelming concentration in the cellular pool, sequestering the miRNA, in doing so , averts the miRNA-mediated de-regulation of original transcripts. In an alternative straightforward scheme, short non-coding RNAs are directed to downregulate the expression of miRNA or precursor miRNA itself. The approach holds much promise in plant genetic engineering by altering the expression of transcription factors as miRNAs are the ultimate regulators of gene expression and consequently the cellular growth and development [21].

Transacting siRNAs (tasiRNAs) are another class of endogenous siRNAs generated with the aid of miRNA-directed cleavage of TAS (Transacting siRNAs) transcripts and are peculiarly known for their functioning in *trans* [22–24]. Thus miRNA target recognition site is a minimal requirement for manipulating the biogenesis of artificial tasiRNAs. The artificial tasiRNAs offer several advantages over other non-coding RNA-based methodologies as generation of multiple functional artificial tasiRNAs from a single genetic construct each targeting different gene is feasible. The scheme allows for manifold targets to be modulated, an attribute suitable for complex metabolic engineering in plants. Furthermore, maneuvering the promoter designs in the artificial tasiRNA constructs, partial silencing is practicable and by directing the tasiRNA into spatio-temporal endogenous miRNA regulation, the exploitation of tasiRNA machinery to accommodate versatile gene silencing is also feasible [25].

Genetically modified (GM) crops obtained either through expression of foreign protein or expression of ncRNAs that have been approved for cultivation are the subject of prior environmental risk assessments (ERAs) through national regulatory agencies for their safe use. The environmental risk assessment (ERA) of GM crops conducted on a case-by-case basis consider any potential, unfavorable impacts the cultivation of modified crop could pose to the cultivator, soil biota, other beneficial insects, organisms in the whole receiving environment. The rationale is to identify any potential risk, the GM crop may pose and to mitigate its effects [26]. Thus, ERA entails integrating the probability and outcome of the exposure to arrive at necessary hypothesis and conclusions, on the principles and practices delineated in the international consensus documents, statutory regulations [27]. Herein, the review is an effort to provide a perspective on considerations while embracing ncRNA-based crop improvement strategies and the predictive ecological risk assessments (ERAs) that go alongside the technology.

Considerations on ncRNA-based Genetic Manipulation

The small non-coding RNA-based genetic engineering differs in modus operandi from earlier generation genetic alterations which are based on protein expression. The RNAi crops involve the repression of gene expression to achieve an advantageous crop phenotype, and hence the following considerations are worthwhile.

Mobile Signals

The RNA gene silencing mechanism has amplification potential as the ncRNA effectors–siRNAs in conjunction with host RNA-dependent RNA Polymerase (RdRP) further generates dsRNAs which in turn leads to build-up of secondary siRNAs resulting in silencing signal amplification [28]. The phenomenon is not only attributed with amplification potential but also with mobility of the signal, as the silencing message is transported to other cells and all through the plant system in due course of time [29]. It was also recognized that the siRNA duplexes function as silencing signals rather than AGO-1-bound siRNA single strands [30]. Though loci-specific induction of siRNA-mediated gene silencing is feasible, its spread to unintended tissues is inevitable; hence, tissue-specific silencing of gene expression is impracticable with siRNA-mediated RNAi [31, 32].

Viral Derived Suppressor Proteins (VSPs)

Since siRNA-mediated gene silencing is an ancient antiviral defense mechanism, plant infecting viruses have

evolved, viral genome-encoded suppressor of silencing proteins the biological role of which is to disrupt RNAi silencing process at various stages [33–36]. The suppressor proteins have different mode of actions among others like dsRNA binding [37], siRNA binding, and sequestration [38], inhibiting the activity of silencing machinery [39], thus preventing the spread of gene silencing signals [40]. Transgene derived hairpin RNA (hpRNA)-induced gene silencing phenomenon also shares the protein machinery with viral defense pathway, and hence the deliberate suppression of plants defense pathway by expression of VSPs leads to the repression of hpRNA pathway [41]. The prevalence of virus infection in plants and consequently the constant presence of non-selectively functioning viral derived suppressors of silencing attach a new dimension to the efficacy of gene silencing-based transgenics and deserve suitable consideration while formulating the ncRNA-based genetic manipulation. Accordingly, the target genes for generation of virus resistant transgenic plants are viral derived suppressor proteins in order to develop trait-stable transgenics.

In silico Analysis

Unlike the protein-based GM crops of yesteryears, the RNAi-induced trait incorporations are precisely based on nucleic acid sequence identity; hence, the role of bioinformatics analysis is imperative in identifying the risk impacts on potential non-target organisms. Computational investigations on the key parameters like threshold sequence identity, mandatory sequence length, base pair composition etc., indispensable for the effectual gene silencing are supposed to facilitate the identification and exclusion of impending target organisms in the vicinity of RNAi-based crops [42, 43].

Predictive ERA Considerations

In the light of concerns associated with ncRNA-mediated gene manipulation, predictive ERA has become an important component of the regulatory mechanism for GM crops in general and RNAi-based GM crops in particular. In this process, current knowledge and hypothesis drives the scientific research to arrive at potential risks the cultivation of non-coding RNA-mediated genetically modified crops possibly will pose and finding ways to mitigate them. It involves the logical steps like problem formulation which includes potential hazard identification, hazard characterization, identification of exposure pathways and characterization, risk management strategies, prediction of the severity of the harm, and expression of uncertainty. The stakeholders and regulatory authorities are familiar with the potential risks associated with the first generation GM crops that include increased invasiveness and volunteerism of the transgenic crop itself, intraspecific hybridization, interspecific hybridization [44], including the plant to plant gene flow, plant to micro organisms gene transfer, damage to non-target organisms [45], resistance management, impacts on cultivation, management and harvesting techniques, trait stability of the GM crop, effects on human, animal health, post market monitoring and other aspects of ERA that all have already been reviewed in a lucid manner elsewhere [27, 46, 47]. Besides the aforesaid risks, RNAi-derived GM crops pose other predictive ecological threats accompanied with knowledge gaps and assumptions that are as illustrated in instances of ncRNA modifications (Table 1). Off-target effects of the transgene, non-target effects on organisms ingesting GM plant parts, persistence of the small ncRNA molecules, effect of mutations in transgene on the crop and target gene/organisms, methods to detect the presence and persistence of ncRNAs, tracking the ncRNA-based crop and derived products are some of the risk issues associated with the RNAi-generated GM crops.

Secondary (Non-target/off-target) Effects

RNAi-mediated gene silencing functions on the principle of nucleotide sequence homology; consequently, it is anticipated that any transcript, hence the gene, which shares sequence homology with the effector ncRNAs, could become target of the silencing leading to inadvertent secondary effects [42, 43, 48, 49]. The non-target effect is more profound when the silencing is intended against conserved gene sequences to obtain broad spectrum resistance against pathogens/pests. In this case, the likelihood of gene sequence homology with the unintended beneficial microbes or insects is far high and consequently it may lead to non-deliberate devastation of the biodiversity. Insect resistance in maize by downregulation of V-ATPase gene of western corn root worm exhibited potentially adverse effects on non-target organisms sharing sequence homology with the target gene [10]. To circumvent such non-target effects, laboratory feeding experiments on likely non-target insects or search for the homologous gene sequences in the non-target organisms are quite valuable. dsRNA screening experiments deduced that ncRNAs originally intended to target western corn rootworm (WCR) effectively caused the silencing of other coleopterans like potato colorado beetle and southern corn rootworm (SCR) even though WCR and SCR shared 83 and 79 % sequence identity in vATPase A and vATPase E region, respectively [10]. A case in point is further exemplified in an endeavor to achieve reduced allergen P34 in

Table 1 Predictive Ecological Risk Assessment and knowledge gaps in ncRNA-based antiviral resistance and Allergen reduction in crops

S. no	ERA features	Virus resistant transgenics plants (VRTPs)		Allergen reduction in soybean by silencing host P34 expression	
		Predictive risks and assumptions	Knowledge gaps	Predictive risks and assumptions	Knowledge gaps
1	Secondary (Non-target/ off-target) effects	Adverse effects on the host transcripts, soil microbes, or human genes sharing sequence homology with the target viral gene	In silico data on sequence homology Routes of exposure and environmental fate of expressed dsRNAs, siRNAs, artificial microRNAs (amiRNAs)	P34 is homologous to Cysteine proteases (CP); hence its negative regulation may cause inadvertent effects on the CPs of beneficial insects feeding on ncRNA expressing seeds	Information on insects, predators, beneficial organisms feeding on seeds In silico data on sequence homology Toxicity testing on non-target beneficial organisms
2	Altered invasiveness or weediness	Virus resistance trait may confer invasiveness nature	Comparative agronomic and phenotypic performance of conventional and ncRNA-based VRTPs	Reduced allergenicity less likely to confer invasiveness however may reduce its inherent invasiveness	Comparative agronomic and phenotypic performance
3	Transgene introgression	Gene flow to related crop species, family of related viruses, or soil microbes As the resistance mechanism operates at post-transcriptional stage, no chance for virus protein trans-encapsidation	Biology and compatible wild relatives of the crop Role of ncRNAs in viral genome recombination Complementation effect of viral suppressor proteins and its role in counter defense of ncRNA silencing requires investigation	Gene flow to wild relatives or compatible species	Distribution of wild/ compatible species Information on pollination biology
4	Impact on pest and disease management strategies	Inadvertent disruption of host RNAi machinery due to overload from virus resistance Safe cultivation of virus resistance crops generated on the basis of protein expression	Screening for susceptibility to other diseases Studies on disease resurgence Previous experience on approved RNAi-based VRTPs Squash, Plum etc.	Inadvertent susceptibility to *Pseudomonas* as P34 is known to confer disease resistance P34 confers insecticidal property; hence its down regulation may lead to chance susceptibility to other insect pests	Screening for susceptibility to *Pseudomonas* and other insect pests
5	Impact on cultivation practices	Adverse changes in the cultivation practices	No change envisioned except for reduced use of insecticides for vector control Ecological/economic benefits accrual due to reduction in chemical spray	Changes in cultivation practices due to reduced allergen soybean	Biology and cultivation of soybean and product characterization of soybean
6	Food and feed safety	Presence of DNA and ncRNAs (dsRNA, siRNA, amiRNA) in edible part	Data on food/feed safety assessment History of safe consumption of virus infected plant parts, economic parts derived from virus resistant transgenic plants	Adverse effects on Oil derived for consumption and De-oiled cake for livestock feed Unfavorable changes in the metabolite composition of the GMO	Data on food/feed safety assessment Substantial equivalence study on overall nutrient and mineral metabolite composition

Table 1 continued

S. no	ERA features	Virus resistant transgenics plants (VRTPs)		Allergen reduction in soybean by silencinghost P34 expression	
		Predictive risks and assumptions	Knowledge gaps	Predictive risks and assumptions	Knowledge gaps
7	Effect of mutations	Breakdown of virus resistance Effects on related viruses infecting the crop Effect of altered siRNAs on the host transcriptome Molecular cross-talk with other cellular processes	Computational studies on sequence homology with related viruses as virus genome information is available in public domain Molecular cross-talk studies using microarray	Expression of allergen Probability of altered expression of transcript (s) of host or insect origin	Adverse effects on the developmental biology of insects infecting soybean Molecular cross-talk studies as soybean transcriptome is relatively well characterized
8	Detection of ncRNA-derived food products	Detection of siRNAs, amiRNA in food products	Discrimination of transgene-derived siRNAs and naturally occurring siRNAs due to host defense mechanism	Detection of siRNAs targeting P 34 transcript in soybean-derived food products.	Differentiation of host-derived small ncRNAs targeting P 34 and ncRNAs originating from transgene.
9	Environmental persistence of RNA	Persistence of various forms of ncRNAs in harvest produce and soil.	Degradation time to 50 % loss (DT_{50}) of small ncRNAs Environmental stability studies on perfect or imperfect hairpin RNA molecules	Persistence of dsRNA, siRNAs in seed, food and derived products	Degradation time to 50 % loss (DT_{50}) of dsRNAs Environmental stability studies on perfect hairpin RNA molecules

soybean [50]. Since P34, allergenic protein, is a kind of cysteine protease (CP) and shares sequence homology with the CPs of beneficial insects, any unintended silencing effect on the CPs of beneficial insects would have devastating implications in the receiving environment. Similarly, the inadvertent effects of silencing may affect the beneficial microorganisms in the vicinity of the ncRNA expression. For instance, root tissues expressing siRNAs targeting soybean cyst nematode may affect the micro organisms dwelling in the vicinity. However, studies have proven that virus resistant transgenics like *Papaya ring spot virus* (PRSV) resistant papaya have very little effect on the actinomycetes diversity and count in the soil [51]; similarly, no significant changes in the insect vector species were observed with the cultivation of Plum resistant to *Plum pox virus* (PPV) upon expression of viral derived coat protein gene [52]. Knowledge gaps regarding biology, species abundance of non-target beneficial insects, herbivores, soil organisms, birds, and other organisms are necessitated to be plugged. Comprehensive in silico data on sequence homology wherever the sequence information is available, routes of exposure, and environmental fate of ncRNA on the potential target organisms are mandatory. Nevertheless, the non-availability of the genomic resources for many of the plausible non-target organisms is an impediment. The stage of ncRNA expression in modified plant identifies potential crop growth stage which demands additional contemplation, in order to monitor changes in the species composition of the environment or off-target effects within the host. Subsequently, standard operating procedure to carry out toxicity testing on non-target beneficial organisms is obligatory once the inventory on probable insects, predators feeding on genetically modified crops is acquired. In this regard, the proposed modification in current ERA model to incorporate case-specific inclusion of test-species depending on their ecological functions in the receiving environment as against the standard testing species based on OECD guidelines is remarkable [53]. Equally significant are the ncRNA-triggered off-target effects on the host transcriptome which are attributed to the six or seven consecutive matches between the transgene-derived siRNA guide strand and off -target RNA [42, 43]. Pleiotropic effects of the off-target activity are found in the transformed tomato plants with short-hairpin RNAs targeting replicase protein of *Tomato leaf curl New Delhi virus* (ToLCNDV). Phenotypic abnormalities like needle-shaped leaves, radial rooting pattern, agravitropic roots, apical apoptosis, uneven leaf shape or curvature, decreased lateral rooting, shortened petiole, reduced stature etc. [54]. Similarly, in an effort to silence soybean myoinositol-1-phosphate synthase gene (GmMIPS1), besides the on-target effect of reduced phytate content, impaired development of seeds is also observed as off-target effect [55]. The off-targets effects can be diminished to a great extent by

screening for sequence homology between the transgene and the genome/transcriptome (wherever available) of the host crop intended to be modified [56, 57]. This way, the transgene comprising potent siRNAs cognate to the host genome, predicted in silico, can be precluded from incorporating it into the genetic constructs for plant transformation. With the other plant species, the off-target effects within host could possibly be studied only as a post-transformation evaluation of the transgenic. Apart from the sequence homology studies, characteristics like amenability and appropriateness of the target mRNA for ncRNA silencing is mandatory to augment the probability of on-target silencing.

Transgene Introgression and Invasiveness

In case of transgenics with selective advantage in the environment like insect or disease resistance trait, the transgene flow to wild relatives or compatible species converts them from being weeds to super weeds. Moreover, the plausibility of development of new routes of exposure of plant incorporated protectants (PIPs) to non-target organisms through such newly created super weeds cannot be completely disregarded. In the development of virus resistant transgenics, viral nucleic acids are expressed as dsRNA to confer the disease resistance. It is mandatory to explore the prospect of recombination between the transgene nucleic acid and naturally infecting viral genome. In an investigational set up, recombination between RNA of transgene-derived viral transcript and naturally infecting RNA viruses was demonstrated [58–62]; however, no empirical evidence up till now is observed in the field conditions. Further RNAi-based antiviral defense mechanism of host had been implicated in the RNA virus recombination in plants [63]. A review on the safety assessment studies of virus resistant transgenic plants operating on the principle of pathogen derived resistance (PDR)-viral protein expression-based strategy over past 15 years has revealed limited impacts on ERA issues such as heteroencapsidation, recombination, allergenicity etc. [64]. Therefore, it is rational to conclude that plants expressing viral derived genome sequences do not pose considerably novel or greater risks. However, ERA investigations on the virus resistant transgenics which are based on RNA silencing are little; hence, the knowledge gaps regarding the role of small ncRNAs in viral genome recombination, the complementation role of viral suppressor proteins, in an otherwise restrictive host, from two different viruses [65] are to be elucidated to assist the regulatory frameworks for the safe release of genetically modified antiviral plants. Integrating the robustness of RNAi and low temperature effectiveness of amiRNA silencing methodologies, an integrated RNAi–amiRNA (inRAM) strategy was attempted to contain the transgene flow via suppression of meiosis-critical gene [66]. The approach could provide for effective means of transgene containment in vegetatively propagated crops, but their application in field crops remains to be seen evidently with necessary amendments. Considerations on the invasiveness or weediness of the transgenic are necessitated to be investigated depending on the trait modified rather than the process of modification. Comparative analysis of conventional and ncRNA-based GM crop on agronomic, phenotypic performance, and germination studies provides information on the altered invasiveness or weedy nature of the modified crop.

Impact on Plant Protection Measures and Cultivation Practices

Inadvertent disruptions of host RNA silencing mechanism owing to saturation of RNAi machinery by constitutively expressing ncRNAs targeting various genes require to be tackled with reference to the plant protection measures. As the induced resistance mechanism utilizes the host defense machinery [41], investigations over any unintentional disruption of host defense capability is mandatory. Down-regulation of P34 allergenic protein expression in soybean, results in unintentional susceptibility to pathogen like *Pseudomonas*. Moreover, P34 is known to confer insecticidal property; hence, in all likelihood, its silencing may lead to inadvertent vulnerability to other insect pests. Hence, addressing the impact of ncRNAs on the plant protection measure ought to remain a major research priority with the knowledge gaps to be narrowed down by screening the GM crop for its susceptibility to other pest and diseases and on the emergence of secondary pests or disease resurgence etc. In this regard, the prior experience gained on cultivation of Virus resistant transgenic plants (VRTPs) like Plum [67] and Squash that are based on ncRNA-mediated resistant trait expression, would be supportive to frame ERA regulations. Similarly, adverse changes in the cultivation practices owing to ncRNA-based pest or disease resistance or altered metabolic profile in crops are also to be ascertained. In case of insect or pest resistant crops, no change is anticipated but for reduction in use of chemical pesticides conventionally used in the pest or insect vector control. However, in plants modified for improved phenotype through metabolic engineering, the information on biology of crop and product characterization is mandatory to arrive at an informed decision. Successful demonstration of crude dsRNA sprays in control of plant viral infection [68, 69], insect control [70, 71] appends another perspective to the ncRNA-based plant

protection measures, consequently to the cultivation practices.

Persistence of ncRNAs

Nucleic acids have been exempted from the tolerance levels on the persistence front, as they are widely being consumed without any demonstrable ill-effects. However, environmental fate of dsRNA, siRNAs, and miRNAs derived from RNAi-based plant incorporated protectants (PIPs) remains to be determined. The unintended toxicity effects of the *in planta* generated dsRNA and its transmission to insects through food have been well documented [10, 72, 73]. Besides the primary toxicity effect, the amplification of the toxicity is also demonstrated. Inside the insects body, dsRNA is further primed to produce secondary dsRNAs which are ultimately cleaved into siRNAs with unpredictable targets [10, 73]. The environmental fate of dsRNA in the receiving environments like soil, water and its effect on sensitive non-target organism required to be considered by determining the degradation time to 50 % loss (DT_{50}) for dsRNAs. The influence of hairpins and other secondary structures resulting from RNA sequence mismatches is not known relative to environmental stability and requires further investigation. The practical difficulty in assessing environmental outcome of RNAi effectors is that the precursor molecules, either perfect (generating siRNA) or imperfect hairpin (generates amiRNA) RNAs, are rapidly processed in vivo into small effector RNA molecules making their detection very difficult. However, the role of transgene derived hairpin RNA structures relative to environmental stability necessitates additional research. Surrogate molecules like plant infecting viroids that mimic the secondary structures of small RNAs are explored to study the environmental stability, and the initiative was redundant as viroids are circular molecules as against the open-ended small RNA effectors [74]. Hence suitable laboratory experiments are required to explore the models for studying the environmental stability of the RNA. The process of dsRNA transfer from various transgenic plants through food to insects and ultimately effecting gene silencing in animals has been patented by Commonwealth Scientific and Industrial Research Organization (CSIRO) which adds to the growing body of evidence for the chemical stability and biological activity of dsRNAs [75].

Food and Feed Safety

In view of the fact that the RNA silencing-based transgenic plants do not produce heterologous proteins, food and feed safety assessments requiring toxicity and allergenicity testing can be eliminated. However, the transgene DNA and its various transcript forms like dsRNA, siRNA, amiRNA, and atasiRNAs are essentially being analyzed for food and feed safety concerns. DNA has been an integral component of any human diet; consumption of DNA has been proven to be safe without any undesirable effects and it is also established that the transgenic DNA differs in no physical and intrinsic properties from the DNA already known to be present in the diet. Thus far feeding experiments have not detected the transgene derived DNA fractions in the vertebrate tissues [76]. Furthermore, it is also observed that no plant origin DNA or DNA from any source has been found to enter the vertebrate system through gastro intestinal tract leading to incorporations in their genomes. Thus it can be inferred that the RNAi-based transgene DNA can be safe for the human consumption. RNA present in the human diet, analogous to DNA, also has safe history of consumption. However, in vitro generated dsRNAs have been demonstrated to be taken up by organisms like nematodes and are found to be implicated in the horizontal RNA transfers to other soil dwelling organisms like bacteria. This creates a problem with ncRNA-based transgenics like nematode resistant soybean wherein expression of sRNAs in roots targeting soybean cyst nematode (SCN) could plausibly cause the transfer of gene to other microorganisms [77]. Small non-coding RNAs present in common human diet derived from crops like soybean, maize among others have been found to exhibit safe history of consumption regardless of the sequence homology they share with the human transcripts [78]. However, investigations are required to ascertain the same for transgene derived small RNAs as they are constitutively expressed hence are relatively abundant when compared with plant diet-derived small RNAs. Moreover, the observation that rice derived miRNA is found in mammalian extra-cellular system as an exogenous ncRNA and modulates the expression of mammalian liver low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA further warrants thorough investigation on the persistence of non-coding RNA. The revelation of not sheer cross-kingdom presence of ncRNAs but also their biologically active participation in gene suppression further emphasizes our deprived understanding on sRNA persistence and their mode of action [78, 79].

Nutritional Composition and Equivalency

Many instances of altered nutritional profile in crops have been attained with the aid of RNAi in recent times [80, 81]. Nutritional composition of the GM crops when compared to that of the conventional counterparts provides greater

insights into the not deliberate modifications the crop has undergone at the metabolites level. The principle of substantial equivalence proposes that should the GM crops be comparable to the non-modified conventional crops in terms of analytes studied, they are inferred to be safe for human food and livestock feed purposes. Any potential hazards observed in the GM crops could straightforwardly be corroborated with the altered composition of nutritional and anti-nutrient factors in the transgenic plant. Investigations on nutritional composition elsewhere have revealed no substantial alterations due to genetic modification, and the studies were predominantly on the GM crops conferring insect resistance or herbicide tolerance [82]. However, the crops modified through genetic engineering for altered metabolite levels are expected to show changes in the nutritional composition. Investigation on canola modified to express MUFA provides an example of food composition studies based on RNA gene silencing. It provides way forward in composition analysis by comparing traits of modified plant to the qualities of the plants, the compositional profile of which, is sought to be achieved through genetic modification. Consequently, herein modified canola is compared for compositional analysis with olive oil which the genetic modification sought to replace, rather than with isogenic line or non-transformed variety. The use of transcriptome study is rational in ncRNA-based transgenics for analyzing the food and other compositional profiles of the modified plant in the wake of revelation that mutagenesis causes more transcriptome changes than the transgenic protein expression in rice [83]. However, use of molecular profiling studies involving transcriptome, proteome, and metabolome in comparing the compositional changes of GM and non-GM crop is premature as these global investigations entail robust platforms to analyze, sift, and prepare the database; for this reason, the comparator data are relatively few. However, with the advent and potential utilization of ncRNAs in crop genetic modification omics utility, particularly, the use of transcriptome studies, is a pre-requisite and would form an integral part of ERA considerations sooner [84].

Effect of Mutations

As the entire RNA silencing process is sequence-dependent, it is conceivable to consider the effect of mutations on the efficacy of non-coding RNAs-mediated silencing approach. The number of scenarios are foreseen with respect to the ncRNA-mediated gene silencing viz., (i) any mutation or polymorphic changes in the nucleotides sequence of the transgene could lead to the breakdown of silencing effect in the genetically modified plant; additionally, off-targets silencing effects of such mutated transgenes cannot be completely disregarded (ii) On the other hand, mutations in the target genes especially of plant pathogen or pest origin not only leaves the silencing system ineffectual but also results in development of resurgent pests and diseases which by then develop into a predicament beyond the management of plant protection measures. Single or double mutation with loss or retention of suppression activity has been well documented in siRNA studies [85, 86]. Hairpin design strategies targeting the mutation or allele-specific silencing of human disorder genes are deployed as a viable therapeutic alternative to discourage mutational effects [87–89]. The flexibility of such effective design must be explored in the context of ncRNAs application in crop improvement. The prospect of breakdown of miRNA-based silencing is moderately high when compared to siRNA-mediated silencing because a point mutation in the target transcript is more than adequate to impair the specificity of miRNA-based transgene-induced silencing. Virus escape studies in an amiRNA-based virus resistant plant identified every-site variants of the virus genome under the pressure of ncRNA resistance. The molecular dynamics for the viral genome evolution in response to amiRNA silencing is identified to be a mix of mutation, drift, and selection [90]. Many other predictive effects are envisioned with the accumulated mutations in ncRNA inducing transgene with plausible adverse effects on beneficial insects or other organisms, off-target effects on the host transcriptome, effects on other viruses infecting the crop in case of VRTPs, aberrant ncRNAs in molecular cross-talk with other cellular processes etc. Hence in silico studies on sequence homology with apparently possible insects, viruses, organisms in the environs of the modified crop wherever the information is available is mandatory. Similarly, employing global expression investigatory strategies like microarray in delineating any probable molecular cross-talk, or adverse effects on the developmental biology of host etc. is advisable in analyzing the effect of mutations in ncRNA-based transgenics.

Detection of ncRNA Derived Food Products

Detection of the movement of RNAi-based genetically modified crops is imperative for technology suppliers, producers, cultivators, consumers, regulatory agencies alike for effective monitoring, tracking, and labeling of products to meet any untoward eventualities arising out of the technology [91, 92]. Unlike the transgenics expressing heterologous proteins which are generally identified based on serologic methods, advanced nucleic acid detection methods are required for the detection of transgene derived small non-coding RNAs in food products. However, effective PCR, ELISA, and LFD-based detection of marker

or reporter genes is standardized in a cost effective manner to circumvent the issue, but even then the question of specificity and discrimination among GM crops needs to be addressed. Given that the genetic modifications function at the nucleic acid level in gene expression pathways, it is inevitable that simple, cost-effective lateral flow devices (LFDs)/test strips or ELISA widely employed for the detection of protein-based modifications in the GM crops become futile. The system warrants nucleic acid detection-based alternative DNA amplification methodologies [93]. In an advancement of GM detection, bioluminescent real time reporter (BART) technology and loop-mediated isothermal amplification (LAMP) (BART-LAMP) procedure which allows for detection of 0.1 % GM maize contamination even in field condition are worth mentioning [94]. An ideal detection technique for RNAi-derived products should be nucleic acid-based, with high specificity, low detection limit (as 21 nt long ncRNAs are effector molecules) in a cost-effective manner. The discrimination of transgene derived siRNAs and naturally occurring ncRNAs in the plant due to plant's antiviral defense mechanism or ncRNAs present naturally in the system to downregulate particular gene of interest.

NGS and ERA

The Environmental risk assessment scenario is rapidly changing with the introduction of high-throughput DNA sequencing technologies called as Next Generation Sequencing (NGS). Particularly the effectiveness of NGS in new biological inquiries like whole transcriptome studies and epigenome analysis, transcripts quantification and characterization of ecological diversity, etc. is noteworthy for its potential application in ERA of ncRNA-based transgenics. Understanding the transcriptome of the ncRNA-modified plant is essential for delineating the cellular functions of the modified genome and help appreciating the plant development in the context of ncRNA modification. NGS in Arabidopsis and genomes elsewhere has already demonstrated the presence of hitherto unknown transcripts and novel splicing variants adding to the complexity of organisms' transcriptome repertoire. In the background of ncRNA-mediated gene regulatory modifications, even vast transcriptome changes are envisioned. The potential of NGS in decoding novel small RNAs and profiling stage/tissue-specific sRNA inventory appends to its utility in the context of ncRNA-based genetic manipulation [95, 96]. The utility of NGS is effectively demonstrated in screening RNAi targets regions of insect *Ostrinia furnalalis* and thus in the selection of candidate target genes for efficient ncRNA-based pest control systems [71]. Another area of NGS application is to investigate on the epigenome modifications arising out of ncRNA expression [97]. Genome level studies on the methylome, other histone modifications, chromatin packaging, identification of transcription factor binding sites etc., when co-related with the gene expression analysis offer profound opportunities to enhance our understanding on the spatio-temporal aspects of ncRNA-mediated gene regulation [98] in GM crop and their conventional counterpart. Characterization of microbial biodiversity in the vicinity of GM crop is of particular interest to explore any unintended real time changes in the species diversity due to the exudes of modified crop expressing ncRNAs. Besides the species richness, direct RNA isolation coupled with NGS sequencing would help in decoding the metabolic pathways that constitute the environment when compared to control environment. In a recent approach called as NexGen Sequencing and Junction Sequence Analysis (NGS-JSA) Bioinformatics, successful amalgamation of speed and simplicity of NGS coupled with the comprehensiveness of in silico analysis was demonstrated in characterizing the transgenic event of soybean [99]. It precludes the role of Southern blotting technique and consequently, the use of radioactive materials in analyzing the copy number of transgene in a GM crop. In an endeavor to determine the genome variations of an RNA virus targeted under ami-RNA silencing, Illumina deep sequencing had assisted not only in identifying the very low frequency virus genome variations but also temporal deviations in the virus genome [90]. NGS alone or in combination with other techniques provides a viable alternative for GM characterization and aids in resolving the key challenges encountered in environmental risk assessments procedures in the wake of ncRNA application in crop improvement.

ERA Regulatory Frameworks of Different Countries

Risk assessment frameworks for the regulation of living modified organisms in countries representing various geographic regions, socio-economic developmental stages reveal that the regulatory framework of most of the countries differs considerably with the Annex III of the Cartagena Protocol [100]. It is because of the varied nature of their protection goals and socio-economic considerations, and hence the outcome of ERA in these countries differs to great extent. However, the evaluation methodologies, various states generally follow are based on sound scientific principles. The trigger of the risk assessment process is generally agreed upon that whether the organism has been genetically modified i.e., process based approach; however, Canada's approach seeks novel trait in the organism regardless of the process of generation of the product. In the context of emerging viewpoints negating the inherent

risky nature of genetic modification, it is noteworthy that the trigger for the risk assessment should move toward 'product-based' approach from 'process-based', one in vogue in most of the countries. Regulatory agenda in USA does not consider whether the gene product has been part of human or animal diet in consonance with the Cartagena protocol. In USA, the potential food safety risk is taken care of by the FDA which is involved in the food safety standards including foods derived from transgenic crops. Nucleic acids are considered to be potentially safe by US Environmental Protection Agency (USEPA) as they are consumed for long time without any verifiable ill-effects; however, the EPA is deciding the course of action and preparing the guidelines and requisite tests to be performed to determine the DT_{50} (i.e., degradation time to 50 % loss) for dsRNA [74]. The regulatory framework of countries like Australia, Canada, UK, Japan, and USA revealed the mandatory data requirement for direct or indirect effect of gene product which affects metabolism, growth, development or reproduction of plants, animals, or microbes along with potential behavioral and physiological effects on the non-target organisms. Thus, the national regulatory framework provides for thorough examination of potential direct or indirect non-target effects arising not only from protein/toxin expression but also provides window for evaluation of ncRNAs causing such adverse effects. Another area of debate is the application of precautionary principle in the circumstance of insufficient scientific data or consensus to the risk management procedures. Regulatory framework in countries like Australia, Germany etc. have explicitly mentioned the use of precautionary approach as the absence of scientific information should not refrain the national authorities from preventing the environmental damage. Since global status of commercialization of GM crops indicates that biotech crops based on expression of heterologous proteins occupy more than 99 % of the area under GM crops [101], the advent and spread of ncRNA-based GM crops invite the attention of ERA regulators to consider the use of precautionary principle on sound scientific footing.

Conclusions

Regulatory mechanisms for the approval and conduct of ERA of GM crops based on the activity of ncRNAs call for a comprehensive transformation because the ERA procedures that are in vogue for more than past 20 years cater to the requirement of genetically modified crops based on archetypal protein expression. The potential and tangible realizations of ncRNA-mediated genetic engineering have made rapid strides. The RNA-based modulations were initially dominated by development of virus resistant transgenic plants, nevertheless, other spheres of influence like incorporation of resistance against insect pests and pathogens likewise in the development of nutritionally superior genotypes. The frontier areas of research in plant small RNAome (sRNAome) and novel RNA-based silencing platforms not only offer optimism for enhanced global food supply but also lend hand in assessing the validity of research hypothesis as an advanced functional genomics tools. The achievements and the impending possibilities of ncRNA-based genetic modifications in crops warrant sound ERA measures. The necessity is more so emphasized with our current deprived state of understanding on the sRNA-based gene silencing especially on the frontiers of ncRNAs persistence, off-target effects, non-target effects on beneficial organisms, role of RNAi food derived sRNA species on human metabolism, nature of RNAi silencing machinery in crops other than model plants so that we should not lose sight on the process of translating the research findings from model plants into cultivable crops. Even though the current paradigm of transgenic plants safety assessment is adequate to address the concerns cropping up due to ncRNA-based gene manipulations, embracing alternative tests like the reliability on bioinformatics approach addresses risk questions with regard to the off-target or non-target effects of ncRNAs. Environmental fate of transgene derived various forms of ncRNAs is an intense area of research to facilitate an informed decision on exposure analysis of target and non-target organisms. The experts while finalizing the ERA problem formulations pertaining to RNAi-based genetic engineering are required to discuss and identify new or additional risk hypothesis so that they are equipped with scientific response to a new scenario. The major goal of the research is to obtain information that would help the regulatory frameworks across various countries to take decisions based on the scientific principles to assist in the safe release of transgenics.

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