7

RNA INTERFERENCE (RNAi) FOR PLANTS

Nur Afiqah Md Samad, Nur Hanis Abdul Rahim,Mariam Firdhaus binti Mad Nordin, Angzzas Sari binti Mohd Kassim*

Faculty of Engineering Technology, Universiti Tun Hussein Onn Malaysia,

Pagoh Higher Education Hub, KM 1, Jalan Panchor, 84600 Muar, Johor, Malaysia.

*Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia Kuala Lumpur, Jalan Sultan Yahya Petra, 54100 Kuala Lumpur

Abstract—As we are facing global population development, strategies are required to improve agricultural production in the battle against hunger and poverty. Agricultural biotechnology provides a powerful method in combination of conventional breeding, new innovations and enhanced management of resources which improves the productivity of livestock, aquaculture, and crops. After the finding of RNA interference (RNAi), researchers have made considerable growth in improving this remarkable crop especially in defence technology. RNA interference is a vital plant growth, development and reaction regulator to various types of stresses. This technology leads to higher efficiency and potency of gene silencing, thus becoming the highly promising technology for crop improvements at a rapid rate with some advantages. Nowadays, RNAi has been widely used for the improvement in agricultural biotechnology and seems to be applicable and commercialized in other fields too.

Keywords— *Agricultural biotechnology, RNAi, breeding technology, crop improvements, crop resistance.*

7.1 Introduction

Over 10,000 years, the development of wild animals and plants in research discoveries and breeding of suitable traits have been established. By this breeding, naturalized plants and farm animals are widely used in livestock and crop agriculture [1]. During the domestication process, people began to choose better plant materials for propagation and animals for breeding. At first, this happened unintentionally, but once the better results have been witnessed, many start to believe the production of food crops and livestock can be improved. Crop varieties which are cultivars with larger seeds and fruits, better quality, nutritional value, shorter growing seasons, longer shelf life, etc were deemed desirable traits [2]. In the twentieth century, technology was rapidly established, and breeding became more sophisticated by using modern technologies including biotechnology. Biotechnology is typically any method or procedure that incorporates life-related research which involves all types of species. These include bacteria, viruses, plants and animals for solving problems or making products that are beneficial to humans with added value [3]. Thus, biotechnology became a main sector of industry, modern medicine and agriculture which are also known as red, white and green areas respectively [4].

7.1.1 Agricultural biotechnology

In agriculture, biotechnology has been utilized in a wide range. A set of scientific techniques in agricultural biotechnology is used to enhance microorganisms, plants and animals functions. Based on the knowledge in DNA, scientists have established various approaches to improve productivity in the agriculture sector. Starting with the potential to recognize genes which impart benefits on other crops and the capability to manage these traits specifically, biotechnology increases the potential of breeders to develop crops and livestock that are not practicable on their own through conventional cross breeding of related organisms [1]. Agricultural biotechnology is predicted to be a possible resource for ensuring food security in the region.

According to records by the United Nations, the world population exceeded 7.3 billion in 2015 and 60% of the global population lives in Asia (4.4 billion). The population is expected to exceed 8.1 billion by 2030. The growth in population directly resulted in a rise of per capita food consumption. Urbanization and increase of the country's Gross Domestic Product (GDP) also lead to a move towards greater consumption of meat and dairy products, while growing demand for grains for the livestock and poultry industry is expected [5].

As we are facing global population development, strategies are required to improve agricultural production in the battle against hunger and poverty. Agricultural biotechnology provides a powerful method in combination of conventional breeding, recent innovations and enhanced management of resources which improves the productivity of aquaculture, livestock, and crops. Agricultural productivity will be improved in an ethical, environmental and sustainable manner. For instance, the reduction of excess fertilizer and pesticide usage which can harm health and biodiversity of organisms. Apart from that, there are many recent crop varieties which are immune to climate changes, pests, and plant diseases are being developed, allowing more efficient vaccines for livestock and also better livestock diseases diagnosis among others [6].

Biotechnology is now used to tackle issues in other fields of agricultural processing and production. This involves plant breeding to conserve while increasing the yield in order to enhance tolerance towards diseases, pests and abiotic stress resistances such as drought with the increase on food nutritional content too. Agricultural biotechnology is also used in planting materials which are low in value and free from any disease for crops such as cassava in **Figure 7.1**, bananas and potatoes. Other than that, it also develops modern methods for the treatment and diagnosis of plant and animal diseases as well as assessment and conservation of genetic resources [4].

Figure 7.1: Cassava [3]

Besides, agricultural biotechnology will improve breeding programs for plants, and livestock and expand the diversity of valuable characteristics. Improvement by agricultural biotechnology of animal feeds and feeding practices are done to reduce environmental waste and enhance animal nutrition. Biotechnology is used to treat diseases and to develop vaccinations against livestock diseases. Normally, people assume agricultural biotechnology is about genetic engineering manipulation only, but it is more than that. For example, it also involves molecular markers for targeted breeding, quick diagnosis of diseases and resistances, identifying plant varieties with specific traits, tissue culture, and vaccine development for plants and animals [6]. There are many undiscovered techniques in agricultural biotechnology which are under research. However, it is significant and economically beneficial to consider how biotechnology complements and broadens certain strategies if it is to be used wisely [4].

Agricultural biotechnology trend in Malaysia

An analysis released by Transparency Market Research (2015) stated that the global agricultural biotechnology industry was worth US$ 15.3 billion in 2012, and is projected to increase by 2019, rising at a compound annual growth rate (CAGR) of 9.5% between 2013 and 2019 [5]. In Malaysia, agricultural biotechnology focuses primarily on sustainable growth, sustainable pest and disease control, nutritional security and efficiency, and safe food. It plays a key function in global development and in sustaining the productivity of the agricultural industry. Given the reality that the application of biotechnology to agriculture has been in operation for several years, the development of agricultural biotechnology in Malaysia is considered to be notable albeit slowly. When the government initiated the National Biotechnology Plan in the early 1900s, the goal was to boost national food security and encourage industrial development as well as to increase the quality of agricultural goods on international markets [3].

On the other hand, Malaysia has a large sector in agricultural biotechnology with lively research and development (R&D) and commercial practice which is tissue culture. This is proven by having the total output of clonal oil palm planting materials with 2.5 million ramets per year in Malaysia. This is projected to rise to 5 million by 2010 from 11 commercial tissue culture laboratories and the Malaysian Palm Oil Board (MPOB).

However, this is still a distant amount to provide 40 million ramets by 2017. Besides oil palm, tissue culture is vigorously sought and commercialized for jatropha, orchids, banana, pineapple, vanilla, papaya, *tongkat ali* (Eurycoma longifolia), rubber and a variety of forest trees such as *gaharu* and teak. There are also other areas in the Malaysia's agriculture sector involving biotechnology such as diagnostics and biologics, animal breeding and reproduction, animal health, plant genomics, fertilizer and soil enhancer technology. Overall, the Malaysian agricultural biotechnology subsector was reported to be worth US$ 67 million in 2009 [5].

However, plant pathogens such as pests and insects may be a major problem in managing the agriculture sector. These organisms can cause a disaster to the agriculture sector if not handled correctly and effectively. Bakar et. al., stated that the usage of pesticides in Malaysia rose from RM 289 million in 1995 to RM 403 million in 2008 (a 39% increase over 14 years). Herbicides account for higher than 76% of the total market. This shows the relative importance of weeds as dominant pests in Malaysian agriculture as well as the country's reliance on herbicides for weed control especially in estates and orchards. These patterns of rising pesticides reliance in Malaysia are a major source of environmental concern, with records of parallel increase in incidences of pesticide resistance pests and millennial weeds. A country may have a major negative impact caused by pathogens and can lead to drastic reduction in crop production and even attempt to wipe out all plant organisms [8]. If this situation persists, there would be a potential extinction of beneficial species and the almost complete disappearance of freshwater fish in rice granaries and intensified environmental pollution in rivers and waterways [7]. Nevertheless, there are options on how to handle these plant pathogens which are environmentally safe such as vaccination instead of using chemicals.

7.1.2 RNA interference (RNAi)

Different techniques have been recorded and developed from time to time and there is a need for modern and effective disease prevention approaches. There is also continuous research in various areas of the world to establish technology that can aid in maintaining the crops and not impede the habitat and condition of the environment. Triggered by this, modern methods and technologies are developed to control the plant diseases and act as alternative safe techniques to chemicals, pesticides and fungicides. Many scientists have made considerable progress in improving this remarkable crop defence technology after discovering the RNA interference. Other than that, RNA interference (RNAi) is important for plant growth, progress and reaction regulators to various types of stresses. The technology has been widely investigated to establish the resistance against plant pathogens such as viruses, bacteria, etc [9].

Petunia hybrida L. plants was the first observed introduction in RNAi technique by adding the chalcone synthase gene (chsA) which is pigment producing gene to improve anthocyanin pigment [8]. The expected colour of the flower was deep purple but appeared variegated or even white [10]. An over expression of chsA gene that encodes significant enzymes in anthocyanin biosynthesis pathways has made a new flower colour pattern in transgenic Petunia [8]. This is where a RNAi term was found.

Double stranded RNA (dsRNA) transportation is a recent innovative technique with a strong potential for plant defence. It is an activating agent for the gene silencing process which refers to RNAi. In addition, RNAi acts as an important character of organisms growth and progression which is a natural and greatly conserved technique in eukaryotic species. It can provide virus resistance to the host and transpose on inactivation through various lineages such as animals, plants and even fungi [9]. Apart from that, it applies to a variety of RNA-based mechanisms, which all result in sequence specific gene expression inhibition at either transcription or translational stages.

It is important to note that RNAi is an effective tool that has high potential for cells to destroy foreign genes [11]. RNAi is a gene-silencing mechanism with a series of complex RNA degradation occurring. The RNA breaks down cycle is activated by introducing the double stranded RNAs (dsRNAs) through transgenes. Then, the dicer cleaved dsRNAs. Duplexes of 21-nucleotides (nt) with 2 nt 3' symmetrical overhangs are formed. These duplexes indicate small interfering RNAs (siRNAs) which mediate the mRNA breakdown. Thus, repression or modification of the gene expression occurred [10]. The usage of dsRNA may have various benefits instead of using conventional or chemical compounds. The dsRNAs operate by their specific nucleotide sequence, while chemical compounds operate by a structure-dependent process. In order to operate in homologous sequence, the dsRNAs and siRNAs agents will act only to the desired pathogen. Above all, the dsRNAs are biodegradable and bio-compatible compounds that are natural in nature and occur all over the places at both within and outside of the organisms unlike the chemical pesticides. Similarly, with any RNA molecule, the stability of dsRNAs in water or soil is lower than in plants and other organisms. This can be shown when dsRNAs enter the natural RNA silencing route and break down into tiny RNAs then turnover through natural degradation mechanisms [12].

In short, RNAi is a technological advance with the most successful and fast-moving boundaries for the genetic advancement of crops. The application of RNAi has been widely used such as reduction of lachrymatory factor synthase production in onions, reduction of alkaloid production in tobacco plants and many more [10].

7.2 Biotechnology on RNA interference (RNAi) for Plants

RNAi is used in plants to monitor transposon (a chromosomal segment that can undergo transposition, especially a segment of bacterial DNA that can be translocated as a whole between chromosomal, phage, and plasmid DNA in the absence of a complementary sequence in the host DNA) and to apply tight control of the developmental processes such as leaf development and flower organ. Besides, plants can defend themselves against virus infection by the application of RNAi. The degradation of sequence specific RNA is known as gene silencing. A natural cellular defence method of RNAi will act against transposon genomic confinement, viruses and other processes [13].

7.2.1 Mechanisms of RNAi

The participation of small RNA molecules is a central feature of the RNA interference mechanism. [14]. The pathway of RNAi can be divided into four stages as in **Figure 7.2**, which are the cleavage of dsRNA by the dicer, the formation of RNA induced silencing complex (RISC), the activation of silencing complex and the mRNA breakdown [10]. The transportation of dsRNA in a cell which is completely homologous in sequence to the target gene occurred in the beginning stage. There are two types of small RNA molecules which are small interfering RNAs (siRNA) and microRNA (miRNA) [14]. siRNA and miRNA are generated by cultivating longer dsRNA and stem loop precursors [15]. Dicer enzymes or known as Endonuclease Rnase III act as an important part in the formation. The dicer enzyme recognizes 22-24 dsRNA and further processes dsRNA in an ATP dependent reaction into double stranded siRNA 21–25 nucleotides in length depending on the species [10].

Next, the dicer enzymes which are DCL2, DCL3 and DCL4, process siRNA from a long double strand of RNA. On the other hand, the precursors of miRNA exported from the nucleus are processed by DCL1 [14]. By fractionation, an extract of a specific nuclease from *Drosophila Melanogaster*, a RNA induced silencing complex (RISC) that is a member of the Argonaut family was found. It is small RNA molecules or short interfering RNAs (siRNAs) which are merged in a multisubunit complex. RISC is inactive in this form to conduct RNAi [10,16]. The RNA specific sequence in the cell is directed and cleaved by RISC. This can be obtained by cleaving the target mRNA at a complimentary region of ten nucleotides upstream of a 5′ residue of the RNA [14]. The RISC helicase unwinds the duplex siRNA. Then, it pairs with its unwound antisense strand to the messenger RNAs (mRNAs) that carries a high degree of sequencing complementarity with the siRNA [15]. In the final stage, the recognition and cleavage of mRNA complementary to the siRNA strand present in RISC. The target mRNA is cleaved into fragments of approximately 22 nucleotides in length. After the cleavage is fully completed, the RISC departs, and the siRNA can be used for the newest mRNA recognition and cleavage cycle [10].

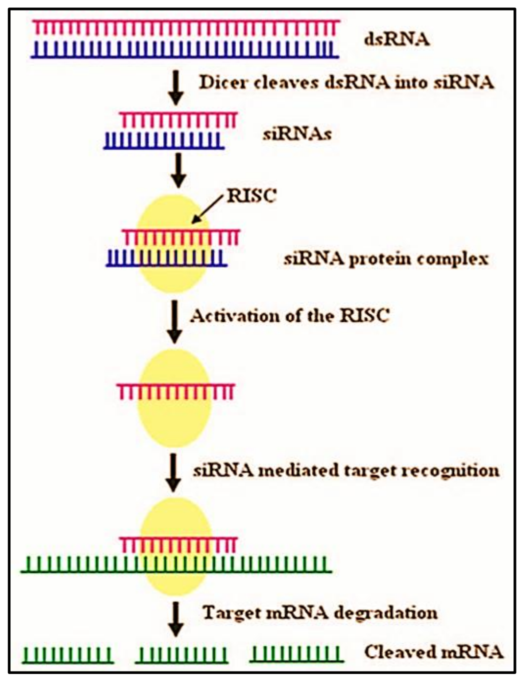

Figure 7.2: RNAi pathway [10]

7.2.2 Strategies of delivering RNAi

In the RNAi field, there are many challenges to ensure the successfulness of the technique such as the transportation of active molecules that will activate the RNAi route in plants. For example, infiltration of tobacco rattle virus (TRV) based vectors in tomato plants, delivery of dsRNA into tobacco suspension cells by cationic oligopeptide polyarginine-siRNA complex etc. These can be done by agro-infiltration, micro-bombardment, virus induced gene silencing (VIGS), host induced gene silencing (HIGS) or spray induced gene silencing (SIGS).

A Agro-infiltration

Agro-infiltration is an effective tool for researching processes relevant to RNAi. It has been proven as an important method for expression of transient genes in plants. Rapid analysis of gene function and post transcriptional gene silencing and interaction of genes between plant resistance and pathogen can be achieved by agro-infiltration. A low pressure needs to be used to force an Agrobacterium cell suspension in a standard agro-infiltration. The Agrobacterium carries the required gene structure to enter the leaf air space via the stomata [17]. In plants, cytoplasmic RNAi can be induced effectively by agro-infiltration which is identical to a technique for transient expression of T-DNA vectors after transmission by *Agrobacterium tumefaciens*. The transiently expressed DNA encodes either a single strand (ss-) or ds-RNA which is normally a hairpin (hp) RNA. The successfulness of hp constructs infiltration depends on the dsRNA translation into siRNAs. Meanwhile, the dsRNA constructs are also useful for inducing silencing and dissecting the mechanism of gene silencing especially concerned with its suppressors, simple protein purification and systemic silencing signal [18]. However, this agro-infiltration method is still unsuccessful in some plant applications such as soybean [17].

B Micro-bombardment

The technique in micro-bombardment is based on hp construct consisting of dsRNA/siRNA or DNA transportation. The hairpin construct enters the plants by application of ballistic pressure. For example, green fluorescent protein (GFP) expression is silenced by micro-bombardment [9]. The RNAi pathway will be activated by bombarding cells with particles coated with dsRNA, siRNA or DNA that encode hairpin constructs as well as sense or antisense RNA. Occasionally, RNAi silencing impact can be shown very qucik which is the day after the bombardment process and it lasts up until four days after the process. After two weeks, systemic spread of the GFP gene silencing occurred. The biolistically delivered siRNAs are considered successful when the RNA blot hybridization with systemic leaves. Hence, there is a new development of siRNAs which accumulated to cause silencing [18].

C Virus induced gene silencing (VIGS)

A process that takes advantage of the plant RNAi-mediated antiviral defence mechanism is called virus induced gene silencing (VIGS). This process is directly aimed against the viral genome when plants are contaminated with unmodified viruses. Plant functional genomics is done by VIGS as it is a fast and efficient method. The mechanism is operated by *Agrobacterium tumefaciens*, a plant pathogen to deliver via its Ti-plasmid. It is a recombinant virus that carries the whole or a part of the targeted gene sequence for silencing. Furthermore, VIGS acts as an effective strategy in targeting putative candidates' genes to target through RNAi. The secondary amplification of the RNAi signal is enabled by VIGS to increase the siRNA and promotes the transfer of siRNA by means of viral movement proteins [19] [20].

D Host induced gene silencing (HIGS)

A successful method to enhance the resistance of plants to insects and nematodes by aiming the important genes to these plant pathogens is called as host induced gene silencing (HIGS) [21]. The production of transgenic plants in order to monitor diseases from fungi and research on the possibility of pathogenicity genes' role in pathogenic fungi are done by RNAi technology. This technology is commercialized to the world today as virus resistant for plants through the viral sequence's expression as transgenes. This technique was successful in some fungal pathogens by in-planta expression of RNAi constructs specifically silencing certain fungal genes including *Blumeria graminis, Fusarium culmorum, Fusarium graminearum* and *Puccinia triticina* [22]. For instance, *Blumeria graminis* is the causative agent of powdery mildew in barley and wheat. Induction of RNAi effects in the binding biotrophic plant pathogen *Blumeria graminis* is done via HIGS. B. *graminis* is blocked closely by the host cell as it has a close connection between them. In the meantime, nutrients are also transported into it. The siRNAs have high possibilities to be transported from the plant into the *B. Graminis* haustorium. When it is in the cell, siRNA extracted from the plant are likely to bind to the fungal RNAi components. Then, this RNAi will induce the effects against the target fungal miRNAs [23].

E Spray induced gene silencing (SIGS)

As the name suggests, this process uses a spray method to silence the gene without modifying the DNA of the plants. The core SIGS concept is the exogenous usage of long dsRNAs and siRNAs. These small interferences manipulate the system of

RNAi in controlling plant pathogens such as pests. SIGS is the recent innovation which is so efficient as it protects the habitat and biodiversity and also targets the desired pathogen only without causing any harm to the surrounding. SIGS acts as a direct disease control agent and resistance repository. Other than that, this method is known as a promising and high possibility technology against multiple crops. Resistance breakdown also can be countered and control any problem associated with transgenic acceptance [9]. SIGS is an efficient, quick and environmentally friendly strategy which avoids the problems related with genetically modified organisms (GMOs) production [24].

7.2.3 Applications of RNAi

Pathogens are doing major damage to crops and create a significant risk to global food guarantee. The development of dsRNA production provides an effective, greatly versatile, non-transgenic and environmentally friendly molecule of dsRNA to the viruses and other plant pathogens [12].

A RNAi for plant disease resistance

Plant pathogens have an immense effect on crop production which can have a huge negative economic effect. These diseases may also kill out all plant organisms. RNAi-induced silencing of the gene has emerged as an effective method of cultivating pathogen tolerant plants. The activation of homologous mRNAs is achieved by dsRNA. This process will impede the translation and transcription in silencing the vulnerable genes. Development of resistances against some diseases triggered by viruses, bacteria and fungi are successful by application of this method [8].

i. Virus

Pathogen mediated resistance (PDR) is the most effective method for fighting plant virus infections. The PDR principle also helped to grow virus-resistant plants. The RNAi build is produced from Rice Stripe Virus which contains coat protein (CP) gene and disease-specific protein gene sequences. The development of RNAi has converted two susceptible japonica varieties that are Suyunuo and Guangling xiangjing to develop resistance to Rice Stripe Disease. The result revealed that after self-fertilization, the homozygous brood of which rice plants were produced by RNAi in the generations T5 and T7 were highly resistant to viral infection with no developmental variations and morphological [25].

ii. Bacteria

Bacteria diseases are also the cause of reduction in crop yields in any crop field such as tomato, soybean or banana. Prevention of bacterial infections is the only way to manage the bacterial diseases as they are difficult to handle and move very fast [25]. In rice, RNAi can inhibit OsSI2 (OsSI2-kd), a fatty acid desaturation process that induces increased resistance of the leaf blight bacterial pathogens (*Xanthomonas oryzae pv. Oryzae*) and blast fungus (*Magnaporthe grisea*). Lycopersicum, Arabidopsis and Nicotiana species are all susceptible to plant pathogen *Agrobacterium tumefaciens*. Transforming repetitive inverted pathogen genes ipt and iaaM by siRNA has been operated in encoding biosynthesis precursors for cytokinin and auxin. This technique has been proved to treat crown gall disease at plants [8].

iii. Fungal

Past studies have reported an increase in protection against fungi using genetically modified RNAi based crops. In 2010, 24 miRNAs responded to infection of fungal diseases from the binding of biotrophic fungus *Blumeria graminis f. sp. tritici* and 149 target genes were further predicted to be modulated by the novel miRNA wheat [25].

B RNAi for plant insects or pests' resistances

Various insect or pest resistance cultivars have usually been produced by conventional breeders. Nevertheless, because of its complexity which demanded many additional features, this solution is cumbersome and time consuming. The use of chemicals to combat pests has been a very popular practice worldwide but this is not environmentally sustainable and safe. So, biotechnologists developed a different approach to handle the pests [8]. The secret to success for this application incorporates knowledge and applications of the (a) insect species and their life cycles (b) exogenous RNA type such as dsRNA, siRNA, miRNA etc. (c) dosage and treatment phase (d) class of target gene and its expression profile (e) gene activity and tissue class (f) nucleotide sequence and dsRNA duration (g) silencing effect durability and (h) gut physiology [11]. *Bacillus thuringiensis* or Bt-based toxins have been found to be successful in transgenic strategies to combat particular insects or pests and have substituted chemical insecticides in many crops. Many biotechnological approaches that are commonly used to control insects or pests in crops. The epithelial cell membrane of insects is permeated with help of Bt insecticidal proteins. Nevertheless, this method is capped to certain crops for the control of particular pests. In fact, there is a high possibility that Bt will not work on some organisms as they can develop resistance. As the transgene encoded RNAi induction in plants is successful, biotechnologists hypothesized that genetic manipulation for exhibiting target insect genes for dsRNAs for crop protection from insects is a feasible approach [8].

C RNAi for crop quality improvement

Conventional breeding has produced a substantial improvement in crop production, but this approach is inefficient because it is time consuming and labour-intensive. Biotechnologists have been able to use this strategy along with the more developments of genetic engineering to improve crop production and nutritional status. Improved quality characteristics and nutritional gain in various crops can be changed by the RNAi approach as it has a promising potential. This approach encourages the identification of intent genes and corresponding pathways and the creation of RNAi vectors for consistency trait screening lines transformation and evaluation [8]. Other than that, the market for plants is growing with a fast population and at the same time, the world is experiencing food security, malnutrition and drought issues. This issue includes the integration of genetic engineering with genomics, plant physiology, and proteomics. This has been shown to be successful in terms of high yielding varieties with enhanced characteristics. The RNAi method and its commitment to obtain the target traits by controlling genetic expression have shown the ability for crop improvement [25].

For example, RNAi application in the cultivation of banana varieties. This is to prevent the bananas from being infected by Banana Bract Mosaic Virus (BBrMV), which has destroyed the Southeast Asian and Indian banana population. The whole banana crop is lost due to the attack of BBrMV in certain areas. This virus infects bananas that destroy the bract region fruit growing area that is useless to farmers. The infection travels through small plants eating any kind of insects called as aphids, and through plant products that are infected. Scientists could grow a variety of bananas that is immune to BBrMV by developing an RNAi vector that aims to silence the virus' Coat Protein (CP) region, but this is still risky to consume. The silencing process of the banana does not have any issue as the CP region of the various virus strains is heavily protected. Besides, the usage of an inducible promoter mechanism is also a creative method developed by scientists. This is to ensure the dsRNA production works only on the infection and not in a constitutive manner [15].

D RNAi for abiotic stress resistance

Plant growth and production are affected by the abiotic stress. This stress is a major risk not only for plants but also other lives on earth. Abiotic stress caused crop damage as the price and quantity losses. Nearly 70% of crop yield loss has been estimated to be a direct result of abiotic stress [8]. The goal in crop plants was at first to give protection against herbicide and pests resistance. Many plant miRNAs play a crucial role in the homeostasis of nutrients, growth cycles, abiotic stresses and pathogen reactions [25]. Some of the stress tolerances are drought, cold and heat, mechanical stress, etc. Furthermore, traditional crop plant breeding methods with greater resistance to abiotic stress have been performed with insufficient successful outcomes. This is attributed to a few factors; (a) yield was the breeders' main concern rather than specific characteristics (b) complexities in the breeding of resistance characteristics, including problems induced by genotype x environment and (c) the desired characteristics could only be introduced from the closely associated organisms [8].

The miRNA plays important regulatory roles in plant growth and stress response by negatively affecting expression of post-transcription genes. At the level of the global genome, it is important to identify miRNAs by means of high-throughput sequencing in order to classify miRNAs genes in plants. In model plants, for example A thaliana, rice and other plants, several extensive studies of miRNAs have also been done. Based on past studies, miRNAs involved in seed germination at a very early stage and reported that miRNA-controlled gene regulation is present in imbibed seed maize. For example, miRNAs in toxicity and tolerance to Al3+. It was discovered that 18 miRNAs had an Al3+ treatment response in 4 hours, 4 miRNAs belonging to 4 families had an Al3+ treatment response in both 4 hours and 24 hours. The miR390 was monitored for 24 hours with Al3+ treatment and was found to be miRNAs with the late reaction. In addition, miR390 is the only one which is known to modulate the auxin response factor responsible for the growth of lateral root [25].

7.3 Advantages of RNA interference (RNAi) for Plants

RNAi is the first potentially great gene silencing technique with a specific sequence mechanism. This mechanism leads to the higher efficiency and potency of gene silencing, thus becoming the highly promising technology for crop improvements at a rapid rate with some advantages.

A User-friendly

RNAi is a user-friendly technology where it can be delivered to plants externally by using any user-friendly tools that are available such as stem injection, spray, seed treatment or root drenching without applying any genetic engineering. For example, dsRNA can be supplied to the irrigation water in plants thus, the molecules will absorb into the vascular system of plants and kill the insects feeding on them. This makes RNAi a flexible vaccine to the plants. Pests and pathogens mutate continually to adapt with the climate changing, so developing an RNAi for the plants is the fastest and sustainable way rather than wasting time for complicated procedures needed in gene editing. In addition, it will be an advantage for perennial crops like raspberry, apple and citrus trees which are expensive to genetically modify and often require years of experiments [26].

B Improving lifespan of a plant

In addition, RNAi can also improve the lifespan of a plant. Plants especially vegetables and fruits tend to expose more to spoilage due to its nature and composition rather than cereal. Thus, this spoilage results in waste and cannot be sold and eaten by humans. In 2004, India, which is the largest producer of vegetables and fruits, lost 30% of their produce due to spoilage [25]. So, it is essential to improve lifespan of these plants as another agronomic characteristic where it can reduce the spoilage and worsening of vegetables and fruits. Xiong et al. (2019) implemented RNAi technology to improve the lifespan of tomatoes [25]. They initiated a dsRNA unit and blocked the expression of the oxidase gene 1-aminocyclopropane-1-carboxylate (ACC) in tomato. The gene ACC oxidase is a large protein in plant cells which is responsible for synthesis of ethylene where it helps plants to reduce water loss. However, Xiong et al. (2019) prevents the production rate of ethylene in ripened plants and fruits to ensure a long lifespan of the tomato.

C High throughput

VIGS and hp RNA's most successful and strongest induction of RNAi gives a chance to explore expression of plant genes on a genome-wide scale [26]. VIGS gives advantages based on agrobacterium-based approaches regarding the vector development as the formation of inverted repeat structure is unnecessary by the insertion of the target sequence while an inverted repeat showed that the efficacy of RNA silencing increases. Research on genome-scale of RNAi depends on high-throughput screening technology. The main advantages of RNAi sequencing genome-scale are its ability to identify and synchronize thousands of genes with the different phenotypes. The RNAi has been commonly used for functional genomics in *C elegans.* High-throughput analysis was performed on most of the ~19,000 genes [30]. Utilizing a huge set of data is a critical challenge for humans which requires statistics or bioinformatics methods. Genome-scale RNAi screening has been causing a rapid increase of data generation rates with its ability to generate a huge number of data experiments. The basic RNAi screening cycle involves the therapeutic targets, RNAi collection, reliable and healthy cell types, RNAi agent transfection, signal recognition, diagnosis, analysis and identification of essential genes. A RNAi library, containing roughly 86% of *C. elegans* genes has been developed that fast forward the research in applying this technique. This technique has been successfully attempted in many species, including humans, as the model organisms [30].

D Less Toxic Side Effect

People have been using chemical pesticides over the years to kill vectors of disease and pests that damage the plants. However, pesticides are practically toxic and poisonous to all organisms when it is exposed directly including humans and animals thus it needs to be safely and properly disposed of. In addition, these chemical pesticides remain in the environment for decades and surely accumulate with our food chain. Nowadays, RNAi acts as a biopesticide instead of chemical pesticides as it applies genetic engineering or topical applications to the plants without harming other organisms it comes in contact with [25]. Moreover, RNAi which are environmentally-friendly can be very specific to the target organisms by its dsRNA without attacking other insects or animals, this gives benefit to the plant. Topical application of dsRNA to the crops will not expose the environment to a hazard or pollution as dsRNA is rapidly degraded in soil and water. Besides, the RNAi method has been practically used in laboratory settings to control insects and most studies show that expression of dsRNA in transgenic plants and direct applications of dsRNA as pesticides come with a promising result [25]. Therefore, RNAi will naturally reduce the usage of toxic pesticides in addition to reducing insect infestations and other plant diseases.

E Highly Specific Action

Another important factor of RNAi in gene silencing is its ability to switch the target gene on and off. This factor makes RNAi a specific and effective technology in biology development. RNAi acts by affecting only target genes or pathogens by treatments and other organisms that are not in target will not be affected. However, RNAi does not work with bacteria that infect the plants because bacteria does not use RNAi [27]. RNAi may then be introduced by regulating their insect vectors that take the bacteria with them and converting the plants from contaminated plants to stable ones.

In addition, this element is useful in the case of bacterium *Xylella Fastidiosa*, which induces a variety of diseases in different plant species, such as grapevine disease, citrus variegated chlorosis, and rapid decline syndrome of olive trees. *X. Fastidiosa* is spread by sap-fed insects, often sharpshooter leafhoppers in America and a spittle meadow bug in Europe. *X. Fastidiosa* has destroyed centuries old olive trees and threatened the local economy in the southern part of Italy. There is no cure for this disease unless the prevention and containment measures are applied. Reports also showed, however, that the RNAi system has been able to shield crops from *X. Fastidiosa* insect vectors and eliminate certain bacterial infections.

Deeper studies are needed to understand more on how RNAi can be applied in large scale especially for crop industry. There must be so many questions on how dsRNA will affect the target and off-target genes, and this is a crucial thing when RNAi is commercialized as biodegradable pesticides [27].

7.4 Effect of RNA interference (RNAi) on Today Society

World's food crop loses 20 – 40% every year to plant pests and pathogens [26]. This affects the global economy. Moreover, this loss becomes worse with the change of climate which may induce the pests and pathogens to grow well and spread faster to new plants. Thus, RNAi are used to protect and improve plants' health from the severe threats to crops. Nowadays, RNAi has been used widely in today's society for the improvement in agricultural biotechnology and seems to be applied and commercialized in other fields too.

A Phenotype Alteration

Since the Ancient Era about 3000 years ago, fancy and decorative plants and flowers have been improved by adjusting most traits including their colour, form, flower size and plant architecture [25]. This alteration leads to the increasing of crop yield over a year thus, improving the world's economy around the world. So, improvement of plant and flower phenotype are crucial in order to attract suppliers and customers to buy.

People nowadays are concerned about looking beautiful and having a good smell. Moreover, most older people love to stay at home planting a lot of plants and flowers. Some of them love to decorate their internal house with beautiful and colourful flowers which will make them comfortable and love to be at home. Besides, some of them put plants or flowers in the house for the purpose of its good aromatherapy. So, the demand for flowers has increased day by day and there is a need to create more colourful and scented plants and flowers at a rapid rate. Today, a lot of plants and flowers have been created with different colours and scents such as petunia, rose, poppy and tulip [25].

The appearance of blue roses today shows that RNAi is the great method of gene silencing in creating different kinds of colours as the blue colour of roses has never been grown anywhere around the world. Roses, which are popular among people especially during Valentine's Day, have grown with a lot of beautiful colours such as red, pink, orange, yellow and even white. However, blue roses cannot be created through traditional breeding [10]. A gene involved in producing the colour of flowers is dihydroflavonol reductase (DFR) where it activates enzyme DFR to produce pigment that makes colour to the flowers. The DFR gene in roses is better at producing red pigment and it is the common colour seen anywhere. In 1997, the first silencing gene to generate blue roses was created by a government department responsible for medical science called the Commonwealth Scientific and Industrial Research Organization (CSIRO). [10]. CSIRO scientists have shut off DFR gene operation that produces red pigment for roses and inserted DFR gene from an iris that is perfect for blue pigment production. The iris DFR gene reveals an unusual result provided by a 100% blue rose.

B Seedless Fruit Development

Besides, RNAi brings seedless fruit development to the world. Consumers are so appreciative with the development of seedless plants especially fruits for fresh consumption or processed fruit products. It has been shown that seedless fruits can improve the texture and lifespan of fruits such as watermelon, cucumber and tomato. Moreover, seedless fruits can achieve high yield even under unwanted climate conditions for pollination and fertilization. This case happens because RNAi develop a fruit development process from ovary of a plant without pollination and fertilization. It was discovered that the seeds in watermelon are the signs of fruit worsening [25]. So, it is desirable to replace the seeds and seed cavities with the fruit tissue that can be eaten and continue to be a great value for all types of customers.

In tomato plants, seedless fruits were detected when the auxin response factor 7 (ARF7) role with RNA silencing was switched off [28]. ARF7 is a phytohormone auxin involved in giving auxin signal to modify the expression of target genes. hpRNA-induced silencing will suppress the expression of AUCSIA genes coding to the ovary in transgenic tomato plants thus, it produced a seedless tomato after emasculation of flower. Seedless tomato is achieved by reducing the flavonoids level. Chalcone synthase (CHS) is the first enzyme in the biosynthetic tract of flavonoids. So, down-regulating CHS will effectively generate a seedless tomato from altered auxin delivery in CHS-silenced tomato.

C Removal of Plant Allergens and Toxins

Plants mostly consist of allergens and toxins which are carcinogenic and unhealthy for human consumption. Allergenic proteins exist all over the plants, even the small one in various fruits, vegetables or tree nuts. However, a lot of studies have proven that the RNAi method can reduce the allergens and toxins in plants and make the plants toxin-free today [28]. Now, RNA silencing method technology cannot be denied its practicability of creating a lot of low-allergenic foods with the results from the studies performed.

Cassava is a major food that is eaten almost daily by humans especially in tropical countries. Yet cassava contains in its tuber a poisonous compound called cyanogenic glucosides. The existence of RNAi method helps the antisense to down-regulate cytochrome P450 enzyme protein which catalyses the first step in linamarin and lotaustralin biosynthesis. Transgenic cassava is thus formed by growing the volume of cyanogenic glucoside in the tuber by more than 90% [28].

Another study on onions was held by producing a tear-less onion. RNA silencing plays its role in down-regulating the conversion of 1-propenylsulfenic acid which is a lachrymatory factor in inducing tear to propanthial-S-oxide.

In the other research on coffee beans, RNAi was down-regulated by shutting off the expression of the 7-Nmethylxanthine methyl-transferase gene (CaMXMT1) for the three distinct methylation stages of the caffeine biosynthetic pathway. This study showed that caffeine content on transgenic coffee beans has been reduced by more than 70% which produced a decaffeinated coffee bean.

D Male Fertility and Sterility Development

One of the most crucial factors to ensure the integrity for plants growing hybrid seed to function is to maintain its male sterility. RNAi technology is used to develop male sterility which is valuable in the industry of hybrid seed [29]. A lot of methods have been used by conventional method for the pollen's abortion from variety of crop species. However, RNAi can just target any genes involved in pollen production which are expressed completely in tissues [29]. Currently genetic manipulation is used by RNAi to generate variants of male-sterile plants such as tobacco and tomato. In plants, MutS HOMOLOG 1 (Msh1) is a member of the nuclear gene that preserves stable genomes in both mitochondria and plastids. The suppression of Msh1 under abiotic stress causes a fluid reaction that requires the inheritance of non-genetic and modified metabolic pathways of the plants [25]. The RNAi technology has been used to break down Msh1 expression in tobacco and tomato that resulted in rearrangement of the mitochondrial DNA associated with normal cytoplasmic male sterility [25]. In addition, the RNAi method also applied to plants for restoring the fertility of male-sterile plants. In the other studies, male-sterile tobacco line has been generated by scientists by restricting the TA29 expression, a gene needed for development of pollen [29].

E Therapeutics and Personalized Medicine

RNAi is also used for medical approaches to down-regulate gene expression concerning pathogenesis of the disease [30]. Besides, RNAi is capable of being an effective medical solution to guided and personalized medicine. The number of diseases and conditions it might deal with is unprecedented, from cancer to infectious diseases, neurodegenerative disorders and even HIV [29]. Diverse therapeutics and nutraceuticals are developed using molecular pharming. The development of possible medicinal substances is known as biopharming or molecular pharming, using methods of genetic manipulation and transient expression like virus penetration, agro-infiltration, and magnification. Primarily, it focuses on secondary metabolites and protein biosynthesis that are extremely beneficial to people but costly on the market. Many papers mentioned RNAi's development capacity for those applications [25].

Antiviral therapy is one of the first known RNAi-focused therapeutic treatments and has produced two distinct types. The first type targets viral RNAs. Various studies have shown that attacking viral RNAs is capable of inhibiting the replication of many viruses. The other way is to block the initial viral inputs by targeting the host cell genes. For example, chemokine receptor inhibition (CXCR4 and CCR5) on the host cells would prevent HIV from entering. Numerous experiments have demonstrated that by targeting genes related to cancer, RNAi may offer a more targeted way to suppress tumour development. It has also been proposed that RNAi can raise cancer cell response to the chemotherapeutic agents and provide chemotherapy with a combinatorial therapeutic approach. The possible therapy dependent on RNAi is for inhibiting invasion and migration of cells.

There is a new finding which showed the RNAi part in therapeutics. Zhang et al. (2019) stated a startling discovery that miRNAs are obtained directly from foods that are found in different animals' plasma and tissues and can modulate the expression of target genes in mammals [25]. MIR168a of rice is considered extremely boosted miRNA in plants in the plasma of stable Chinese males and females. MIR168a which has been proven by in-vivo and in-vitro experiments is efficient of binding low density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA to the human or mouse, inhibiting LDLRAP1 production in the liver reducing the elimination of LDL from the plasma of mouse contributing to increased content of the LDL. This discovery demonstrates the therapeutic ability of miRNA exogenous supply derived from plants to control the expression of different genes in cross-kingdoms like humans [25].

CONCLUSION

Agricultural biotechnology is a sustainable method that increases values and benefits to the crop sector, especially plants while increasing the output from agricultural crops to meet world’s demand for food, fibre and fuel. Developing a variety of plants that can tolerate and resist pathogens and pest insects with the climate changing is important to ensure a healthy supply of food sources to humans and animals. In the early years of crop protection, chemical pesticides were widely used against pathogens and pest insects. The amount of toxic chemicals used have resulted in seen and unseen negative impacts to the human population as well as the environment. The need to replace these toxic chemical pesticides to a safe biological material for crop and environment is very important and in dire need.

RNAi has shown a lot of advantages to the world of agricultural biotechnology to solve the serious cases of malnutrition, food security and shortage. RNAi also proved its highly engineered sustainable method to protect plants and improve lifespan of plants. This study also revealed how RNAi applied on plants for agricultural improvement through its mechanism. RNAi has also shown some possibilities for applications of it in crop improvement such as nutrient fortified, hypoallergenic crops, decaffeinated coffee beans, and nicotine-free tobacco [25]. However, it is still very early to observe the impacts of this technology in both pros and cons. The products produced through RNAi should be assessed in terms of food safety to human or animals and environmental protection before it can be widely claimed and used all over the world. Overall, RNAi has a huge potential to improve agricultural yield in the next few decades.

References

[1] What is Agricultural Biotechnology? (2004) U.S. Agency for International Development, Agricultural Biotechnology Support Project II, and the Program for Biosafety Systems.

[2] Rozhan Abu Darak. (2019). An Outlook of Agricultural Biotechnology in Malaysia. *FFTC Agricultural Policy Platform (FFTC-AP).*

[3] Wieczorek Ania and Wright Mark. (2012). History of Agricultural Biotechnology: How Crop has Evolved. *Nature Education Knowledge*. 3(10): 9

[4] Genetically Modified Crops. Retrieved April 26, 2020 from https://www.greenfacts.org/en/gmo/3-genetically-engineered-food/1-agricultural-biotechnology.html.

[5] Mahaletchumy Arujanan. (2016). Development of Agricultural Biotechnology in Malaysia in Comparison to Global Status. *The Planter, Kuala Lumpur*. 92 (1087): 689-697.

[6] Overview: Malaysian Agricultural Biotechnology. Malaysian Biotechnology Corporation (2009) .

[7] Baki B. Bakar. The Malaysia Agricultural Industry in the New Millennium- Issues and Challenges.

[8] Adnan Younis, Muhammad Irfan Siddique, Chang-Kil Kim and Ki-Byung Lim. (2014). RNA Interference (RNAi) Induced Gene Silencing: A Promising Approach of Hi-tech Plant Breeding. *International Journal of Biological Sciences*. 10 (10): 1150-1158. https://doi.org/10.1007/ 10.7150/ijbs.10452.

[9] Imran Ul Haq and Siddra Ijaz. Plant Disease Management Strategies for Sustainable Agriculture through Traditional and Modern Approaches: Sustainability in Plant and Crop Protection. https://doi.org/10.1007/978-3-030-35955-3.

[10] Nusrat Ali, Swapan K. Datta and Karabi Datta. (2010). RNA interference in designing transgenic

crops: GM Crops. 1:4, 207-213. https://doi.org/10.4161/gmcr.1.4.13344.

[11] Munmi Borah and Naga Charan Konakalla. (2019). RNA Interference and its Application Crop Protection. *International Journal of Current Microbiology and Applied Sciences*. 8(11): 1100-1111. https://doi.org/10.20546/ ijcmas.2019.811.130.

[12] Annette Niehl, Marjukka Soinimen, Minna M. Poranen and Manfred Heinlein. Synthetic biology approach for plant protection using dsRNA. https://doi.org/10.1111/pbi.12904.

[13] Andra C. Velasquez, Suma Chakravarthy and Gregory B. Martin. (2009). Virus-induced Gene Silencing (VIGS) in *Nicotiana benthamiana* and Tomato. *Journal of Visualized Experiments*. (28), e1292.

[14] Francisco J.L. Aragao, Abdulrazak B. Ibrahim and Maria Laine P. Tinoco. (2014). RNA Interference: Omics in Plant Breeding. First Edition.

[15] S. Abdolhamid Angaji, Sara Sadate Hedayati, Reihane Hosein poor, Sanaz Samad poor, Shima Shiravi and Safoura Madani. (2010). Application of RNA interference in plants. *Plant Omics Journal*. 3(3): 77-84.

[16] T.L. Dheemanth, R.K. Ramachandra, Mohan Chavan and B.G. Prakash. (2017). RNA Interference in Crop Improvement. *Trends in Biosciences*. 10(46): 9322-9335.

[17] Vimal Kumar Dubey, Ung Gyu Lee, Deok Ho Kwon and Si Hyeock Lee. (2017). Agroinfiltration-based expression of hairpin RNA in soybean plants for RNA interference against *Tetranychus urticae*. *Pesticide Biochemistry and Physiology*. http://dx.doi.org/10.1016/j.pestbp.2017.01.004.

[18] V K Sharma, Gulzar S Sanghera, Prem L Kashyap, Brij Bihari Sharma and Chandresh Chandel. (2013). RNA: A novel tool for plant disease management. African Journal of Biotechnology. 12(18): 2302-2312. https://doi.org/10.5897/ AJB12.2791.

[19] Dinah Kiriga, Steven Runo and Amos Alakonya. (2014). A virus-induced gene silencing (VIGS) system for functional genomics in the parasitic plant *Striga hermonthica*. *Plant Methods*. 10:16.

[20] A. C. Velasquez et al. (2009). Virus-induced Gene Silencing (VIGS) in *Nicotiana benthamiana* and Tomato. *Journal of Visualized Experiments*. (28), e1292. https://doi.org/10.3791/ 1292.

[21] Chuntao Yin and Scot Hulbert. (2015). Host Induced Gene Silencing (HIGS), a Promising Strategy for Developing Disease Resistant Crops. *Gene Technology.* 4:130. https://doi.org/10.4172/2329-6682.1000130.

[22] Xiao-Yi Guo, Jing Fan, Hong Xiong, Fu-Xian, Jun Shi, Yi Shi, Ji-Qun Zhao, Yi-Fu Wang, Xiao-Long Cao and Wen-Ming Wang. (2019). Host-Induced Gene Silencing of MoAP1 Confers Broad-Spectrum Resistance to *Magnaporthe oryzae*. *Frontiers Plant Science*. 10:433. https://doi.org/10.3389/fpls.2019.00433.

[23] Cristina Rosa, Yen-Wen Kuo, Hada Wuriyanghan and Bryce W. Falk. (2018). RNA Interference Mechanisms and Applications in Plant Pathology. Annual Review of Phytopathology. 56: 26.1-26.3. https://doi.org/10.1146/ annurev-phyto-080417-050044.

[24] Ming Wang and Hailing Jin. (2017). Spray-Induced Gene Silencing: a Powerful Innovative Strategy for Crop Protection. *Trends Microbiol*. 25(1): 4-6. https://doi.org/ 10.1016/ j.tim.2016.11.011.

[25] Satyajit Saurabh, Ambarish S. Vidyarthi and Dinesh Prasad. (2014). RNA interference: concept to reality in crop improvement. *Planta*. 239: 543-564. https://doi.org/10.1007/ s00425-013-2019-5.

[26] Horiguchi, G. (2004). RNA silencing in plants: a shortcut to functional analysis. Differentiation, 72(2-3), 65–73. doi: 10.1111/j.1432-0436.2004.07202005x.

[27] Lundgren, J. G., & Duan, J. J. (2013). RNAi-Based Insecticidal Crops: Potential Effects on Nontarget Species. BioScience, 63(8), 657–665. doi:10.1525/bio.2013.63.8.8.

[28] Guo, Q., Liu, Q., A. Smith, N., Liang, G., & Wang, M.-B. (2016). RNA Silencing in Plants: Mechanisms, Technologies and Applications in Horticultural Crops. Current Genomics, 17(6), 476–489. doi:10.2174/1389202917666160520103117.

[29] Pocket K No. 34: RNAi for Crop Improvement. (n.d.). Retrieved from https://isaaa.org/resources/publications/ pocketk/34/default.asp

[30] Singh, D., Chaudhary, S., Kumar, R., Sirohi, P., Mehla, K., Sirohi, A., Singh, P. K. (2016). RNA Interference Technology — Applications and Limitations. RNA Interference. doi:10.5772/61760.

[31] Cagliari, D., Avila dos Santos, E., Dias, N., Smagghe, G., & Zotti, M. (2019). Nontransformative Strategies for RNAi in Crop Protection. Modulating Gene Expression - Abridging the RNAi and CRISPR-Cas9 Technologies. doi:10.5772/ intechopen.80874.

[32] Zhang, D., Goodfellow, S., Wang, M.-B., & Zhang, R. (2019). Bacterium-Mediated RNA Interference: Potential Application in Plant Protection. Plants, 8(12), 572. doi:10.3390/plants8120572

[33] Brianna Elliott. (2017, March 24). Cassava: Benefits and Dangers. Retrieved April 26, 2020, from https://www. healthline.com/nutrition/cassava