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RNAi mediated gene silencing in crop improvement for quality food and environment

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ABSTRACT

RNA interference (RNAi) is an effective tool for gene silencing and a promising gene regulatory approach in functional genomics that has a significant impact on crop improvement which permits down-regulation in the gene expression with a greater precise manner without affecting the expression of other genes. RNAi gene silencing mechanism is expedited by small molecules of interfering RNA to suppress a gene of interest effectively. It has several advantages over traditional transgenic approaches as genetically modified RNAi plants do not contain transgene proteins. This novel approach has witnessed a variety of potential applications in agriculture for crop improvement, including the development of disease-resistant, abiotic or biotic stress tolerant, and high-yielding elite varieties, nutritional quality improvement, removal of allergens and toxins, delayed ripening of fruit and vegetables for longer storage period and enhancement of secondary products. This revolutionary technology could be further exploited to open many new avenues in the development of eco-friendly biotech approaches for crop protection and improvement. There is a great need to strengthen research and development activities in this promising area for proper, effective, and safe utilization of these tools as sustainable solutions for crop improvement.

Key words: Crop improvement, disease resistance, functional genomics, gene silencing, nutritional quality, RNA interference

INTRODUCTION

Gene silencing phenomenon can occur either through repression of transcription, termed transcriptional gene silencing (TGS), or through mRNA degradation, termed post-transcriptional gene silencing (PTGS). TGS results in the reduction of transcription whereas PTGS results in sequence-specific mRNA degradation in the cytoplasm without dramatic changes in the transcription of the corresponding gene in the nucleus. Both TGS and PTGS are used to regulate the endogenous genes. Interestingly, mechanisms of the gene silencing also protect the organism's genome from transposons, and viruses (Angaji et al., 2010). Andrew Fire and Craig Mello have received Nobel Prize in Physiology/Medicine in 2006 for this important breakthrough discovery of RNA interference-mediated gene silencing in 1998 (Fire et al., 1998). RNAi-mediated gene silencing has proven to be novel and a potential reverse genetics tool for functional genomics to decipher the function of genes through genome-wide screening in different eukaryotic organisms, including plants (McGinnis, 2010). It is a powerful strategy for silencing genes for the genetic improvement of several desirable plant characteristics including biotic and abiotic stress tolerance (Saurabh et al., 2014). The pesticides

which are deliberately used in agricultural practices for protection of the crops from diseases and pests are highly toxic thus create potential risk to the environment and human health. It has been generally accepted that the world population is expected to exceed nine billion people by 2050. Supplying this population with safe, secure, and healthy food will challenge the world's agricultural resources and production systems (Kebreab, 2015). In order to meet the projected demand for food production, we have to adopt various promising technologies like RNA interference (RNAi)-based strategies for the improvement of crop yield and quality by protecting crop plants against biotic (pathogens, insects and nematodes) and abiotic (salinity, drought, etc.) stresses, by eliminating/reducing toxins and allergens in plant food materials.

RNAi AND GENE SILENCING MECHANISM

RNA interference is a highly sequence-specific gene silencing mediated by double-stranded RNA (dsRNA). The dsRNA can trigger the RNAi pathway. RNAi pathways are evolutionary conserved across eukaryotic systems and are involved in defense against viruses, transposons, and transgenes, and regulation of genes associated with plant developmental processes and stress responses (Mamta and Rajam, 2017). Two types of RNA interference (RNAi) can be involved in plants: small interfering RNA (siRNA) and microRNA (miRNA). The miRNA is single-stranded (Bartel, 2004; Carthew and Sontheimer, 2009), whereas the siRNA functions as a small dsRNA (21-27 nt) produced from cleavage of a larger double-stranded RNA (dsRNA), designated as a precursor (Nakahara and Carthew, 2004). The precursor dsRNA is cleaved to produce the effectors small RNAs (sRNAs) of 21-24nt size by RNAase III enzyme Dicer. The sRNAs include small interfering RNAs (siRNAs) and/or micro RNAs (miRNAs). These sRNAs are then involved in a multiprotein complex known as RNA Induced Silencing Complex (RISC) in which Argonaut protein is present and it degrades the passenger (sense) strand of sRNAs. Then, the activated RISC along with the guide (antisense) strand of sRNAs can find the cognate target messenger RNA based on the complementary sequence and degrade or repress the translation based on the extent of sequence similarity as a result there is no protein (Fig. 1).

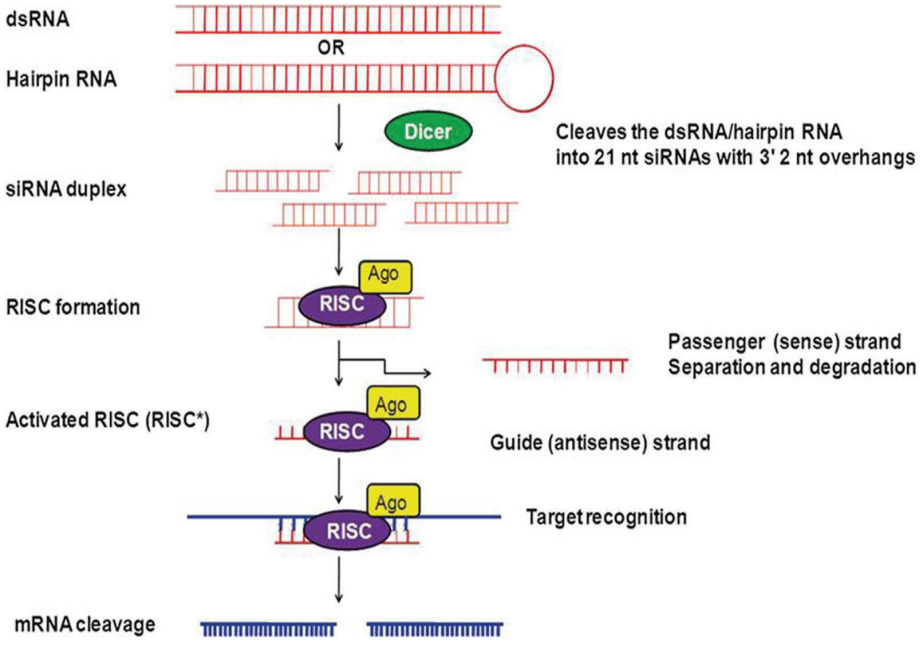

Fig. 1. Biogenesis and mechanism of action of siRNAs for gene silencing (Yogindran and Rajam, 2015)

The siRNA pathway is induced by dsRNA formed due to viruses, transposons, or introduced transgenes, and is involved in defense against such invasive nucleic acids. On the other hand, miRNAs are generated from endogenous genes, which are mostly transcribed by the RNA polymerase II (rarely RNA polymerase III) to produce the primary miRNA (primiRNA), which is then processed into precursor miRNA (pre-miRNA) by Dicer like-1 (DCL1). The pre-miRNA is then cleaved again by DCL1 to form miRNA/miRNA* duplex, which is transported to the cytoplasm by an exportin-like protein. The miRNA duplex is then transferred to RISC, where the passenger strand is selectively degraded by argonaut protein, and the single-stranded mature miRNA (guide strand) along with RISC is involved in the degradation or translational repression of the target mRNA (Rajam, 2020), presented in Fig. 2.

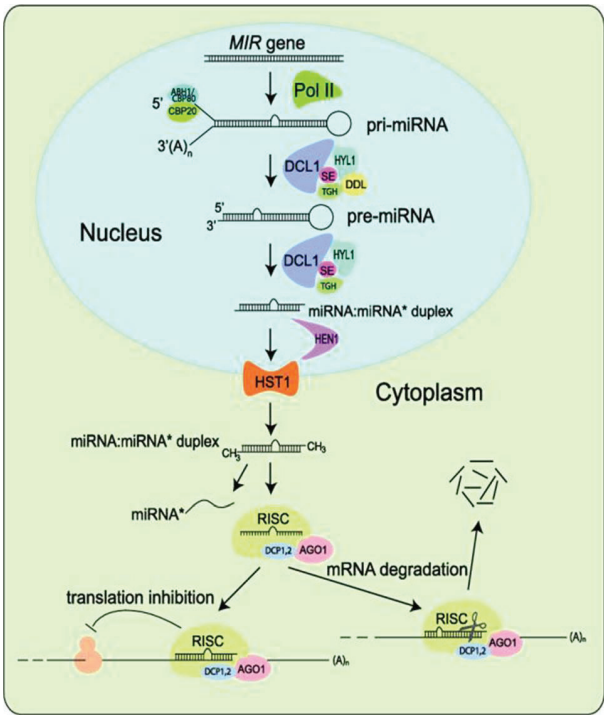

Fig. 2. miRNA biogenesis pathway in plants: miRNA genes are transcribed by RNA Polymerase II into pri-miRNAs that fold back to form hairpin structure.

Splicing and further processing in nuclear involve the interactive functions of HYL1, DDL, TGH and SE and of the cap-binding proteins CBP20 and CBP80. Pri-miRNAs and pre-miRNAs are sequentially processed by DCL1 to yield one or several phased miRNA/ miRNA* duplexes, which are methylated by HEN1 and transported to the cytoplasm by HST1. The miRNA is selected and incorporated into dedicated AGO1-containing RISC that directs cleavage or translation inhibition of the target mRNA transcript. (Yang and Li, 2012)

RNAi IN CROP IMPROVEMENT

RNAi for the development of plant disease resistance

Plant breeders have adopted different approaches for developing pathogen-resistant genotypes are tedious and time-consuming but in the last decade, RNAi-induced gene silencing emerged as an effective tool to create resistance against many diseases of economic importance caused by bacteria, fungi, and viruses and opened new avenues in the development of eco-friendly techniques for plant improvement as specific genes are suppressed which cause disease. In the RNAi approach, double-stranded RNA (dsRNA) acts as an igniter in the RNA interference and activates the homologous mRNAs to inhibit its translation and transcription to silence the susceptible genes (Almeida and Allshire, 2005). Host gene silencing-hairpin RNAi (HGS-hpRNAi) is reported as a more stable gene silencing method that can be employed to increase fungal and bacterial disease resistance by changing the gene expression against pathogens through genetic engineering in the host plant (Senthil-Kumar and Mysore, 2010). In rice, RNAi can knockdown OsSSI2 (OsSSI2-kd) that cause increased resistance against the bacterial pathogen (*Xanthomonas oryzae* pv. *oryzae*) of leaf blight which is one of the most destructive diseases of rice that causes a significant yield loss (Jiang et al., 2009; Das et al., 2021). The siRNAs proved effective against the crown gall disease in *Nicotiana* and *Lycopersicum* species caused by a pathogen *Agrobacterium tumefaciens* by the transformation of inverted repeats of this pathogen genes ipt and iaaM to encode precursors of biosynthesis for auxin and cytokinin (Escobar et al., 2001). Gene silencing can be obtained by a host-induced gene (Avra10), which results in limited fungal disease attached in

wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) through a transient gene expression resistant to RNAi because of the silent point mutations which suggest that the transfer of RNA from the host plant to the fungal pathogen *Blumeria graminis*, leads to RNAi-based plant protection. RNA silencing also employs as a natural antiviral defense mechanism to develop resistance in plants against viral diseases by virus-induced gene silencing (Ding, 2010). RNA silencing hosts target protein translation and process the virus-mediated dsRNA, which results by pathogen replication into virus-mediated siRNAs (vsiRNAs). These vsiRNAs then target and suppress gene expression and protein translation in the virus genes. For stabilization of the defense system, the virus encodes a 'viral suppressor of RNA silencing protein', that has been identified and isolated from various plant viruses (Ding and Voinnet, 2007). Bayer Crop Science has acquired a worldwide license to develop, market, and sell selected crop plant varieties in which the RNAi technology has been successfully applied by the CSIRO scientists. Using this technique this group has developed varieties of barley that are resistant to BYDV (barley yellow dwarf virus) (Wang et al., 2000). Domínguez et al. (2000) have developed transgenic citrus plants expressing the coat protein gene of the *Citrus tristeza* virus. Scorza et al. (2001) revealed for the first time RNAi's role in virus resistance in woody perennial species and produced Plum pox virus (PPV) resistant genotypes containing the PPV coat protein gene. Viss et al. (2003) successfully developed crown-gall-resistant transgenic apple trees that silence *Agrobacterium tumefaciens* oncogenes (iaaM, iaaH, and ipt). Another success is the silencing of multiple genes in the apple scab fungus *Venturia inaequalis* reported by Fitxgerald et al. (2004). The development of Papaya variety resistance to papaya ringspot virus (PRSV) by this technology is a promising example that reflects the success of RNAi (Gonsalves, 2006). In the last decade, many scientists reported the successful application of this technology in different crops for the development of resistance to different pathogens (Table 1).

Table 1. Use of RNAi technology in various crop plants against different pathogens

Pathogens	Targeted genes	Effects	Crop	References
Blumeria graminis	Virulence effector (Avra10)	Reduced fungal development	Wheat	Nowara et al., 2010
Puccinia striiformis f. sp. Tritici	Calcineurin homologue (PsCNA1, PsCNB1)	Slower extension of fungal hyphae	Wheat	Yin et al., 2010
	Mitogen-activated protein kinase (MAPK1), Cyclophilin (CYC1), Calcineurin regulatory subunit (CNB)	Reduced virulence	Wheat	Panwar et al., 2013
B. graminis f. sp. hordei	Ribonuclease-like protein, Virulence effector, Glucanase, Metalloprotease	Reduced virulence	Barley	Pliego et al., 2013
Fusarium graminearum	Cytochrome P450 lanosterol C-14a-demethylase (CYP51	Inhibition of fungal growth	Wheat	Koch et al., 2016
Verticillium wilt	Ave1, Sge1, and NLP1	Reduced virulence	Tomato	Song & Thomma, 2016
Puccinia striiformis f. sp. tritici	Tritici Protein kinase, A catalytic subunit (PsCPK1)	Reduced virulence	Wheat	Qi et al., 2018
	Glycine-serine-rich effector (PstGSRE1)	Reduced virulence and increased H_2O_2 accumulation	Wheat	Qi et al., 2019

RNAi for the development of plant pest resistance

Although plant breeders have developed various insect and nematode resistant cultivars, but these approaches are tedious and time consuming. The common practice of using pesticides to control pests creates many adverse effects on human health and the environment. The transgenic approach although effective and replaced chemical insecticides in many crops, but this approach is limited for some specific crops to manage some specific pests, and there is also a threat that some insects can develop resistance (Gordon and Waterhouse, 2007). RNAi offers robust and more selective pathway for combating various destructive insects and nematodes that cause significant economic losses in agriculture. Baum et al. (2007) demonstrated in western corn rootworm (WCR) ingestion of dsRNAs provided in the diet can trigger the RNAi which results in stunted larva growth and their death. They further confirmed that transgenic corn plants which are engineered for WCR dsRNAs expression exhibited a substantial reduction in insect damage that suggests RNAi pathway is effective and can be exploited further to control coleopteran insects. Mao et al. (2007) reported a new strategy for plant-mediated herbivorous insects RNAi, which describes the suppression of a critical insect gene through insect feeding on plant engineered to develop a specific dsRNA that can prompt dissection of gene functions in these insects. They further reported that the herbivorous insect RNAi efficiency can be stimulated by ingestion of transgenic dsRNA-producing plants that are gene-specific and proved effective against cotton bollworms (*Helicoverpa armigera*) damage. A gene 'CYP6AE14' was identified in *Helicoverpa armigera*. After feeding on plant material exhibiting dsRNA specific to gene 'CYP6AE14', the effect of the transcript decreased in the midgut, and larva growth was also retarded. The gene silencing of 'glutathione-S-transferase' (GST1) can trigger the RNAi process when herbivorous insects feed on plant material expressing dsRNA. RNAi-mediated death of whitefly via oral dissemination of dsRNA, targets ADP/ATP, translocase, a-tubulin, ribosomal protein L9 actin ortholog, and v-ATPase A genes responsible for insect mortality (Upadhyay et. al., 2011). RNA Interference in the expression of the targeted genes results in various phenotype disturbances viz. Stunted growth, moulting defects, and insect mortality (Thakur et al., 2014).

RNAi approach has appeared as a novel tool to control plant parasitic nematodes and has been exploited in plants to develop resistance against nematodes (Fire et al., 1998; Graham et al., 2010). The delivery of dsRNAs from plant to nematode occurs by the ingestion process of plant cytoplasm and it accelerates the RNAi after its entry into the nematode body that results in inactivation of targeted genes through dsRNA (Gheysen and Vanholme, 2007). There are so many successful examples (Table 2) that proved its effectiveness against different insects and pests which have opened new pathways for crop protection by developing pest-resistant cultivars of crop plants. However, the success of the RNAi approach to control notorious pests mainly depends on the proper screening system for target gene selection and an appropriate delivery mechanism (Wang et al., 2011).

RNAi for abiotic stress tolerance

The function of miRNAs (microRNA) in relation to abiotic stress like oxidative stress, cold, drought, and salinity were reported by Sunkar and Zhu (2004) in Arabidopsis plants under various abiotic stress. they also confirmed miR393 was sturdily up regulated when exposed to higher salinity levels, dehydration, cold, and abscisic acid (ABA). Additionally, miR402, miR319c, miR397b, and miR389a were controlled by abiotic stress under varying levels in Arabidopsis. RNAi technology may be a substitute for complex molecular techniques because of containing several benefits: its specificity and sequence-based gene silencing. This ability of RNAi has been efficaciously utilized for incorporating desired traits for abiotic stress tolerance in various plant species (Jagtap et al., 2011).

Table 2. Use of RNAi technology in various plant species against different pests

Pests	Targeted genes	Effects	Crops	References
Insects				
Sitobion avenae	Catalase gene CAT	Mortality	wheat	Deng and Zhao, 2014
	Salivary sheath protein	Mortality/ fecundity/ transgenetional gene silencing	wheat	Abdellatef et al., 2015
	Acetylcholinesterase gene SaAce1	Susceptibility/ fecundity	wheat	Xiao et al., 2015
	Secreted salivary peptide DSR32, salivary protein DSR33, serine protease 1 DSR48	Mortality	wheat	Wang et al., 2015
Sitobion avenae	Olfactory coreceptor gene SaveOrco	Impaired response	wheat	Fan et al., 2015
	Lipase maturation factor 2-like gene	Mortality/ fecundity	wheat	Xu et al., 2017
	Laccase 1 (Lac1) Feeding	Mortality	wheat	Zhang et al., 2018
	Zinc finger protein (SaZFP)	Mortality	wheat	Sun et al., 2019
	Ecdysone receptor (EcR) and ultraspiracle	Mortality/ fecundity	wheat	Yan et al., 2016
Nilaparvata lugens	NlHT1, Nlcar, Nltry	Knockdown of midgut genes	rice	Zha et al., 2011
Diabrotica virgifera virgifera LeConte	Genes encoding proteins	Control of coleopteran insect pests	maize	Baum et al., 2007
Bemisia tabaci	v-ATPaseA	Enhanced whitefly resistance	tobacco	Thakur et al., 2014
Helicoverpa armigera	Cytochrome P450 gene (CYP6AE14)	Develop resistance to bollworm	cotton	Mao et al., 2007
Myzus persicae	Rack1, M. persicae C002 (MpC002)	Develop aphid resistance	tobacco	Pitino et al., 2011
Nematodes				
Meloidogyne incognita	Heat-shock protein 90, isocitrate lyase, Mi-cpl-1	Reduced reproduction	wheat	Lilley et al., 2007
Pratylenchus spp.	Troponin C (pat-10) Calponin (unc-87)	Reduced reproduction	wheat	Tan et al., 2013

Among the genetically engineered plants, the rice exhibited gene expression of RACK1 inhibition caused by RNAi, which explained the potential role of RACK1 in drought stress conditions in rice crops. The transgenic rice was observed with a superior level of tolerance in contrast to non-transgenic rice plants (Jian et al., 2010). Analysis of miRNAs and genome sequencing profiling were executed in drought-studied rice at a various range of growth stages utilizing a microarray platform and 16 miRNAs (miR1126, miR1050, miR1035, miR1030, miR896, miR529, miR408, miR156, miR171, miR170, miR168, miR159, miR397, miR396, miR319, miR172 and miRNA1088) were remarkably involved in down-regulation in response to drought stress (Liu et al., 2008) whereas 14 miRNAs (miR1125, miR159, miR903, miR169, miR901, miR171, miR896, miR319, miR395, miR854, miR851, miR474, miR845, and miRNA1026) were found in up-regulation under drought stress and few miRNA gene families, like miR319, miR896, and miR171 were recognized as both up-and down-regulated groups (Zhou et al., 2010). Upregulation throughout drought stress in maize crop has been studied by Wei et al. (2009) with miR474, which interact with proline dehydrogenase. In recent studies, miRNA-expressing patterns of drought tolerance wild emmer wheat in relation to drought stress were explored by utilizing a plant miRNA microarray platform (Kantar et al., 2010). The miRNA expression has been studied in *Phaseolus vulgaris* and *Brachypodium* spp. for cold stress (Arenas-Huertero et al., 2009; Zhang et al., 2009). In wheat, 32 families of miRNA were distinguished, among them 9 identified miRNAs were supposed heat responsive. The miR172 was distinctly decreased, while miRNAs including (miR827, miR156, miR169, miR159, miR168, miR160, miR166, and miR393) were noticed with up-regulation in response to heat stress (Xin et al., 2010).

Now a days, soil salinity is designated a serious threat to agricultural production. Genetic techniques are presently being used to enhance tolerance against salinity with the help of using breeding methods, induced genetic variations, marker-assisted selection, functional genomics, bioinformatics, and either through selection in stressed environments or via QTLs mapping. In *P. vulgaris*, it was reported that increment in the accumulation of miR159.2 and miRS1 with the addition of NaCl (Arenas-Huertero et al., 2009). In *P. trichocarpa*, miR171l-n, miR530a, miR1446a-e, miR1445, and miR1447 were down regulated; on the other hand, miR1450 and miR482.2 were up-regulated in salt stress period (Lu et al., 2008).

RNAi for the crop quality improvement

Workers on this crop were able to reduce the level of glutenin and produced a rice variety called LGC-1 (low glutenin content 1) by applying RNAi to improve rice plants (Kusaba et al., 2003). RNAi technology has been successfully used to generate a dominant high-lysine maize variant by knocking out the expression of the 22-kD maize zein storage protein, a protein that is poor in Lysine content (Segal et al., 2003). Tang et al. (2004) developed quality and normal maize seeds with high levels of lysine-rich proteins by RNA interference. The RNAi silencing technology can be used to silence the gene(s) responsible for the production of the neurotoxin in Lathyrus sativus called β-oxalylaminoalanine-L-alanine (BOAA) (Williams et al., 2004). RNAi-mediated suppression of the gene DET1 (DE-ETHIOLATED-1) expression under fruit-specific promoters has been shown to improve carotenoid and flavonoid levels in tomato fruits with minimal effects on plant growth and other fruit quality parameters (Davuluri et al., 2005). The RNAi-induced silencing technology has enabled the creation of varieties of Coffee that produces natural coffee with low or very low caffeine content, thus by-pass the need of extraction (Van Uyen, 2006). A RNAi constructs designed by Regina et al. (2006) to silence the genes encoding the two starch branching isozymes of amylopectin synthesis, were expressed under a seed-specific promoter in wheat which resulted in increased grain amylose content to over 70% of the total starch content (Tang et al., 2007). The efficiency of RNAi and the targeted virus RNA

was significantly highlighted in plums (Scorza et al., 2013) and other fruit. In 'Arctic' apple, four genes are silenced that control polyphenol oxidase (PPO) production (Armstrong and Lane, 2009), which causes the production of brown melanin due to oxidation following fruit damage.

CHALLENGES AND FUTURE PROSPECTUS OF RNAi IN CROP PLANTS

While the perspective of using RNAi in agriculture for crop genetic improvement and protection appears to be promising, several challenges need to be resolved for efficient practical applications.

Stability issues of dsRNA

Microorganisms in the environment can degrade dsRNA prior to their uptake by pathogens or pests. Rapid degradation of dsRNA may occur by nucleases in the saliva, gut lumen, and/or haemolymph of pests as well (Allen and Walker, 2012; Katoch and Thakur, 2012; Chung et al., 2018). The high or low pH found in the gut lumens of some pests can also reduce dsRNA stability either directly or indirectly by affecting the activity of gut nucleases (Cooper et al., 2019). Several researchers show that dsRNA is degraded to undetectable levels within 48 h after their application on three types of soil (silt loam, loamy sand, and clay loam) and within 7 days after their addition to aquatic systems containing natural water and various types of sediment (Albright et al., 2017; Fischer et al., 2017). Therefore, dissecting the process of dsRNA degradation is helpful in evaluating the potential effect of dsRNA in various environments target organisms and in the selection of cost-effective methods for dsRNA production.

Costly production of the required amount of dsRNA

The traditional dsRNA production method in the laboratory is expensive and produces only a limited amount of dsRNA and thus is not practical for large-scale application needs (Ahn et al., 2019). Producing dsRNA in bacterial cells with RNaseIII deficiency seems to be an alternative. However, only a handful of works have demonstrated microbial-based dsRNA production. One approach uses the L1440-HT115 (DE3) system that has been successfully applied in the RNAi of *Mythimna separate* (Zhang et al., 2010; Das et al., 2015; Parsons et al., 2018).

Off-target effects

Although RNAi technology has been widely used for functional analysis and development of crop varieties due to its many advantages, non-specific effects often referred to as off-target gene silencing should be considered. The off-target effects introduce uncertainty in gene function studies. Several studies show that siRNA is not always specific and can have off-target effects and thus is problematic in disease management (Mamta and Rajam, 2017). Also, some target genes are highly conserved between species which increases the likelihood of off-targets among them. The sequences of vATPase A and vATPase E from *L. decemlineata,* for instance, shared 83% and 79% nucleotide-sequence identities to their counterparts in Western Corn Rootworm (WCR), respectively. dsRNAs from WCR vATPaseA and vATPaseE could reduce the fitness of Colorado potato beetle (CPB; Leptinotarsa decemlineata) in a bioassay (Baum et al., 2007). Xu et al. (2006) reported that 50-70% of gene transcripts in Arabidopsis plants have potential off-targets when used as a silencing trigger for PTGS and this can obscure experimental results. Moreover, excessive siRNA production could also lead to off-target effects thus the use of appropriate promoters and the suitable number of transgene copies introgressed into the host genome are very important. Another important aspect to consider is that the off targeting could also affect exposed non-target organisms, causing environmental and biosafety issues. The most troublesome situations are arising during the Dicer cleavage and siRNA production, the siRNA amplification, and transitive silencing, and the target gene mRNA recognition and degradation. To prevent them, the gene fragment used for producing dsRNA (the trigger) should be chosen to be as specific as possible, taking into account that a sequence of only 14nt or less can lead to inhibition of gene expression. The use of vectors with tissue-specific and inducible promoters is another solution. Computational prediction tools can be used to design RNAi

constructs and to screen potential off-target effects. A suitable computational design program is needed for the specific and systemic evaluation of non-target and off-target effects which should be further verified by additional bioassays. Fakhr et al. (2016) reviewed various algorithms for efficient siRNA design and listed the pros and cons of different online software, on mammalian gene silencing. Some of these steps could also be applied in plants to identify the more favourable siRNAs, or the use of specific parameters of Basic Local Alignment Search Tool (BLAST) algorithms. The BLAST algorithms could be used to find regions of similarity against the whole genome of the species. The sequencing costs can be reduced by accessing different public databases, like Phytozome (Goodstein et al., 2012), Plaza 4.0 (Van Bel et al., 2018), or PlantGDB (Duvick et al., 2008), among others. Regarding specific RNAi-related databases, PVsiRNAdb holds detailed information related to plant virus-derived small interfering RNAs (vsiRNAs) (Gupta et al., 2018). The discovery of high-throughput sequencing has become an important tool for sRNA discovery and profiling. The UEA small RNA Workbench is a suite of tools for analyzing miRNA and other small RNA data from high-throughput sequencing devices (Stocks et al., 2018). There P-SAMS and si-Fi21 are some tools for siRNA design. The Plant Small RNA Maker Site (P-SAMS) is a web tool for efficient and specific targeted gene silencing in plants using two applications: P-SAMS amiRNA and P-SAMS syn-tasiRNA for the simple and automated design of artificial miRNAs and synthetic trans-acting small interfering RNAs, respectively (Fahlgren et al., 2016). The si-Fi21 offers an efficient prediction of RNAi sequences and off-target search and it is specifically intended for long double-stranded RNAi constructs including virus, microRNA, and host-induced gene silencing (HIGS) (Lück et al., 2019)

RNAi resistance

Pests and pathogens can develop resistance to RNAi-based products through various mechanisms (James, 2010). The RNAi-based gene silencing strategy induces down-regulation of the target gene by incomplete resistance in most of the cases which may reduce the selection pressure on the pathogen that may contribute to durable resistance. The genetic variation in pathogenic organisms may also cause single nucleotide polymorphisms (SNPs) in the target gene. Synonymous SNPs lead to nearly no fitness cost on the pathogens and pests, but the difference between dsRNA and the original gene sequences reduces their complementarities, causing reduced RNAi effect or RNAi resistance (Scott et al., 2013). Thereby, the potential of RNAi resistance should be taken into consideration in the application is very important.

Many researchers reported that they have not found any unexpected or adverse effects during their studies on risk assessment of RNAi-engineered fruit trees (Yien et al., 2011; Scorza et al., 2013). For example, oral feeding of mammals with PPV-resistant plum in experimental models showed no adverse effects on mice and no allergenic reactions. The RNAi-modified papaya did not reveal any genotoxicity in any analyzed gastro-organs in rats (Yien et al., 2011). These results suggest that RNAi does not elicit any unexpected toxic reactions and does not represent any bio-risk to mammals (Scorza et al., 2013). The V-type ATPase based RNAi technology has passed the GM safety evaluation in many countries. It has also been licensed for planting by the US Environmental Protection Agency (Zotti et al., 2018). Technical barriers are being overcome to allow a wide range of applications for crop improvement. The technology of encapsulated dsRNA on leaves with SIGS has significantly promoted dsRNA stability in the environment as well as during its uptake by pests enhancing plant protection. The development of cost-effective approaches for massive production of dsRNA (e.g., bacterial, plant, and synthetic production) can reduce the costs of this technology. This interference strategy occurring in plants can be exploited to change metabolic paths to optimize desired characteristics that could not be achieved with classical breeding. However, in common with the use of new genetic techniques, the introduction of this new RNAi technology raised safety concerns, although the highly selective nature of RNA activity reduces the likelihood of off-target and non-target effects. This has been supported by the genetic and bioinformatics

information obtained through next-generation sequencing (NGS), high-throughput sequencing (HTS), and other techniques (Shendure and Ji, 2008). RNAi provides additional options for plant breeders to improve different food crop varieties compared with other new breeding techniques (NBTs) such as clustered regularly interspaced short palindromic repeats/CRISPR-association protein (CRISPR/Cas) or transcription activator-like effector nucleases (TALENs). Another important feature of RNAi is that the dsRNA molecules can be highly mobile in plants. Therefore, dsRNA produced in the part of the plant (e.g., rootstock) can have the potential to spread into the grafted parts of the plant to confer resistance to disease to the whole plant, including fruit. This results in fruits that are not genetically modified but protected by the presence of target-specific degradable small RNA molecules (Limera et al., 2017). In addition, dsRNA molecules can be formulated and applied as a topical treatment to plants to change their physiology or combat pests and pathogens. In 2016 the iPlanta Cost Action CA15223 'Modifying plants to produce interfering RNA' (available at https:// iplanta. univpm.it, accessed in November 2020) was established with the objective of bringing together experts from a wide range of fields to develop a deeper understanding of the science of RNA, the applications of RNAi, the biosafety of these applications and the socio-economic aspects of these potential applications (Liu et al., 2021). Furthermore, the risk assessment of GM RNAi plants is important in order to ensure the safety of RNAi crop plants, although the available literature suggests that there are no adverse effects of RNAi molecules (Ramon et al., 2014).

CONCLUSION

RNAi technology has emerged as a promising new strategy for crop improvement and has recently become one of the highly effective and powerful tools of functional genomics for silencing the gene expression for genetic improvement of crop plants, which opens new avenues in the agriculture sector by enhancing production and quality with suitable high yielding disease resistant elite varieties with abiotic stress tolerance and improved nutritional quality. There is no doubt that disease and pest control based on RNAi technology is a highly effective method to save crops from pathogens, insects, and nematodes but further focused research needs to understand the exact mechanism and RNAi pathway components that will hopefully resolve the complex plant defence system against various pathogens, insects, and nematodes. Transgenic and non-transgenic plant-based RNAi approaches for different pathogens in different crop plants have shown great promise and new opportunities. Its revolutionary capabilities could be further exploited in different crops to create another "Green Revolution" by producing sufficient quality foods. However, there is a great need to strengthen research and development activities in this promising area for proper, effective, and safe utilization of these tools as sustainable solutions for modern crop protection and improvement to produce sufficient quality food so that in near future everyone can get nutritious food and able to eradicate hunger, malnutrition, and health problems.

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