Chapter 3

Small RNAs in Plant Response to Abiotic Stress

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61834

Abstract

Small noncoding (nc) RNAs (sRNAs) are the important posttranscriptional regulatory factors in gene regulatory networks. They are involved in many important processes of plant development and stress responses. Increasingly research data reveal that microRNAs (miRNAs), heterochromatic small interfering RNAs (hc-siRNAs), trans-acting small interfering RNAs (ta-siRNAs), natural antisense small interfering RNAs (nat-siRNAs), repeat-associated small interfering RNAs (ra-siRNAs), and the piwi-interacting RNAs (piRNAs) are involved in heat stress, salt stress, cold stress, and drought stress, which are found in metazoans. Some small RNAs are required for plant thermotolerance and salt tolerance. These findings facilitate our investigation of the genetic basis of plant adaptability to various environmental stresses and the genetic manipulation of plant tolerance to many abiotic stresses. This chapter highlights the recent advances in understanding the crucial roles of sRNAs in plant responses to heat, drought, salinity, and cold and proposes the potential technologies and strategies used to identify abiotic-stress-regulated sRNAs in addition to the recent advances and methods for validation and analysis of their target genes.

Keywords: Abiotic stress, microRNAs, Plants, Small RNAs, Thermotolerance

1. Introduction

Environmental stresses, such as heat, drought, salinity, nutrient deficiency, and low temperature, are the major natural limiting factors for plant growth and crop productivity and thus are the major causes of crop losses worldwide. In recent years, much progress has been made in unraveling the complex and sophisticated molecular mechanisms by which plants have evolved during periods of environmental stresses, and a great deal of attention has been paid to identifying these stress-responsive proteins and their relevant gene networks. Plant-stress responses depend on the precise expression of the genes and their accurate regulation, which is attained by multiple mechanisms at different levels such as transcriptional, posttranscrip-

tional, and posttranslational regulations. Although studies have been mostly focused on the transcriptional level of regulatory mechanisms so far, recent results lead us to the point that posttranscriptional events also play a very important role in gene expression regulation in major scenarios of a plant life, from developmental processes to stress responses. Small noncoding RNAs (sRNAs) are the important posttranscriptional regulatory factors in gene regulatory networks. They are involved in many important processes of plant development and stress responses.

sRNAs are roughly divided into different categories based on the genomic origins of their precursors: microRNAs (miRNAs), trans-acting small interfering RNAs (ta-siRNAs), and natural antisense small interfering RNAs (nat-siRNAs). These sRNAs are loaded into RNA-induced silencing complexes (RISC) and regulate the expression of their relative target genes negatively by affecting the mRNA levels, chromatin remodeling, and DNA methylation. Understanding of sRNA-guided stress regulatory networks should provide us with new tools and vision for the genetic improvement of plant stress tolerance and eventually developing more stress-resistant plants in future.

This chapter highlights the recent advances in understanding the crucial roles of sRNAs in plant responses to heat, drought, salinity, nutrient deficiency and low-temperature stresses, and proposes potential technologies and strategies used to identify abiotic stress-regulated sRNAs in addition to the recent advances and methods for validation and analysis of their target genes.

2. Small noncoding RNAs

The discovery of RNA interference (RNAi) in the late 1990s has been a tornado for the past decade in terms of surprising geneticists for it changed the earlier understanding of the RNA field and the complexity of posttranscriptional control and epigenetic regulation caused by small RNAs. In 1995, Guo and Kemphues used antisense RNA sequence to block the par-1 mRNA in *Caenorhabditis elegans* when they figured out par-1 mRNA is repressed by par-1 mRNA itself [1].

Posttranscriptional gene silencing (PTGS) by RNAs was first reported in plants in 1996. The lin-4 gene, which is known to be essential for the timing of larval development in *C. elegans*, is controlled by a short RNA, which is not translated to any proteins but has a partially complementary sequence to the 3′ region of lin-4 transcript that inhibited the translation of lin-4 to protein [2]. Hence, this discovery even changed the concept of PTGS mentioned in high school biology textbooks as it questioned the central dogma proposed by Francis Crick in 1956, which stated that RNAs carry the biological information encoded in DNA molecules and they subsequently provide the code for translation into proteins [3-4]. This discovery called so much attention that was introduced as the breakthrough of the year when it was published in the journal *Science* in 2002 by Couzin and changed the basic concepts about the gene expression and RNA functionality [5]. This was in agreement with the results published by three independent labs that discovered miRNAs in the model plant *Arabidopsis thaliana* [6-8]. They reported many miRNAs in plants, most of which have a very conserved sequence among

http://dx.doi.org/10.5772/61834

different species. Small RNAs could be much more than what was thought. They can influence almost all the functions in a cell by targeting the transcription factors and key genes.

One of the main reasons that small RNAs called so much attention was that soon after their discovery, the target genes for these small RNAs were reported to be crucial in leaf or flower development, which was consistent with the previous reports [9-13]. They have been found to have an impact on almost all the biological processes in eukaryotic cells as they have a wide range of target genes, which are corresponded to some of the previously identified regulatory genes and transcription factors that proved to play key roles such as in controlling cellular metabolism, growth and differentiation, phase transition timing and leaf patterning, and defense mechanisms against biotic and abiotic stresses in case of plants. These 18–25-nucleotide (nt) RNAs are categorized into many different classes based on their size, their biogenesis pathway, and their mode of action.

sRNAs are short nucleic acid sequences that give rise to the assembly of protein–RNA complexes, which later are able to repress the expression of their identified target genes by sequence-specific base pairing. This silencing of the target sequence can occur through several ways by (1) reducing their rates of transcription, (2) reducing the stability of their mRNAs in the cell, or (3) reducing the translation of their mRNAs into protein.

Although much of the work on ncRNAs field has been focused on small RNAs of under 40 nucleotides long, there are larger ncRNAs called mRNA-like ncRNAs (or mlncRNAs)that have received much less attention and have been reported to play some roles in some of the plant functions such as phosphate starvation response and nodulation. The article by Rymarquis et al. explains about them [14].

The generation of sRNAs involves a set of evolutionary conserved proteins, such as Dicer (DCR) or Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerase (RDR), which all together form the RNA silencing machinery in plants. The DCLs have been the most studied enzyme so far, which, in *Arabidopsis thaliana*, are classified into four groups: DCL1 acts during miRNA metabolism, DCL2 is responsible for the viral resistance, DCL3 triggers the transcriptional silencing, and DCL4 cooperates in posttranscriptional silencing and ta-siRNA metabolism (dissecting *Arabidopsis thaliana* dicer function in small RNA processing, gene silencing, DNA methylation patterning, and nature genetics). Plant genomes encode only one of the three known classes of AGO proteins, namely AGO1, which is involved in both miRNA and sRNA biogenesis.

The sRNAs are categorized into different classes based on their size, their biogenesis pathway, and their mode of action to at least six groups, including microRNAs (miRNAs), heterochromatic small interfering RNAs (hc-siRNAs), trans-acting small interfering RNAs (ta-siRNAs), natural antisense small interfering RNAs (nat-siRNAs), repeat-associated small interfering RNAs (ra-siRNAs), and the piwi-interacting RNAs (piRNAs), which are found in metazoans.

2.1. miRNAs

Typically, miRNAs are derived from single-stranded RNA precursors that are transcribed by RNA polymerase II from MiRNA genes called primary microRNA transcript (pri-miRNA),

which are capable of forming a self-complementary fold-back structure named hairpin or stem loop in which the mature miRNA could reside on the 3′ or 5′ end (Table 1). This imperfect double-stranded structure is further recognized and processed by DCL1 in association with other protein factors [15]. This gives birth to the miRNA/miRNA* duplex which based on the thermodynamic features will have a different fate but usually the pre-miRNA strand is loaded onto an AGO1-containing, RNA-induced silencing complex (RISC) and the miRNA* strand lives for a short time in the cell. Mature microRNAs (miRNAs), which are the so-called hairpin-derived RNAs, are 20–24 nt long and single stranded while miRNA genes are 70–300 nt long. Mature miRNAs help the target recognition and cleavage in cooperation with AGO1 and miRISC. The first cleavage by DCL1 generates a stem-loop intermediate, called the precursor miRNA (pre-miRNA), and the second cleavage by DCL1 releases the miRNA duplex, one strand of which is known as mature miRNA and the other strand is known as miRNA* (miRNA star).

Class	Full Name	Originating Loci	Function	Biogenesis
miRNA	microRNA	*MIRNA* genes	Repress target gene expression through mRNA cleavage and translational repression	The fold-back structures of long ssRNA transcripts are cleaved by Dicers
siRNA	short-interfering RNA	Repeats, transposons, and retroelements (endogenous). Transgenes and viral RNAs (exogenous)	Silence repeats and transposons through RNA-dependent DNA methylation and chromatin modification	RDR-generated dsRNAs are cleaved by Dicers
ta-siRNA	trans-acting siRNA	TAS loci	Repress target gene expression through mRNA cleavage	TAS transcripts are cleaved by miRNAs, transcribed by RDR into dsRNA, and then processed by Dicers
nat-siRNA	natural antisense transcript-derived siRNA	Loci producing pairs of sense-antisense transcripts	Stressed-induced nat-siRNA to repress target gene expression through mRNA cleavage	The dsRNA derived from overlapping transcripts is cleaved by Dicers
piRNA	piwi-interacting RNA	Repeats, transposons, and retroelements	Germ-line-specific piRNA to suppress repeats and transposons in flies and mammals	ssRNA derived from transposons is cleaved by PIWI protein

Table 1. Small RNAs involved in plant response to abiotic stresses

Mammals use only one class of RNAse III enzyme, Dicer, to generate both miRNAs and siRNAs. In plants, there are a variety of specialized DCL endonucleases, which are classified into 10 categories. DCL1 is involved in miRNA biogenesis pathway while other DCLs

participate in various aspects of sRNA-mediated generation or gene silencing pathway (Figure 1) [16].

All the information about the reported miRNAs and their sequences and annotations are stored in a database called miRBase (www.mirbase.org), which is updated on a regular basis with the new published data in the literature [17]. So far, there are 205 precursors and 384 mature miRNAs reported in the model plant *Arabidopsis thaliana*, which is about 1–2% of its genome. Theoretically, the perfect base pairing between miRNAs and complementary target mRNAs helps the process of finding target genes for each miRNA. By computational methods, there are a plenty of databases that are able to predict the potential target genes for each newly found miRNA.

2.2. siRNAs

siRNAs were first identified in 1999 in plants [18], and later there were many reports about diverse sets of endogenous siRNAs in plants as well as in animals (Table 1) [6] [8][19][20-22]. Most of the plant siRNAs are around 24 nt in length that are excised from the long double-stranded RNA duplexes or transcripts generated from inverted repeat regions [23-24]. The sources of these double-stranded sequences that eventually trigger biogenesis of siRNAs could be endogenous or exogenous. Endogenous plant siRNAs can be classified into several categories, including miRNA-induced trans-acting siRNAs (tasiRNAs), natural antisense siRNAs (nat-siRNAs), cis-acting siRNAs (casiRNAs), heterochromatic siRNAs, and many other unclassified small RNAs [25]. In plants, ta-siRNAs are generated from the genomic loci named TAS genes, which are transcribed by RNA-pol II. The generation of ta-siRNAs is triggered by an miRNA, which cleaves a nonprotein-coding transcript of a tasiRNA gene [26-29]. In plants, there are eight TAS loci reported so far, which belong to four families (TAS1–4). TAS1 and TAS2 families are cleaved by miR173 with the association of AGO1. TAS3 family transcripts are cleaved by the guidance of miR390 and AGO7 and usually target the auxin response factor (ARF) transcripts. TAS4 transcript is cleaved by miR828 guided together with AGO1 and they usually target myeloblastosis (MYB) transcription factors [30]. These cleaved RNAs are then processed by the suppressor of gene silencing 3 (SGS3) and copied into double-stranded RNAs by RNA-dependent RNA polymerase 6 (RDR6). DCL4 cleaves them in multiple rounds so that it finally gives rise to the 21-nt ta-siRNAs. ta-siRNAs are loaded onto AGO1 complex to degrade the target mRNAs [31].

The other class of siRNAs called nat-siRNAs are separated into two groups: cis-nat-siRNAs that are generated from two RNAs, which were transcribed from the same loci but opposite strands, and trans-nat-siRNAs, which were transcripts from different loci [32]. RDR6 and DCL2 are involved in generating 24-nt nat-siRNAs, and RDR6 and DCL1 are involved in generating 21-nt nat-siRNAs. trans-nat-siRNAs are transcripts from different loci but processed by the same proteins (RDR6 and DCL2). ta-siRNAs cleave the target mRNAs by being partially or fully complementary with them.

Heterochromatic siRNAs mostly originate from transposable elements or repeats and their mode of action is slightly different from miRNAs and ta-siRNAs, as they modulate the histone

modification at their homologous regions in the genome and inhibit the gene expression at the transcriptional level.

2.2.1. nat-siRNAs (natural antisense siRNAs)

Natural antisense transcripts (NATs) are small RNA molecules, which are endogenous and show partial or entire complementarity to other transcripts (Table 1). cis-NATs are categorized in the nat-siRNAs group and are transcribed from the same genomic loci but in the opposite strand of DNA as their sense transcripts. This class of NATs is very common in eukaryotes (l7–30% of the genes encode complementary cis-NATs in animals and plants) [33-37]. In animals, NATs are involved in alternative splicing, DNA methylation, RNA editing, and genomic imprinting [38-41]. In plants, several cis-NATs are involved in gene regulatory mechanisms [42-43]. There are already some reports about the identified cis-NATs in *Arabidopsis* and rice on the genome-wide scale [44-46].

2.2.2. ta-siRNA (trans-acting short interfering RNAs)

ta-siRNAs are 21 nt in length and are reported to be found only in plants so far (Table 1). ta-siRNAs originate from a noncoding RNA precursor, which is initially targeted to be cleaved by an miRNA molecule. RNA-dependent RNA polymerase converts the cleaved products into double-stranded RNA molecules, which are later cleaved again into 21-nt ta-siRNAs. Hence, the formation of these RNAs is determined by the presence of both miRNA (Dicer-Like1, Argonaute1, HYPONASTIC LEAVES1, and HUA ENHANCER 1) and siRNA (RNA-dependent RNA polymerase 6 and DCL4) biosynthesis pathways components. ta-siRNAs can guide cleavage of target mRNAs and regulate gene expression at the posttranscriptional level like plant miRNAs.

2.3. Small RNAs in abiotic stress

Abiotic stress is known to be one of the attention-calling factors globally, which causes a considerable yield loss each year. Hence, much effort has been made in understanding the complex stress-response mechanisms, especially in the identification of stress-responsive protein-coding genes. But, in recent years, after the discovery of small noncoding RNAs, they have been found to be involved in plant stress responses and indeed very functional players in these pathways. These small RNAs regulate the gene expression in different levels and hence are entangled within all the vital pathways in the plant development, metabolism, and stress response.

As sessile organisms, plants have evolved their specific adaptation and acclimation mechanisms in order to survive during the hard spell. To do the morphological and physiological adaptations to abiotic stresses, the plant needs to manage the complicated rearrangement of gene expression networks, which are controlled at transcriptional and post-transcriptional levels. The concern about future food shortages makes it imperative to better understand the genetic control of stress tolerance networks and pathways and to use this knowledge to increase the total tolerance of important crop species. As the first step, we have to understand

http://dx.doi.org/10.5772/61834

the complex responses of the plants to stress, from changes in molecular level to physiological level.

With the help of high-throughput gene expression analysis, there have been many reports about the modulated genes and different small RNAs under abiotic stresses. The difference in the expression level of these genes could indicate that it might be responsive to stress condition and, as a result, it can help the plant to survive the hard condition. Many studies have been published in this regard in various plant species, some of which having an economic importance, like rice, wheat, legumes, barley, sugarcane, potato, and tomato as well as many other species. Some of the genes are induced after facing the stress conditions in these studies while some are downregulated, which is connected with the roles these genes play during the stress condition. Also, the respective miRNAs or other sRNAs that target these mRNAs show a different expression pattern during the stress condition. sRNAs, which are accumulated by stress, might downregulate their target genes and act as a negative regulator of stress tolerance; for instance, the genes involved in cell expansion and division should be downregulated as the plant needs to save energy in order to pass through the hard environmental condition. On the other hand, reduction of sRNA level might lead to upregulation of their target genes, mRNAs, which positively regulates the stress tolerance.

The molecular basis of plant tolerance to abiotic stresses and stress regulation of small RNAs has been studied using different methods to observe the altered expression of these molecules and their related target genes; for instance, the sequence analysis of small RNA libraries before and after the stress condition, microarray data analysis, mutagenesis, and RNAi. Their reports have identified numerous genes and sRNAs that are induced by applying different stress conditions, which is the material for the next step: making transgenic plants and check if these overexpressed transgenics could exhibit an improvement in stress tolerance. But the fact is that even though some of the genes and small RNAs show altered expression under stress, they do not play any role to make the plant more tolerant to the stress. And the reason is largely because of the complex genetic interactions underlying the plant tolerance toward stress, which are still to be understood.

From transcriptomic studies, we know that the stress conditions such as heat, drought, cold, and salt evoke the expression of an overlapping set of genes, suggesting that their signaling transduction pathways share common control points. Most of the genes, which are detected to be responsive to abiotic stresses, are usually the genes that regulate plant development and reproduction (as the plant faces the urge to save more energy for producing viable seeds rather than a high biomass), also senescence-related genes (as to recycle the nutrients from the old leaves to younger leaves and reproductive parts and wasting less water and energy for them), as well as the genes that are involved in the abscisic acid (ABA) pathway, which play a crucial role in plant growth and development pathway and redox pathway.

RNA interference technology is one of the potential reverse genetics tools for understanding the functional significance of these genes and their respective regulatory sRNAs. The information about stress-induced genes and sRNAs including the sequence and annotation could be found in the genome databases like National Centre for Biotechnology Information (NCBI) or stress complementary DNA (cDNA) databases. In addition to these stress-induced genes,

the regulatory elements for these genes are also altered during stress condition including small regulatory RNAs. A number of these sRNAs, which are induced in different plant species and under different stress conditions, can be found in some recent review papers [47]. A vast amount of data has been published about the expression profiling of different sRNAs in various stress conditions. Although these expression-profiling experiments can provide us with some clues about the involvement of these sRNAs in gene regulation under those specific circumstances, to find the relevance of each of these sRNAs in imparting stress tolerance in plants can only be studied by functional genomic approaches like gene overexpression or downregulation. RNA interference technology using constructs transcribing self-complementary hairpin RNA is one of the reverse genetics approaches to downregulate genes in plants.

Another powerful technique to learn about gene functions in a developmental or physiological context in plants is by mutagenesis and to isolate the corresponding mutants with altered phenotypes. Various mutagenic agents, including chemical and biological, have been widely used in this regard, each of them with its own advantages and inconveniences.

For *Arabidopsis thaliana*, the genome sequence is publicly available; hence, relying on reverse genetics to understand the relevant roles of genes is currently a common practice. There are specific screening methods used in order to measure the effect of each stress on the overall plant physiology after the treatment as well as the methods used for inducing the specific stress condition in the plant, which have been studied and reviewed many times.

2.4. Drought stress

Drought stress is known as the most significant stress especially with regard to the climate change and global warming. It restricts plant growth and development severely, while tolerant plants are able to survive by several mechanisms such as consuming small amounts of water or keeping their stomata closed at a high rate under drought conditions.

Approximately two-thirds of the potential yield of major crops are lost every year due to the adverse growing environments [48]. A worldwide increase in arid areas, including the Mediterranean basin, has been predicted by the International Plant Protection Convention (IPPC) in 2001 and 2007. Water deficit also leads to salinity stress in many cases, which makes the growth situation even harder for the plants. Therefore, it is regarded as the most important abiotic stress and it is necessary to develop strategies toward sustainable use of water and improve plant-drought resistance [49]. Many genes have already been studied and reported to be involved in the drought-resistance response network in the plants. But in recent years, it has become clear that sRNAs play pivotal roles in stress responses as well in regulating the expression of resistance genes.

MiRNA-expression profiling under drought stress has now been performed in *Arabidopsis*, rice, and *Populus trichocarpa*, and many other plants under drought-stress conditions and some of the miRNAs were shown to be responsive toward this stress in different plants, some of which can be reviewed in the available literature [50-51].

Another group has worked on miRNA expression patterns of drought-resistant *Triticum turgidum* ssp. *Dicoccoides* in response to drought stress, using an miRNA microarray platform

[52]. MiR474, which targets proline dehydrogenase (PDH), was upregulated during drought stress in Zea maize [53]. Zhao et al. worked on miR169g and miR393 under drought condition in rice, while miR393 was conversely induced by drought [54]. Many other recent reports used several different methods to study the expression pattern changes of miRNAs in different plants under the stress condition [47].

2.5. Salt stress

Crops worldwide are threatened by excessive soil salinity due to the accumulation of salt delivered along with irrigation water and by coastal flooding and the high evapotranspiration rates caused by climate change. About 6% of the total arable land in the world is affected by excess salt [55] and it has been predicted to increase to about 30% of the world's arable land by 2025 and 50% by the year 2050 [56]. Several genes and pathways in plants are affected by salt stress [57]. Hence, the promising approach to address the problem of soil salinity is to increase the understanding of response of plants to salinity-related stress. These genes are mostly involved in signal transduction, activation of ion channels, and growth-factor-regulated modification of plant architecture, and, in particular, root morphology.

Besides the genes, numerous differentially regulated miRNAs have also been identified in salt-stressed plants. For instance, miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394, miR396, and miR397 were all reported to be overexpressed in response to salt stress in *Arabidopsis*, while the accumulation of miR398 was downregulated [50].

miR169 was also reported to be induced by high salinity stress [46]. The authors found a cis-acting ABA-responsive element (ABRE) in the upstream region of miR169n, which suggested that miR169n might be regulated by ABA. Another group used microarray experiments as a method to explore the miRNA profile of maize in different lines (salt-tolerant and salt-sensitive); finally, it was reported that the expression levels of miR156, miR164, miR167, and miR396 family members were downregulated considerably, while it was increased in miR162, miR168, miR395, and miR474 families after salt-shock in root tissue [58].

2.6. Cold stress

Some plants increase their tolerance to cold in order to deal with the low temperatures. This phenomenon is known as cold acclimation. In recent years, many cold-regulated genes have been identified in plants under cold stress. The C-repeat binding factor (CBF) cold-responsive pathway was considered as the most known cold tolerance pathway in plants [59]. There are three CBF/DREB1 family members, including CBF1, CBF2, and CBF3 (DREB1b, DREB1c, and DREB1a, respectively), encoding the DNA-binding proteins of Apetala2/ethylene responsive factor (AP2/ERF) family [60]. Also, the expression of many miRNAs in cold stress has been examined in different plants including *Arabidopsis thaliana*. Several miRNAs belonging to different families were reported to be upregulated under cold-stress condition in *Arabidopsis thaliana* (miR165/166, miR393, miR396, and miR408), while some other miRNAs (miR156/157, miR159/319, miR164, miR394, and miR398) were shown to be either transient or mildly regulated under cold-stress treatment condition [19][50]. In another report, the expression

levels of miR168 and miR477 family members were increased after the cold-stress treatment, while miR156, miR475, and miR476 members were downregulated in *Populus* plants [55][19].

2.7. Heat stress

The average temperature of our planet is rising year by year because of the climate change. As a result, changes in the patterns of rainfall, droughts, and submergence stress are induced to the natural environments. Heat stress even alters the distribution and productivity of important crops negatively throughout the earth. A temperature rise of −5°C above the plant's optimum temperature is considered as a heat stress. It disrupts normal functions of cellular processes, may lead to delay in plant growth and development, and it might even result in death of the plant, but, usually, high temperatures result in water deficiency, which eventually leads to increase in salt concentration. Recent studies indicate that the projected global warming in the upcoming years will negatively affect the yield of important crops; hence, the necessity of focusing on gene networks and their regulatory components becomes obvious.

A major component with regard to responding to heat stress is the induction of heat shock proteins (HSPs), which get activated by heat shock transcription factors (HSFs). There are five classes of HSPs based on their molecular weights: HSP100, HSP90, HSP70, HSP60, and small heat shock proteins (sHSPs, 15–30 kDa). On the other hand, HSFs recognize heat stress elements on the promoter of heat stress-responsive genes (HSE: 5′-GAAnnTTC-3′). Plant HSFs are categorized into three classes based on their oligomerization domains (A, B, and C) [61].

However, the more upstream regulators of HSFs remain to be identified. Guan et al. have reported that miR398 is rapidly induced by being subjected to heat stress while its target genes (CSD1, CDS2, and CCS) are downregulated. They further reported that the expression levels of HSF and HSP genes in *csd1-*, *csd2-*, and *ccs*-mutant plants are increased under heat stress, and *csd1*, *csd2*, and *ccs* plants are more tolerant to heat stress than wild-type plants. They identified two HSFs, which act upstream of miR398, suggesting that this pathway is an essential regulatory loop for plant thermotolerance [62].

Based on deep sequencing experiments, Wang et al. suggest that there is a new class of small RNAs that originate from the chloroplast genome, which are responsive to heat stress [63]. They performed RNA sequencing (RNA-seq) and found 1031 cis-NATs in *Brassica rapa* based on the homology with *Arabidopsis* and 303 conserved cis-NATs, which correspond to the ones in *Arabidopsis* [64]. TAS1 (trans-acting siRNA precursor 1) targets, derived from small interfering RNAs named heat-induced TAS1 target1 (*HTT1*) and *HTT2*, are involved in thermotolerance [65]. *HTT1* and *HTT2* genes were highly upregulated in *Arabidopsis thaliana* seedlings in response to heat shock based on their microarray analysis. TAS1a has a trans-acting small interfering RNA, which targets the *HTT* genes. Overexpression of TAS1a accelerated the expression of TAS1-siRNAs and decreased the expression levels of *HTT* genes that eventually led to weaker thermotolerance. Conversely, stronger expression of *HTT1* and *HTT2* genes upregulated various *Hsf* genes, helping the plants to achieve a stronger thermotolerance. In HsfA1a-overexpression transgenic plants, which present a higher tolerance to heat stress, the *HTT* genes were upregulated. In the meantime, HsfA1a was shown to bind to the *HTT1* and

HTT2 promoter regions and activate them directly. Finally, they proposed that HTT1 interacts with Hsp70-14 and Hsp40, nuclear factor Y, and subunit C2 complex.

Wheat miRNAs showed differential expression in response to heat stress; by using Solexa high-throughput sequencing, Xin et al. cloned the small RNAs from wheat leaves treated by heat-stress gene [66]. Stief et al. also reported that miR156 is responsible for heat stress memory in *Arabidopsis* [67].

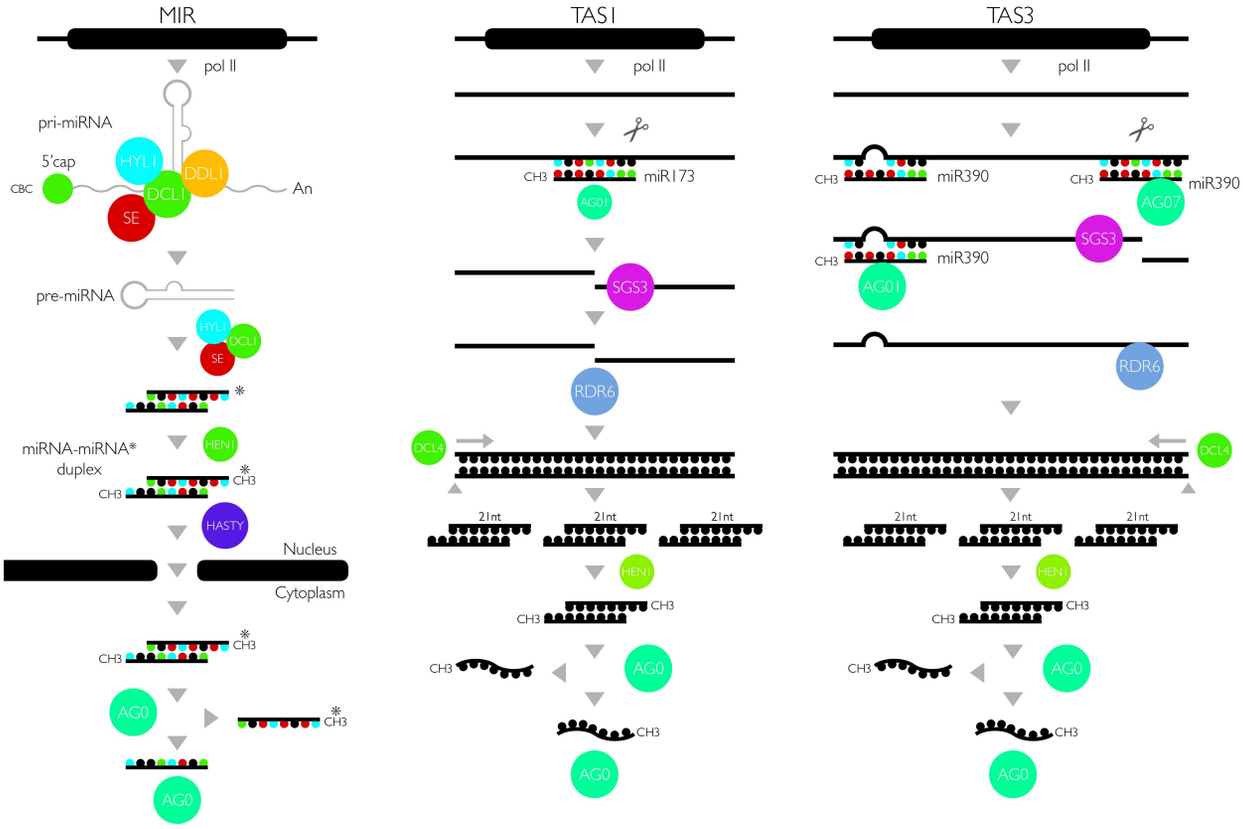

Figure 1. Biogenesis of miRNAs and ta-siRNAs

3. Perspective

Physiological responses to stress are controlled by expression of a large number of genes, many of which are regulated by microRNAs. At the molecular level, identification of stress-responsive genes is an initial step toward understanding plant stress response as pyramiding of different genes in the same plant is an option for achieving better stress tolerance. Although finding genes and sRNAs, which show induction by stress, is an important step toward stress tolerance improvement, most of the studies in which they use transgenics only show the importance of the introduced transgene and not the overall metabolic effects that the trans-host gets exposed to. On the other hand, the new stress-tolerant transgenic lines should have

no or few undesired phenotypic changes plus a minimal yield penalty. In stress-tolerant transgenics, which are introduced so far, a constitutive promoter has been used for expressing the transgene in most of the cases. These transgenes must be utilized to overcome the problem of yield penalty and growth retardation in these experiments. Admittedly, most reports published on stress-tolerant transgenic plants are based on the limited characterization of the stress condition as well as the tolerant phenotypes. Adequate assays for phenotyping of the stress-tolerance trait must be undertaken under natural stress conditions. Overall, there is a lack of uniformity in the stress induction regimes applied by various research groups, which makes the comparisons of the responses among different reports difficult and this fact must be taken into consideration.

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