Chapter

microRNA Utilization as a Potential Tool for Stress Tolerance in Plants

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Abstract

This chapter describe the possibilities of MicroRNAs (miRNAs) in crop plants gene expression regulation in different metabolic pathways. Several current researches have shown different environmental stresses induce abnormal expression of miRNA, thus signifying that miRNAs may be an appropriate tool for genetical improvement in plant for stress tolerance. These miRNAs mainly control gene expression through translational inhibition. Generally, stress induce miRNAs-based inhibition of their target mRNAs, however, positive transcription factors accumulated and become more active after mRNA inhibition. Initially, researchers were mainly focused on miRNA identification, appropriate to specific or multiple environmental condition, expression profiling and recognize their roles in stress tolerance. Transformed miRNA expression studied in some plant species for better understanding of plant development and stress tolerance such as heavy metal, salinity, temperature, drought and nutrient deficiency. All these findings indicate that miRNAs act as a potential tool for genetic engineering and to enhance stress tolerance in crop plants.

Keywords: RNA, miRNA, siRNA, biotic and abiotic stresses, plant development

1. Introduction

Plants form an essential portion of the earth system and used by man as food, shelter, and a great source of medicine. Main threats to plant products and productivity are various human actions, biotic and abiotic stresses like soil toxicity, climate change, water stress, microbial pathogen, insects, herbivores *etc*. Global industrialization and increasing human population are two main factors that promote environmental changes and also enhance the demand for crop production. However, climate change and ever-increasing demand of plant products has the ability to modify the atmospheric properties and modify soils, which can make crop yield, development and growth more difficult. One of the important ways involves to enhance the yield and crop productivity is by using environmentally friendly plant protection measures [1]. Improvement in molecular biology and biotechnology identified the microRNAs role at post transcriptional level in controlling important secondary metabolites synthesis pathway [2]. This chapter gives a brief description on discovery and biogenesis of miRNA. On the basis of current research, it also describes the miRNA-based strategies used as potential tool for gene regulators in biofuel sources, beverages, cereals, fruits, fibers and economically important crops.

Plants are sessile organisms, obstinately face adverse environmental perturbations termed as abiotic stresses, most important being drought, soil salinity, extreme temperatures, and heavy metals [3]. Abiotic stresses have become a major challenge due to their widespread nature and the devastating impacts on plant growth, yields and the quality of plant produce. However, plants have developed intricate mechanisms for sensing and responding to environmental changes [4]. To turn on protective mechanisms, plants trigger a network of genetic regulations including altered expression of large proportion of genes by transcriptional and/or translational regulations [5].

2. miRNA biosynthesis and their conservation in plants

miRNA is ranges from 20 to 24 nucleotide base pair in length and non-coding RNA molecule [6]. MIR gene (miRNA genes) found in intergenic area of genome but some miRNA gene also found within intronic region in both sense or antisense direction. miRNA biomolecules are grouped in the genome and transcribed collectively as long polycistronic RNAs. miRNA synthesis occurs in the nucleus (**Figure 1**).

MIR genes transcribed into a specific primary transcript by the RNA polymerase II [6]. These are distinctive and specific primary transcripts modified at 5′ end by capping with a modified nucleotide and polyadenylated tail at the 3′ end with some adenosines [7]. The miRNAs are further modified by DICER-LIKE (DCL1), serrate (SE) and Hyponastic leaves 1 (HYL1) into precursors miRNA (pre-miRNAs). Pre-miRNA is a hairpin like structure. miRNA: miRNA duplexes produce in nucleus by further modification of pre-miRNAs by DCL1 [7].

S-adenosyl-L-methionine dependent RNA methyltransferase enzyme responsible for duplex methylation and HUA enhancer 1 at their 3′ end (HEN1) [8].

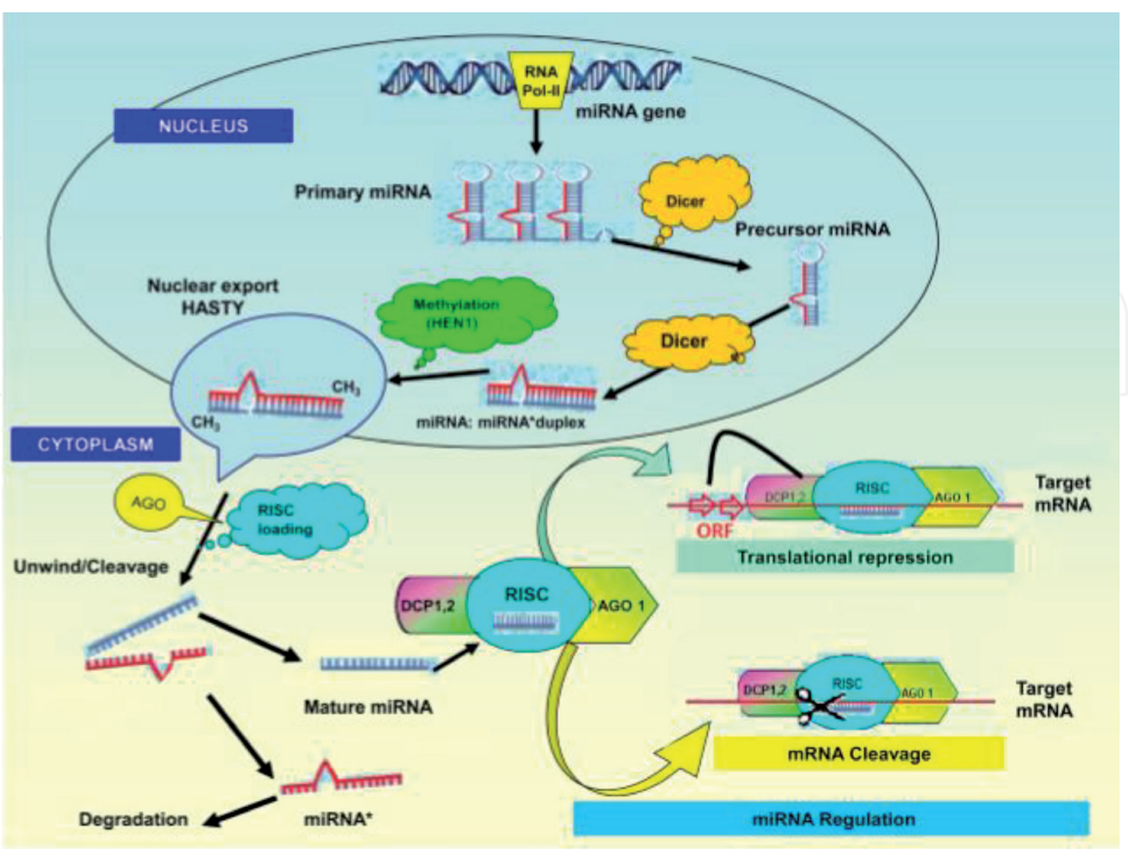

Figure 1.
Biosynthesis and mode of action of miRNA. Source: Djami-Tchatchou et al. [50].

Plant homologous of EXPORTIN 5 and HASTY transport this modified duplex into cytoplasm [9]. After transportation duplex separated by RNA-induced silencing complex (RISC) along with ARGONAUTE (AGO) protein [10, 11]. One strand of the miRNA is inserted into AGO protein comprised RISC complex, while other stand degraded by exosome along with small-RNA degrading nuclease [8]. This miRNA promotes the RISC loading to complementary mRNAs targets. Mature miRNAs showed resemblance with the target mRNA encourages site-specific cleavage of target mRNA while miRNAs along with some damaged base pairing with target mRNA leads inhibition of translation [8].

Some previous studies have shown that few miRNA bio-molecules are evolutionarily conserved among all significant plant lineages, including bryophytes, pteridophytes, gymnosperms, and angiosperms [12, 13]. miRNA families further divided into two different classes on the basis of miRNA diversification and conservation. The young miRNAs are expressed only in specific condition and at very low level although old miRNA is more evolutionarily conserved and highly expressed. In *Arabidopsis* some old studies reported the sign of regular synthesis and degradation of MIRNA genes. MIRNA gene producing loci either by aberrant transposition or replication/recombination from expressed gene sequences. Furthermore, it also showed that few miRNAs are lost during evolution [14].

3. Gene expression regulation by miRNA

For controlling various function of plant biology, specifically process control by transcription actors, miRNA diversity is significant [15]. miRNAs act as a significant controllers of gene expression and investigation on this aspect increasing now a days [2, 13]. Gene expression regulated by miRNA through high sequence similarity at the post-transcriptional level. Proper pairing between miRNA and targeted mRNA promotes the corresponding mRNA degradation and improper pairing between miRNA and target mRNA leads translation inhibition [13]. Poly(A) tail removal induced by mRNA which further promotes the destabilization and degradation of the target mRNAs [16]. Along with post-transcriptional gene expression regulation, miRNAs also decrease arbitrary fluctuation in transcript copy number and promote different metabolic pathways by transcription inhibition. miRNA of different length produces by different gene, as well as varied length miRNAs originated from the same gene. DCL1 enzyme mainly processed plant pre-miRNAs and produce 21 nucleotide base pair long mature miRNAs, but few other DCLs *i.e.* 2–4 can also be involved to produce miRNA of various lengths [17]. Diversity miRNA pool expanded by such miRNA heterogeneity and can efficiently enhance their monitoring possibilities. Additionally, miRNA diversity has practical used for the production of miRNA precursor-based expression casettes designed to produce artificial and sequence specific miRNAs [18].

4. Molecular techniques used for miRNA study

4.1 Techniques used for miRNA isolation, identification, and characterization

For miRNA identification first crucial step is to recognize their roles. Direct cloning, sequencing, genetic screening and some bioinformatic approaches commonly used for miRNAs identification [19]. In *Arabidopsis* first plant miRNAs identified such as miR156, miR159, miR164 and miR171 by isolating, cloning and sequencing of small RNA populations [20]. Small number of miRNAs have been

recognized by genetic screening, mainly due to the redundancy and sequence similarity with other miRNA-coding genes. Genetic screening and activation-tagging approach used for miRNA identification and significant to separate prominent miRNA mutants like miR172a-2 [21]. JAW (jagged and wavy) loci also identified by genetic screening, which generate a microRNA (miR-JAW) that can leads the degradation of mRNAs of TCP genes which control leaf development [22]. Mutations like deletions/insertions/further promotes the loss/gain of miRNA binding sites during co-evolution of miRNAs and their target sites [23]. Mutation also contributes in identification for specific defect during development. In the earlier decade, thousands of plant miRNAs identified by both experimental and computational approaches. The main computational method used for miRNAs identification on the basis of sequence similarity against DNA/genome sequence of some important crop plants is BLAST (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov/blast/) [24–27].

Direct cloning and genetic screening are experimentally old methods used for identification and functional characterization of miRNAs [20]. Sanger sequencing technique used after direct cloning for identification of sequence of base pair. But now a days next generation sequencing technology evolved as a powerful tool for discovery of novel miRNA and target identification in crop plants [9, 28]. Real time PCR and Northern bloting technique used for validation of identified miRNAs expression [29, 30]. Along with this, miRNA identification at protein level possible by using some other methods like mass spectrometry, proteins chromatography, protein foot printing, Western blotting, *etc*. at the protein level. Outcomes from these approaches showed that miRNAs act as rheostats to make fine-scale alterations in protein output. Further, miRNA identification promotes the development of different database which contains searchable evidence on the miRNAs. miRbase (http://www.mirbase.org) is the most significant and crucial bioinformatics tool used for miRNA research which is a searchable and comprehensive miRNA database mainly based on miRNA name, annotation, references and keyword [30]. A another bioinformatic database like The Plant MicroRNA Database (PMRD; http://mirnablog.com/plant-micrornadatabase-goes-online/) also contains information about plant miRNAs, like miRNA and their target(s), expression profiling, genome browser and secondary structure [31]. Several computational tools such as AthaMap (http://www.athamap.de/), CLC Genomic workbench 6 software (CLC Bio, Cambridge, MA, USA) and Next-Gen sequence databases also enhance the NGS performance along with the knowledge about miRNA and their role in Plants (https://mpss.udel.edu/index.php) [32].

4.2 Approaches for miRNA target screening and prediction

Several bioinformatics approaches and tools used for identification of miRNA target gene [33]. Sequence similarity scoring and secondary structure investigation are main bioinformatics criteria used for miRNA and target identification are: miRTarBase, miRTour (http://mirnablog.com/mirtour-plantmirna-and-target-prediction-tool/, psRNATarget (http://plantgrn. noble.org/psRNATarget/) and TAPIR (http://bioinformatics.psb.ugent.be/webtools/tapir/) [30]. Relationship between microRNA and its target confirmed by micRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/) [34]. While the target mRNA expression levels can be observed by real-time PCR, for mapping the target site 5′-Rapid Amplification of cDNA Ends (RACE) used. Now a days, the degradome sequencing technique was developed for identification of comparative profusion of cleaved targets [30].

5. Functional roles of miRNAs

Several previous studies on model plants like *Arabidopsis* and some other plants confirmed that the miRNAs are involved in different biological pathways in which they play significant role in growth and development, upkeep of genome integrity, response to various biotic and abiotic stresses, signal transduction, homeostasis and hormone signaling pathways [35]. Different aspects of the miRNA regulatory roles in development, biosynthetic pathway and adaptive response to stresses have been reviewed [36] and more continues to be reported. Current study revealed that the miRNAs can be used to reprogram various cellular pathways, followed by the formation of microbe-associated molecular pattern (MAMP) molecules during pathogen attack, promote some dynamic changes in microtranscriptome along with differential transcriptional regulation in support of immunity and resistance [2].

6. miRNA-based strategies for improving plant crops

miRNA functional analysis confirmed their essential role in different biological and metabolic pathways in economically important plants [37]. Several studies indicated that miRNAs are act as a riboregulatory which control gene expression during plant growth and development, and response to biotic and abiotic stress. Consequently, miRNA-based genetic engineering technology is one of the most important tools which can play an essential role to enhance agricultural production in order to generate superior crop cultivars [38]. miRNA-based regulation of gene expression manipulated by several transgenic methods like overexpression of miRNA resistant gene, the production of artificial targets [37] is used. When miRNA of interest possesses a negative control on stress factors that miRNA will be an outstanding way for crop improvement, in which transgenic plants overexpressing the semi RNAs are susceptible to stresses [39].

Additional approach in which artificial miRNAs (amiRNAs) designed and used to suppress protein-coding mRNA expression. This is an advance gene silencing technique at post-transcriptional level which has been used efficiently in different plant species [40]. The artificial miRNAs technology was used to suppress the expression of the cucumber mosaic virus suppressor 2b [18]. After efficiently inhibiting 2b expression it also enhances resistance to transgenic tobacco plants against this virus [41]. This method was found an essential way to generate advance transgenic plants with high yield and improving crop tolerance to biotic and abiotic stresses [42].

6.1 Improve crop tolerance to abiotic and biotic stresses using microRNA-based approaches

Plant through molecular pathway replies to abiotic stresses involve interaction and interlinkage of different biosynthetic pathways involving gene expression regulation by miRNA and miRNA regulation [43]. Therefore, novel plant varieties produced with better environmental stress tolerance is essential for increasing crop productivity and quality. Previous studies proved that in tomato, the overexpression of miR169 increased water stress tolerance by inhibiting stomatal opening, which decreasing transpiration rate and water loss [44]. Salt stress tolerance in rice improved by decreasing expression of osa-MIR396c [45]. Similarly, transgenic rice lines produce by increasing the expression of miR398-resistant miRNA, due to this transgenic rice with more Cu- or Zn superoxide dismutases enzyme exhibited

more tolerance to high water and salinity stress [46]. Induced expression of miR319 improve cold stress tolerance and also modify leaf morphology in rice [47].

Modernisation and advancement in miRNA research have also contributed in biotic stress tolerance in several plant species. Some previous research investigated that the Osa-miR7696 miRNA overexpression produce blast resistance transgenic rice [48]. In several plant species it was studied that the overexpression of miR393 effectively hinders the microbial growth and providing a disease resistant tool [3]. Few investigations showed that the miR160a overexpression positively regulate callose deposition induced by MAMP, while miR773 and miR398b negatively control MAMP-induced callose deposition and provide specific protection to bacterial infection [49]. Numerous studies conducted on model plants like Arabidopsis to investigate the miRNA role in plants [30], but till now only few researches were done on the significant role of miRNAs. Therefore, it is concluded after detailed analysis of old literature that miRNAs and its regulation play a crucial role in stress tolerance in plants.

6.2 microRNA-based approaches to improve plant growth and development

miRNAs play a crucial role in plant growth and development pathways such as leaf morphogenesis, apical dominance and plant biomass. Several miRNAs based new method used for production of transgenic plants for improving growth and development like plant morphology, fruit quality improvement, grain yield and more shelf life [50]. Overexpression of miR319 caused continuous growth of leaf margins and larger leaflets in tomato [51]. Previous studies in *Arabidopsis* indicated that the miR156 overexpression results in the increase in number of leaves, shape and size which can be 10 times higher than normal wild-type [52]. Recent investigation on switchgrass showed that the overexpression of miR156 repressed apical dominance which results into the increase in biomass and number of tiller by 58–100% in genetic modified plants [53]. Correspondingly, in tomato the more expression of miR156, increased the number of branching and leaves, and further enhance the plant biomass but supress the apical dominance [54].

Overexpression of OsmiR397 microRNA enhance rice productivity by increasing number of panicle branching and grain size [55]. Overexpression of miR319 in rice also increases the number of small and longitudinal veins [47]. In rice overstimulation of miR390 miRNA increasing the lateral root formation [56] by decreasing the gene expression of several lateral root growth repressors such as ARF2, ARF3, and ARF4. In *Medicago truncatula*, it was studeid that the overexpression of miR160, regulate the expression of gene which were significant for gravitropic movement and root development and induce several defects in root growth, root apical meristem organization and root nodule formation [57]. It has also been well-documented that miRNAs also play an essential role in controlling transition from vegetative to floral meristem in few crop plants such as *Arabidopsis*, maize and rice. Glossy 15 (APETALA-like gene) in maize mainly control the transition from vegetative to reproductive phase along with the leaf morphogenesis. An additional study on maize demonstrated that the miR172 overexpression leads to the inhibition of glossy15 gene expression which delayed phase transition from vegetative to reproductive [58]. Furthermore, [59] demonstrates that overexpression of miR172 and miR156 promotes the adult reproductive phase however low miR172 and high miR156 expression promote juvenility. Till now various role of miRNAs are studied in economically important crop plants either by increasing or decreasing miRNA expression, the manipulation of miRNA expression can be used for confirmation of miRNA functions and provide an effective way for improving plant growth, development, fruit, and seed development as well as plant biomass and productivity [50].

6.3 miRNA manipulation by genome editing technologies

To access the diverse roles of miRNAs in crops, a number of investigations conducted on miRNAs [45]. Although, due to small size of miRNA, the effectiveness of the well-known approaches for functional loss and inhibiting miRNA expression are comparatively less strong. Mainly, two genome editing techniques with modified nuclease enzyme are significant for selecting appropriate genome alteration [60]. These techniques mainly depend on the production of double-stranded breaks at target sequences by TALENs (Transcription Activator-Like Effector Nucleases) and CRISPR (Clustered, Regularly Interspaced Short Palindromic Repeats) DNA modification methods [61]. CRISPR-Cas9 technology developed as an advance RNA-dependent gene and genome editing tool due to its suitability to a variety of organisms [62]. A previous study on rice demonstrated that Cas9 also guided by modified gRNA for appropriate cut and genome editing of some selected crops [63]. One more study in soybean showed that the CRISPR/Cas9 system is very effective for removing a green fluorescent tagged protein transgene and modifying nine different endogenous loci [64].

CRISPR/Cas9 genome editing technology specifically and strongly decreases the 96% expression of miRNAs [62]. This method has transformed gene editing abilities and has been useful in several model plants such as *Arabidopsis*, tobacco *etc*. and other crops plant such as rice, wheat, maize, tomato and sorghum. But non-coding RNA editing by CRISPR/Cas method in plants is still emerging [65]. Due to the effectiveness of CRISPR/Cas technology, it can be also used as an influential genome-editing tool for genetic modification and functional characterization of plant genes/miRNAs and for genome modification to improve agricultural crops [66].

7. Conclusion and future perspectives

Above mentions exemplified case studies indicated that currently miRNAs viewed as a most essential gene regulator tool. This chapter also focused on the studies that describe the multipurpose role of miRNAs in plants. Recent advancement in biological science made to access and characterize miRNAs in crop plants, with a growing number of researches on the significant function of miRNAs [49]. As described, in plants few important processes like homeostasis, growth and development, vegetative to reproductive phase transition and signal transduction and response to various stress are regulated by miRNA [13]. Few recent studies also showed that in plants miRNAs biomolecule act as plant defense and organizers of immune responses [67]. In this regard, the supervision of miRNA expression levels would recognize a crucial way for enhancing the plant growth and development as well as various biotic and abiotic stresses tolerance.

Various transgenic approaches, focused on miRNA role and its importance and identification on corresponding target genes. These includes miRNA overexpression, tissue-specific expression of the targets or miRNAs or stress induce, artificial miRNA, expression of miRNA resistant target gene and artificial target mimic [68]. In cases where the natural target gene has a harmful effect on plant, the overexpression of the regulatory miRNA decreasing the corresponding mRNA expression [47]. Where the target gene has a positive effect, approaches tracked can involve the artificial target mimics or selection of miRNA resistant target gene and the overexpression of the target genes [37]. Although this approach generally successful, the applied agricultural application of the miRNA methods is interesting since the modification and alteration in complicated multi-genic traits such as productivity

may require alteration in expression of different genes during different developmental stages of plant. However, for improving crop stress tolerance several new strategies used for improving miRNA bases gene regulation in model plants [69].

It is also possible that few miRNAs affect expression of target gene only in some specific cell, and exclusively under specific environmental conditions as several miRNAs may have complicated expression pattern. In this circumstance, analysis of effect of miRNA on expression in whole organs would uncertain; therefore, the experiment should be specifically and carefully designed for improved results. Several artificial miRNAs designed and used to suppress expression of a target gene and protein-coding mRNA of interest is one of the among valuable and suitable approach for plant improvement [70]. Due to insufficient concentration and expression the effect of some crucial miRNAs may not visible in living tissue. For producing plants with desirable characters, it would be essential to execute some quantitative analysis of the natural miRNA(s) of the cells, before designing the artificial miRNAs.

Although, old artificial miRNA approach used as important tool for genetic modification [70], but still there is some more knowledge required for its appropriate use in the differentiation and translational inhibition by miRNA. Single miRNA produced by artificial nucleic acid with the ability to inhibit target loci, avoid and predict all the unnecessary genes for experiment design [52]. By modifying miRNA regulatory pathways, miRNA activity also altered for attaining a desired trait [71]. miRNA action negatively controlled by endogenous RNAs, it is a more flexible and fast way to understand the miRNA function, as well as for manipulation of target gene in plants [72]. This technique offers the way to attenuated miRNA inactivation by significant regulation of native miRNA targets and produce a wide range of phenotypes by change the miRNA decoy site sequence. However, for appropriate crop improvement and proper utilization of this approach, miRNA inactivation at appropriate level and to avoid off-site effects of miRNA which generate some false positive results [73]. Also, the interaction between miRNA and miRNA decoy, as well as the several complex produce by this interaction, is not well known and can causes the miRNA destabilization in organisms. Consequently, for the practical uses of this method in plants, some new and advance modification is needed on the procedure basic decoy-associated regulation of gene expression and miRNA turnover. miRNA and miRNA variants act as important tool in plant improvement, it is also nullifying the side effect produced accidently by genetic engineering technology [73]. If the targeted gene and expression of miRNA is modified, it can produce some pleiotropic alteration in plant development and morphogenesis. Therefore, it is very essential to recognize the miRNA regulation method for plant development, growth and plant responses to different biotic and abiotic stresses. This will simplify the plan of appropriate strategies resulting in the desired traits but with minimum trade-offs in the modified crops.

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