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Review

RNA silencing technology: A boon for crop improvement

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RNA interference (RNAi) is a powerful tool for gene silencing in different organisms, including plants. It is being used in functional genomics to decipher the function of genes. This technology has also witnessed a variety of potential applications in agriculture for crop improvement, including the development of crops for resistance against biotic (weeds, pathogens, insect pests and nematode parasites) and abiotic stresses (drought, high and low temperature, etc.), nutritional quality improvement, healthier oils, delayed ripening, male sterility, modification of flowering time and flower colour, alteration of plant architecture, enhancement of secondary products, and removal of allergens and toxins. RNAi has several advantages over traditional transgenic approaches as genetically modified RNAi plants do not contain transgene protein, however the risk assessment of these plants should be examined to rule out any off-target effects.

Keywords. Crop improvement; gene silencing; nutritional quality; RNA interference; stress tolerance

1. Introduction

The current world population size is 7.7 billion and it is projected to reach 9.7 billion by 2050. India expected to be the largest country as far as population is concerned, surpassing China by next 4 years or so (UN DESA Report 2015). Therefore, feeding the growing mass is a big task as there is already a huge gap between food production and population size. In fact, it has been projected that there will be about 40% increase in global demand for cereals, roots and tubers by around 2020, and approximately 70% increase in food production would be needed by 2050 to feed the world population of 9.7 billion (FAO 2009). As we have to double the food production sustainably on the same cultivated land by 2050, we definitely have to witness a second Green Revolution. In order to meet the projected demand for food production, we have to adopt various technology options, particularly plant breeding and biotechnology, including RNA interference (RNAi)-based strategies for the improvement of crop yield and quality by protecting crop plants against biotic (pathogens and pests) and abiotic (salinity, drought, etc.) stresses. In this short review article, an overview of the potential of RNA silencing for crop improvement is given.

2. RNAi and gene silencing

RNAi was discovered as a potential tool for gene silencing by Andrew Fire and Craig Mello in *Caenorhabtitis elegans* in 1998 (Fire *et al.* 1998) and they have received Nobel Prize in Physiology or Medicine in 2006 for this important break-through discovery. RNAi is highly sequence-specific gene silencing mediated by double-stranded RNA (dsRNA). Previously, a similar sequence-specific gene silencing was reported in Petunia, which is called as Co-suppression or Post-transcriptional Gene Silencing (PTGS) (Napoli *et al.* 1990) and Quelling (or PTGS) in a fungus (*Neurospora crassa*) (Romano and Macino 1992).

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RNAi pathways are evolutionary conserved across eukayotic systems, and are involved in defense against viruses, transposans and transgenes, and regulation of genes associated with plant developmental processes and stress responses (Mamta and Rajam 2018). dsRNA can trigger RNAi pathway. The precursor dsRNA is then cleaved to produce the effector small RNAs (sRNAs) of 21–24 nt size by RNAase III enzyme Dicer. The sRNAs include small interfering RNAs (siRNAs) or micro RNAs (miRNAs). These sRNAs are then recruited 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 translation based on the extend of sequence similarity as a result there is no protein (figure 1). 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 RNA polymerase II (rarely RNA polymerase III) to produce primary miRNA (pri-miRNA), 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 cytoplasm by exportin-like protein. The miRNA duplex is then transferred to RISC, where the passenger strand is selectively degraded by Argonaut protein, and now single-stranded mature miRNA (guide strand) along with RISC is involved in the degradation or translational repression of the target mRNA (Yogindran and Rajam 2015, 2016) (figure 2).

For stable silencing of genes, hair-pin RNAi constructs (RNA silencing constructs) harbouring the partial target gene sequence (200–300 bp) in sense and antisense orientation with a small intron as a spacer DNA between them under the control of an appropriate promoter are designed to express dsRNA in transgenic plants. The expressed dsRNA produces multiple siRNAs in plants by Dicer. The synthetic dsRNAs/siRNAs are used for transient gene silencing (Yogindran and Rajam 2015). Alternatively, artificial miRNAs (amiRNAs), which use the endogenous pre-miRNAs, where the native miRNA/miRNA* sequences are replaced with the desired amiRNA/amiRNA* sequences. These amiRNAs lead to an efficient and specific silencing of the target gene (Yogindran and Rajam 2016).

3. RNAi and crop improvement

RNA 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), *C. elegans* and *Drosophila*. Also, it is a powerful strategy for silencing of genes for the improvement of several agronomically important

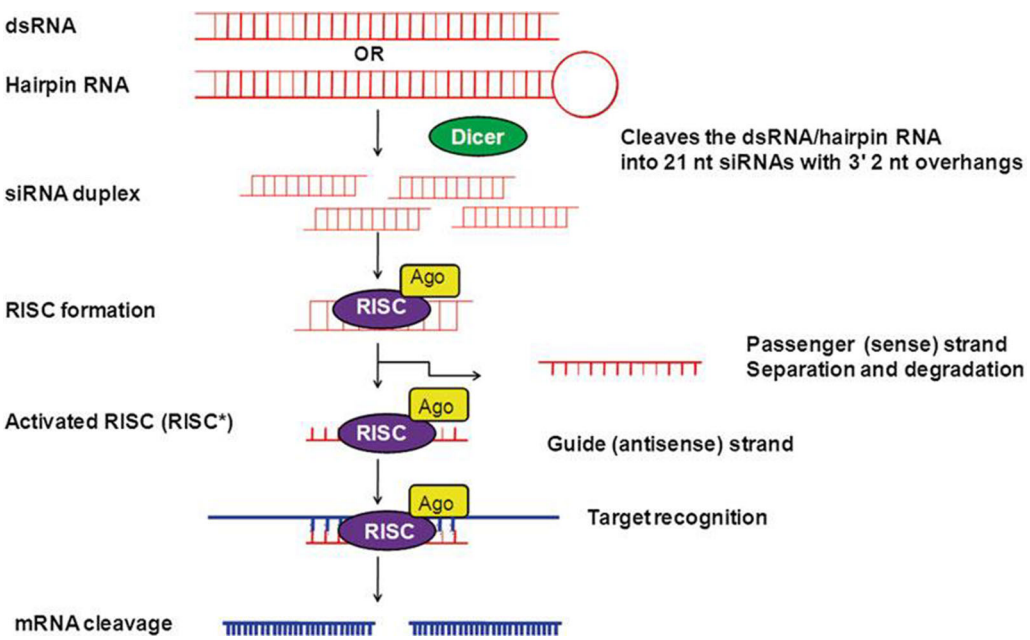

Figure 1. Biogenesis and mechanism of action of siRNAs for gene silencing. (Adapted from Yogindran and Rajam 2015).

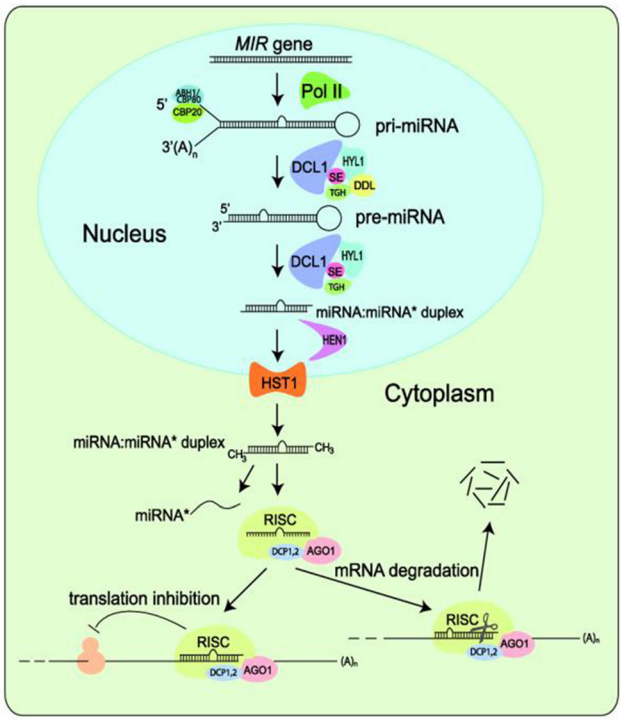

Figure 2. miRNA biogenesis pathway in plants. miRNA genes are transcribed by RNA Polymerase II into pri-miRNAs that fold back to form hairpin structure. Splicing and further processing in nuclear involve the interactive functions of HYL1, DDL, TGH and SE and of the cap-binding proteins CBP20 and CBP80. 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 (Adapted from Yang and Li 2012).

traits, including biotic and abiotic stress tolerance (Saurabh *et al.* 2014; Mamta and Rajam 2018).

3.1 *Stress tolerance*

Crop plants are infested by insect pests, which cause a significant loss of crop yield. The conventional methods like the use of insecticides for insect control are met with certain limitations. Recently, RNAi has been successfully applied for the development of insect resistant crops. This involves the identification of a vital gene of the target pest and its silencing through host plant mediated RNAi or host induced gene silencing (HIGS). As the vital gene product is absolutely needed for insect growth and development, the suppression of its expression will lead to insect mortality. Mao *et al.* (2007) and Baum *et al.* (2007) were first to use RNAi for the control of cotton bollworm (*Helicoverpa armigera*) and a coleopteran insect pest (Western corn rootworm) by targeting P450 monoxygenase (*CYP6AE14*) and Vacuole ATPase A (*V-ATPase A*) respectively. Subsequently, RNAi- and miRNA-based approaches were used by many researchers for the control of different insect pests (Younis *et al.* 2014; Yogindran and Rajam 2016; Zotti *et al.* 2018; Mamta and Rajam 2018; Saini *et al.* 2018). Similarly, RNAi was also used to target the essential genes of nematodes (reviewed by Tamilarasan and Rajam 2013; Dutta *et al.* 2015; Banerjee *et al.* 2017), viruses (reviewed by Duan *et al.* 2012; Khalid *et al.* 2017), and fungal (reviewed by Mcloughlin *et al.* 2018) and bacterial (reviewed by Saurabh *et al.* 2014) pathogens for resistance against these pathogens/pests. Abiotic stress tolerant plants were also developed by RNAi-based strategies (Saurabh *et al.* 2014; Younis *et al.* 2014).

3.2 *Other applications*

RNAi-based approaches were also used for many other important applications in agriculture. Plants with new traits such as the alteration of plant architecture and flowering time, delayed ripening for prolonged shelf-life of fruits, induction of male sterility, improvement of nutritional quality, healthier oil production, seedless fruit development, low gluten wheat, low lignin content in jute, high lysine containing rice, control of mycotoxin contamination in crops like peanut, modification of flower colour (e.g., blue rose) and scent of flowers, enhancement of pharmaceutically important secondary metabolites, low nicotine tobacco, decaffeinated coffee, low narcotic opium, elimination of allergens and toxicity from some plants like peanut and *Lytherus* respectively, and therapeutics (reviewed by Koch and Kogel 2014; Saurabh *et al.* 2014; Younis *et al.* 2014; Guo *et al.* 2016; Pathak and Gogoi 2016; Majumdar *et al.* 2017).

4. Off-target effects of transgenic RNAi plants

RNAi technology is commonly used in functional genomics for silencing gene expression in plants. Until recently, it was believed that siRNAs are extremely specific, but the recent work with functional genomics has revealed that siRNAs often suppress the expression of unintended genes (called as off-target effects) in

plants, which may lead to false conclusions (Senthil-Kumar and Mysore 2011). However, there are many ways to mitigate the off-target effects of siRNAs. For instance, using a minimal length of target gene sequence (usually 200–300 bp) for preparation of hp-RNAi constructs, no or minimal homology of the selected target gene sequence with the genes of the host plant or beneficial organisms and humans.

The main concern about transgenic RNAi plants is the off-target effects of the expressed siRNAs, i.e., non-specific silencing of plant RNAs and affecting non-target organisms (Mamta and Rajam 2018). Therefore, the assessment of transgenic RNAi plants for off-target effects on the host plant and other beneficial organisms and the persistence of siRNAs in the environment is very important (Lundgren and Duan 2013).

5. Conclusions

RNAi phenomenon was discovered about 20 years ago by Fire *et al.* (1998) in *C. elegans,* and this work won the Nobel Prize in 2006. Since then, RNAi technology has revolutionized the field of plant biology not only for functional genomics but also for a variety of potential applications in agriculture, including the development of crops resistant to viruses, fungal pathogens, insect pests and nematode parasites. RNAi-based strategies continue to contribute many more exciting applications in agriculture. In recent years, a number of patent filings on RNAi-derived crops were witnessed, particularly in US, European and Chinese patent offices with multinational giant firms as the most prolific patent filers (Jalaluddin *et al.* 2019).

RNAi transgenic plants have several advantages over traditional transgenic plants. For instance, RNAi-mediated gene silencing does not involve the protein machinery of the host plant as it occurs at the post-transcriptional level, and thus there is no transgene protein. In the absence of transgene protein, it is very unlikely that these RNAi GM plants would cause allerginicity and toxicity, the target pathogens/pests would gain resistance against RNAi and there could be minimal bio-safety issues (Rajam 2012; Yogindran and Rajam 2015). However, there is one limitation that siRNAs produced from the expressed long dsRNA can have off-target effects, i.e. silencing of unintended or non-target genes if there is a substantial homology. Thus, the risk assessment of GM RNAi plants is important in order to ensure the safety of RNAi crop plants, although the available literature suggest that there are no adverse effects of RNAi molecules (Ramon *et al.* 2014).

Transgenic RNAi crops are still considered as GM crops globally, which means that they have to undergo rigorous evaluation before approval is granted. Interestingly, the recent developments where the exogenous dsRNA is being used as spray for the control of viruses, fungal pathogens and insect pests (called as environment RNAi), and this has provided a non-transgenic alternative to GM crops. However, the technical hurdles and regulatory ambiguities surrounding this emerging technology still remain challenging, and its full potential remains to be realized (Jalaluddin *et al.* 2019).

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