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## Stress Responsive Non-protein Coding RNAs

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Additional information is available at the end of the chapter

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### Abstract

Environmental or growth conditions are constantly influencing the development and maturation of organisms. To ensure precise control of gene expression under these normal as well as stress conditions, organisms developed very accurate molecular regulatory mechanisms at transcriptional and posttranscriptional levels. Key roles of small noncoding RNAs (miRNAs and siRNAs) in regulation of gene expression are no longer astonishing us nor are constantly emerging new classes of non-protein coding RNAs (tRNA-derived small RNAs, snoRNA-derived small RNAs, mRNA-derived small RNAs, and long non-coding RNAs). Although there is evidence of involvement of those various classes of noncoding RNAs in response to stress conditions, in most cases, we are still lacking basic information on their targets and interplay between different regulatory pathways. In this review, we focus on “canonical” and “noncanonical” noncoding RNAs and their involvement in abiotic stress responses.

**Keywords:** Noncoding RNAs, abiotic stress, crops, yeast

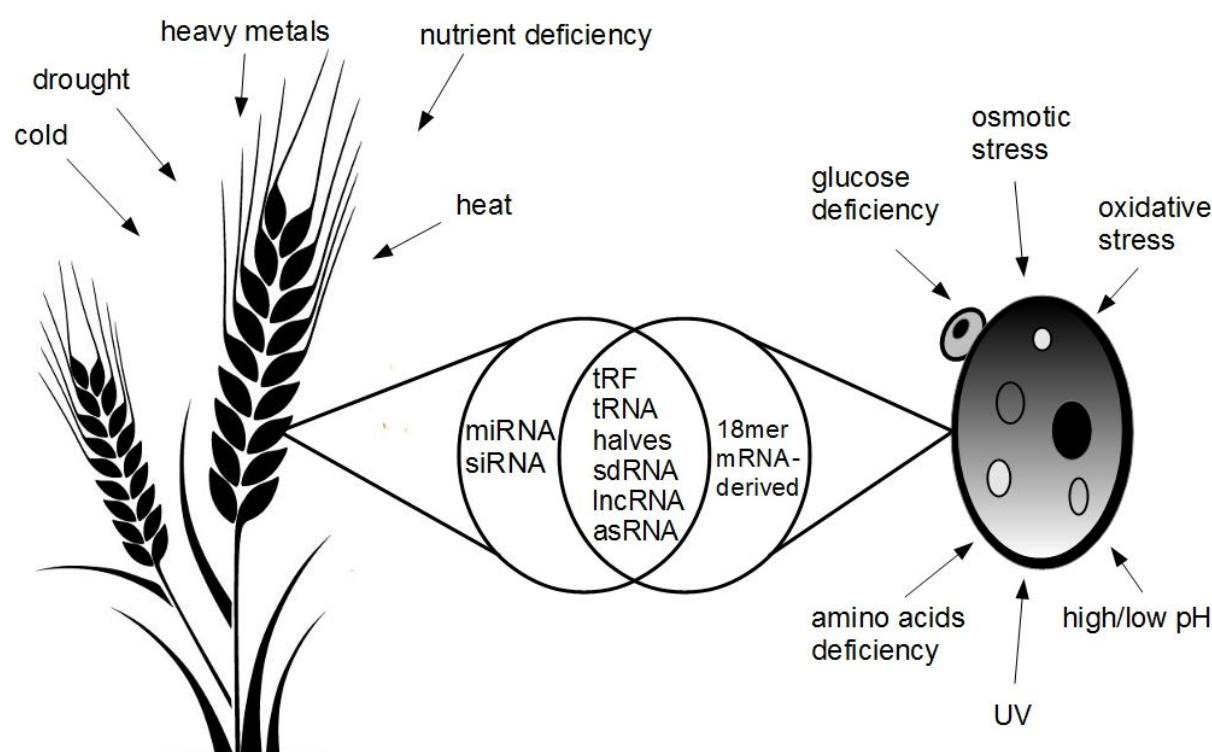
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### 1. Introduction

Biological organisms exposed to any external circumstances that exert a detrimental influence on them are capable of establishing mechanisms of protection and adaptation. To minimize stress influence, several different strategies can be applied, these are tolerance, resistance, and avoidance or ultimately escape. Because of their sedentary lifestyle, plants are restricted to tolerance, resistance, and avoidance mechanisms only, and thus require short-term strategies to quickly and efficiently readapt their metabolism [1]. Therefore, to ensure their survival under unfavorable conditions plants have established refined adjustments to stresses at all levels (anatomical, morphological, cellular, biochemical, and molecular) [2]. On the other hand, microorganisms, such as industrially important baking yeast, have specific and delicately

balanced internal conditions for optimal growth and function. Industrial applications expose yeast to multiple unfavorable environmental conditions, therefore accurate stress response is key for yeast cells. When environmental conditions change abruptly, yeast cells rapidly adjust its internal balance to that required for growth in the new conditions.

The physiological and biochemical changes in plants under stress conditions are a result of alteration in abundance of many transcripts and therefore proteins [3] pinpointing the role of TGS (transcriptional gene expression) and PTGS (post-transcriptional gene expression) in the adaptation to environmental changes. To achieve precise and timely regulation of stress-responsive gene networks in addition to regulatory DNA sequences and different types of proteins, plants, as well as many other organisms, including yeast, have employed the gene expression regulation by RNA molecules.



Differentially expressed RNAs are involved in regulation of plant and yeast metabolism pathways during development as well as stress conditions. Baking yeast are depleted of RNAi dependent molecules (miRNA and siRNA) that are present in plants.

**Figure 1.** Overview of different classes of ncRNAs associated with response to abiotic stress conditions in plants and yeast.

For many decades RNA was considered to be a passive intermediate in the flow of information from DNA to protein, apart from other well-known and described functions in translation (tRNAs, rRNAs) or splicing (UsnRNA). In 1998, its new role has been discovered and regulatory capacity of RNA molecules immensely broadened our understanding of gene expression processes. Moreover, new sequencing technologies and high-resolution microarray analysis

have revealed genome-wide pervasive transcription in many eukaryotes, generating a large number of RNAs of no coding capacity. Now, nearly 20 years after the first discovery of the RNA interference, we know that small non-coding RNAs not only regulate gene expression on post-transcriptional and transcriptional levels, but can also affect the organization and modification of chromatin. Moreover, they regulate growth and development of organisms, organ development, hormone signalling, and the defense against pathogens [4, 5]. They also function during environmental stress adaptation, providing precise regulation of gene expression.

Herein, we wish to describe the roles of non-protein coding RNA molecules during abiotic stresses in industrially important organisms, such as crops and yeasts. We wish to focus on “canonical” small RNAs, such as miRNAs and siRNAs, but also new classes such as mRNA-derived RNAs, tRNA-derived small RNAs, snoRNA-derived small RNAs (sdRNAs), mRNA-derived small RNAs, and long non-coding RNAs (lncRNAs) (Figure 1).

## 2. Canonical small RNAs

In plants, small RNAs exhibit unexpected complexity, but two main categories can be distinguished based on their biogenesis and function and these are miRNAs (microRNAs) and siRNAs (short interfering RNAs). Baking yeast lack these two categories of small RNAs since their biogenesis and functions involve RNAi machinery, which is absent in *S. cerevisiae*.

### 2.1. miRNAs

miRNAs are predominantly 21 nucleotides in length [6] and they are derived from 70–500 nucleotide long, imperfect, hairpin-like structured primary transcripts called pri-miRNAs by the activity of RNase III dicer-like (DCL) enzyme [7]. DCL1 processes primary miRNA transcript into an miRNA-miRNA\* duplex with 2nt overhangs at the 3' end [6]. With the help from hyponastic leaves (HYL1) and SERRATE proteins miRNA duplex is released and then Hua Enhancer 1 (HEN1) methylates 3' ends of the duplex and therefore stabilizes it by preventing uridylation and subsequent degradation. HASTY, the plant homolog of exportin 5, transports the miRNA duplex into the cytoplasm [8, 9]. The mature miRNA is loaded into RISC (RNA-induced silencing complex) and guides the translational inhibition or cleavage of target mRNAs through the action of AGO proteins in a sequence-specific manner [8–10].

To date, many stress-responsive miRNAs have been identified and the vast majority of their targets are transcriptional factors or proteins playing important roles not only in developmental and growth processes but also in biotic and abiotic stress responses [11]. There appears to exist common pathways of stress responses based on the induction/reduction of similar small noncoding RNA entities. It has been shown that different stress conditions trigger similar responses in various plant species; but on the other hand, the plant's response to similar stress condition could trigger the expression of different pools of miRNAs even in different varieties of the same plant species.

Drought and salinity are major external factors influencing crop productivity and seed quality worldwide. Understanding plant's responses to these stresses is therefore of high importance and one of the main research topics nowadays. Several studies showed differential expression profiles of many genes during water deficit conditions [12, 13]. Recent efforts have also led to the identification of many miRNAs altered in response to drought in many plant species such as cowpea [14], soybean [15], or *Triticum dicoccoides* [16]. Strikingly, the plant response varies in different species, depending on the habitat or plant family, resulting in majority of miRNAs having differential accumulation patterns; nevertheless, some miRNAs share common accumulation pattern as shown below. The differences observed are not only attributable to plant species or growth conditions but could also result from different genotypes in the same species. Soybean seedlings representing two different genotypes (drought-sensitive and tolerant) showed an increase in the level of five miRNAs (miR166-5p, miR169f-3p, miR1513c, miR397ab, and miR-seq13) [15]. In wild emmer wheat, 13 miRNAs were found to be differentially regulated upon drought (miR1867, miR896, miR398, miR528, miR474, miR396, miR894, miR156, miR1432, miR166, and miR171) [16]. Interestingly, some miRNAs share common accumulation pattern; for example, miR474 has also been shown to be upregulated in maize under water deficit [17]. Genome-wide approach allowed to identify 30 miRNA families significantly down- (16: miR156, miR159, miR168, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088, and miR1126) or upregulated (14: miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026, and miR1125) under the drought stress in *Oryza sativa*; strikingly 9 miRNAs (miR156, miR168, miR170, miR171, miR172, miR319, miR396, miR397, and miR408) showed opposite expression patterns to previously described in drought-stressed *Arabidopsis* [18]. Some miRNA gene families were identified in both down- and upregulated groups (miR171, miR319, miR896). One miRNA that is consistently being upregulated during drought is miR393. The increase in the level of this microRNA has been observed for rice, *Arabidopsis*, *Medicago truncatula*, and *Phaseolus vulgaris* [19–23].

It is established that somewhere between 6% to 20% of worldwide cultivated arable land is affected by excessive concentrations of salt [24–26]. Some cellular and metabolic processes observed in plants during salt stress conditions (as a result of the limitation of the plant's ability to uptake water) are similar to those observed during drought [24]. Nevertheless, there are many genes and pathways that discriminate these two stress conditions [12, 28]. In salt-tolerant and salt-sensitive *Zea mays* lines, microarray hybridization experiments led to the identification of 98 salt-responsive miRNAs representing 27 families. Several microRNAs showed differential accumulation levels upon salinity in roots: members of miR156, miR164, miR167, and miR396 families were downregulated while miR162, miR168, miR395 and miR474 were upregulated [28]. In rice, other microRNAs appear to be involved in response to salt stress, these are miR169g, miR169n, miR169o, as well as miR393 [29, 30]. These microRNAs target the transcript of NF-YA gene encoding for a subunit A of a conserved transcription factor previously shown to be downregulated in drought-affected wheat [31]. Three microRNAs (miR414, miR164e, and miR408) have been established to be downregulated under salinity in rice [32]. Although, their target genes OsABP (ATP-binding protein), OsDBH (DEAD-box helicase), and OsDSHCT (DOB1/SK12/hely-like DEAD-box helicase), were for the first time



validated and upregulated in response to salt stress their functions still need to be experimentally confirmed.

Cold stress is one of the most severe abiotic stresses. The disorders triggered by low temperatures depend on the duration of stress condition and the rate of temperature decrease. Cold stress includes chilling (above 0°C) that inhibits water uptake and freezing (below 0°C) that induces cellular dehydration and thus osmotic stress and hyper accumulation of reactive oxygen species [33]. There are several factors (genes and transcription factors) playing important roles in cold stress acclimatization [10, 34–37]. Post-transcriptional mechanisms of cold acclimation include pre-mRNA processing, mRNA stability, and export from the nucleus. During the last few years, an emerging role of microRNAs in cold stress acclimatization and responses began to unveil. Cold, as well as ABA, dehydration, and salt stress upregulate the expression of miR393, miR397b, and miR402. In accordance with the upregulation of miR393 under cold stress, its target, putative E3 ubiquitin ligase SCF complex F-box protein, appears to be downregulated by cold stress in Arabidopsis [38]. Thus, during cold acclimation, the cleavage of E3 ubiquitin ligase mRNA would lead to diminished proteolysis of its targets (probably positive regulators of cold stress). Moreover, one of the targets of miR393 encodes an F-box protein (At4g03190) [39, 40], which is similar to glucose repression resistance 1 (GRR1), a yeast protein involved in glucose repression. Plants can use sugar status as a signal to modulate growth and development in response to abiotic stresses since it has been observed that sugar metabolism is affected by various abiotic stresses. It has been speculated therefore that miR393 could integrate sugar signaling with cold stress responses [41]. Moreover, in sugarcane, it has been observed that miR319 is differentially expressed during cold treatment for different periods of time, in both shoots and roots [42]. These data are in accordance with previous reports showing that miR319 was upregulated in response to cold stress in Arabidopsis [39, 40]. Genome-wide experiments in *Prunus persica* led to the identification of miR156, miR159, miR164, miR167, miR172, miR393, miR396, miR414, miR2275, and miR5021 as cold responsive [43, 44]. They are regulating genes involved in flower and leaf developmental processes. In rice, 18 miRNAs have been shown to respond rapidly to cold stress conditions (miR156, miR166, miR166m, miR167a,b,c, miR168b, miR169e, miR169f, miR169h, miR171a, miR535, miR319a,b, miR1884b, miR444a.1, miR1850, miR1868, miR1320, miR1435, and miR1876). Most of them are downregulated, which suggests that their targets are being upregulated in adaptation to cold stress. Interestingly, there were four miRNAs (miR1435, miR1876, miR1320, and miR1884) present in rice, but not Arabidopsis, in response to cold stress, which suggests a species-specific mechanisms [10]. miR171 is a large family consisting of 12 known members. In Arabidopsis, miR171a was induced 6 h after cold treatment [40]. On the other hand, miR171a in rice (rice and Arabidopsis share the same mature sequence) was significantly downregulated. Moreover, the expression levels of the other three miRNAs (e, f, i) were also decreased in rice in response to cold. In contrast, miR171 c, d, and h were induced after 6 h [10]. The difference in expression pattern as in the case of miR171 could be reflective of divergence in response of particular plant species to cold stress.

miRNAs also appear to play crucial roles in regulation of nutrient homeostasis. There are 14 mineral elements essential for plant growth and development. Phosphate (Pi) is one of the most prominent ones; it is involved in phosphorylation reactions, synthesis of nucleic acids,

membrane lipids, but also in energy delivery and often is a limiting nutrient for plant growth. The phosphate acquisition from soil particles requires the secretion of plant enzymes that release phosphate, then phosphate transport through Pht1 family of transporters (phosphate transporters protein family 1). Phosphate homeostasis in cells is regulated through the action of PHO1 and PHO2 proteins, transcription factors PHR1, WRK75, ZAT6, bHLH32, MYB62, in *Arabidopsis* and PTF1 in rice and microRNAs. MiR399, upregulated in low-phosphate stressed plants [45–47] targets two genes; a phosphate transporter PHO2 and a putative ubiquitin conjugating enzyme UBC24 transcript [6, 48] have been isolated from the phloem and their level increases in the phloem upon phosphate starvation, suggesting their role as phosphate starvation signals being translocated from shoots to roots where they promote phosphate uptake by downregulating PHO2 [49, 50]. Recently, miR-827-3p was shown to be upregulated in nitrogen and phosphorus deficiency conditions [51, 52]. In rice, it has been shown to target two genes encoding proteins containing SPX and MFS (major facilitator superfamily) domains. SPX-domain proteins are involved in Pi sensing and/or Pi transport [53]; whereas MFS-domain proteins are membrane proteins functioning in small molecule transport [54]. It has been suggested that SPX-MFS proteins might be involved in intracellular or intercellular Pi transport through regulating the expression of Pi transporter (PT) genes [55] and/or Pi storage or metabolism [56]. Similarly, in *Arabidopsis*, it targets the nitrogen/phosphate balance regulator Nitrogen Limitation Adaptation (NLA, AT1g02860) transcripts [57]. Overexpression of miR827 under Pi-starvation conditions causes the downregulation of NLA (together with downregulation of PHO2 through the action of miR399) leading to increased levels of phosphate transporter 1 (PHT1) and elevated activity of Pi uptake at the plasma membrane.

Sulfur (S) is another fundamental macronutrient necessary for proper growth and development. For plants, it is available from the soil in sulfate form, it is taken up by the roots, and distributed within the whole plant by sulfate transporters. It is found in amino acids, peptides and proteins, iron-sulfur clusters, and several co-factors, but also in an oxidized form in polysaccharides, lipids, and modified proteins [58, 59]. Sulfate deprivation induces the accumulation of miR395 that targets ATP sulfurylases (APSs) and sulfate transporter AST68 [6, 60], thus reducing their levels. In Brassica plants, deprived of sulfur, the level of miR395 increases in the root, stem, and leaf tissues, it is however strongest in the phloem [61, 62].

The regulation of copper (Cu) homeostasis is also managed through the action of microRNAs. This micronutrient is indispensable for photosynthesis and plant protection against reactive oxygen species [63, 64]. Under copper-deficient conditions, upregulation of miR398 leads to the downregulation of cytosol and chloroplastic Cu/Zn superoxidase dismutase transcripts level (CSD1 and CSD2) and copper chaperone for superoxide dismutase CCS1 [65, 67]. Cu/Zn SOD can be, therefore, replaced with FeSOD in chloroplasts [68]. The level of miR398 is elevated in Brassica under copper deprivation in leaf, stem, and root tissue but most importantly in the phloem [62], such as in the case of sulfur deprivation, which is in accordance with other research suggesting that the phloem has a distinct set of microRNAs from leaves and roots and responds specifically to stress conditions [63]. Several other microRNAs have also been recently linked with copper-deficient conditions (miR397, miR408, and miR857). They are predicted to target Cu-containing proteins (laccases and plantacyanin) [66].

Under high, heavy metal concentrations, plants are subjected to cation imbalances that lead to alterations in plant physiology and biochemistry. It is not surprising, therefore, that in response to these kind of stresses a set of microRNAs is differentially regulated. It has been observed in rice [68], *M. truncatula* [69], *Brassica napus* [70], and *Arabidopsis* [71]. In *Brassica*, several microRNAs have been linked with cadmium exposure (miR156, miR160, miR164b, miR171, miR393, miR394a,b,c and miR396a,) [70, 72]. In rice, assorted microRNAs have been upregulated in roots (miR601, miR602, and miR603) while some were downregulated in leaves (miR602 and miR606) or in roots (miR604) [68]. The levels of miR171, miR319, miR393, and miR529 were all heightened in response to mercury (Hg), cadmium (Cd), and aluminium (Al) exposure in *Medicago* [69].

## 2.2. siRNAs

siRNAs are derived from double-stranded RNAs that result from transcription of inverted-repeat sequences, natural cis-antisense transcript pairs, or by the action of RNA-dependent RNA polymerases (RDRs) that convert single-stranded RNA transcripts (e.g., virus replication) into double-stranded RNAs [8]. They also derive from heterochromatic regions and DNA repeats and mediate the silencing maintenance of the regions from which they originate [73]. These are then cleaved by dicer-like (DCL) enzymes to produce 21-24-nucleotide long species, the size depends on the specific catalytic activity of the respective DCL protein. Similarly to miRNAs, siRNAs are then loaded into Argonaute (AGO) protein-containing RISC that guides target regulation at the transcriptional level via the so called RNA-directed DNA methylation (RdDM) or at post-transcriptional level [8, 26, 74]. Several classes of siRNA can be distinguished, such as trans-acting siRNAs (tasiRNA), heterochromatic RNAs (hc-siRNA), and natural antisense siRNAs (nat-siRNA).

Tasi-RNAs are derived from specific genetic loci called TAS genes through a microRNA-dependent pathway [75, 76]. Cleaved TAS transcripts are converted into dsRNAs by RNA-dependent RNA polymerase 6 (RDR6), which are in turn cleaved into 21-nt siRNAs. Four families of TAS genes have been identified in *Arabidopsis*. TAS2 siRNAs target PPR mRNAs (pentatricopeptide repeat); TAS3 siRNAs target ARF2, 3 and 4 transcription factors; TAS4 siRNAs decrease the level of MYB transcription factors [78]. TAS1 and TAS2 are recognized by miR173, TAS3 by miR390 and TAS4 by miR828 [64]. TasiRNAs derived from TAS4 have been implicated in Pi deficiency conditions. They are directly involved in the biosynthesis of anthocyanins in response to low Pi [78]. A putative siRNA pta22 from *Pinus taeda* has been implicated in the response to pathogens, targeting transcripts of two genes encoding for disease resistance proteins [79]. The levels of TAS1, TAS2, and TAS3 tasiRNAs have been elevated in hypoxia-treated samples in *Arabidopsis* suggesting their role in stress responses [80]. These changes in tasiRNA levels have been proved to be correlated with the levels of TAS-targeting miRNAs (miR173 and miR390). The decrease in PPR transcripts level can be associated with the protection of mitochondria during hypoxia stress.

The usage of high-throughput sequencing methods allowed to identify thousands of genes in convergent overlapping pairs that can generate complementary transcripts in rice and *Arabidopsis* [41, 81, 82]. A natural cis-antisense transcript pair SRO5-P5CDH nat-siRNA plays



an important role in osmoprotection and oxidative stress management that results from high salinity [83]. P5CDH (pyrroline-5-carboxylate dehydrogenase) and SRO5 (similar to RCD One) are both involved in regulation of proline metabolism. The downregulation of P5CDH transcripts causes accumulation of proline, which is an important mechanism involved in the increased tolerance to salt stress conditions. But the decrease in the level of P5CDH causes an increase in a toxic metabolite P5C and reactive oxygen species that are detoxified by SRO5 proteins in the mitochondria [83].

There has also been a report showing changes in 4 siRNA levels in wheat seedlings under cold, heat, salinity, and dehydration stresses. Two of them (named 002061\_0636\_3054.1 and 005047\_0654\_1904.1) were downregulated in heat, salinity, and dehydration. The latter one was also responding to the cold stress by being upregulated. The level of another one (080621\_1340\_0098.1) was decreased under heat but upregulated under cold stress. The fourth one (997927\_0100\_2975.1) was not differentiating only under heat stress, but downregulated when plants were treated with salinity, cold, and dehydration conditions [84].

Heterochromatic 24-nt siRNAs are essential components of RdDM pathway [85]. The pathway has been established to protect plants from invasive nucleic acids, such as transposable elements and repetitive sequences, through DNA methylation and chromatin modifications [86–88]. It has been shown that 21-nt and 24-nt hcsiRNAs are the most abundant class (80%) of all small RNAs in plants [60, 89, 90]. The role of hcsiRNAs in abiotic stress responses is not well understood; although in the 1980s, McClintock has already suggested that all kinds of stresses could potentially reshape a plant genome via transposon activation [91]. Global analysis in plants such as *Arabidopsis* and rice suggests that the vast majority of transposons are inactive, methylated, and targeted by siRNAs [92]. Recently, few researches suggested that hcsiRNAs could play an important role in the adaptation to stress conditions. In *Arabidopsis* seedlings subjected to heat stress, *ONSEN* (copia-type retrotransposon) levels have been shown to increase in mutants affected in RdDM pathway [93]. After the recovery period, the level of *ONSEN* transcripts decreased gradually suggesting a role for hcsiRNAs. What is interesting is that the insertion of new *ONSEN* copies has been observed during flower development but before gametogenesis. Surprisingly, *ONSEN* insertions were also able to confer heat stress responsiveness to nearby genes. Together, all these data suggest that hcsiRNAs can contribute to control of transgenerational retrotransposition triggered by abiotic stresses and can participate in new regulatory networks established to protect plants against stress conditions. The detection of mPing transposition into a rice homologue of flowering time gene-*CONSTANS* in stressed cultivars supported this intriguing hypothesis. It turns out that stress-activated transposons could positively contribute to genome adaptation to growth in colder climates (reviewed in [1]). Moreover, stress-mediated induction was shown for *Tos17* (rice), *Tto1* (tobacco), *Tnt1* (tobacco), and *BARE-1* (barley) retrotransposons. Low temperature treatment was shown to decrease methylation and increase the excision rate of *Tam3* transposon by binding its transposase to GCHCG sites immediately after DNA replication, thus preventing de novo sequence methylation (reviewed in [1]).

### 3. Non-canonical small RNAs

In plants, RNAi-dependent as well long ncRNAs-dependent mechanisms constitute the vast majority of stress response mechanisms. However, other non-protein coding RNAs are continually being discovered. At the same time, *S. cerevisiae* lacks the RNA interference pathway, and thus the ncRNA-based gene regulation is performed solely by the RNAi-independent mechanisms. Newly identified small non-coding RNAs other than miRNAs and siRNAs, their functions in gene regulation in yeast and plants, as well as possible mechanisms of action are discussed below.

#### 3.1. tRNA-derived small RNAs

In the expanding repertoire of small noncoding RNAs, tRNA-derived RNA fragments have been identified in all domains of life [reviewed in 94]. According to the nomenclature based on tRNA fragment size and the part of the tRNA molecule from which fragments are derived [95], tRNA fragments can be separated in two major classes: tRNA halves and smaller tRNA fragments (tRFs). In most of the cases reported till now, full-length tRNA levels do not decline significantly and tRNA fragment levels are consistently lower than those of full-length tRNAs, suggesting that only a small proportion of tRNAs is subjected to cleavage. This is in contrast to the complete depletion of tRNAs targeted by colicins in bacteria [96]. Recent evidences suggest that tRNA-derived fragments are not just a random degradation products but rather stable entities that may have major biological functions. The direct influence on protein biosynthesis has been revealed since: (i) tRF binds to 30S and inhibit translation in *Archaea* [97] and (ii) tRNA halves in human cell lines inhibit protein synthesis by displacing eIF4G/eIF4A from uncapped>capped RNAs [98].

So far, only few reports on plant tRNA-derived fragments are published. Such small RNAs have been observed under non-stress conditions in plants [99]; however, the basal levels of tRNA halves are low and often increase during stress conditions. In 2009, it has been reported that tRNA fragments are present abundantly under phosphate (Pi) deficiency in *Arabidopsis* [78]. In this study, a significantly higher amount (almost six-fold) of small RNAs derived from tRNAs was present in the roots than in the shoots. The accumulation of tRNA fragments in the -Pi roots was 1.4 times higher than that in the +Pi roots. When the origin of these RNAs was further analyzed, no correlation was observed between the abundance of small RNAs from specific tRNA species and their codon usage. What gained a special interest is the observation that a 19-nucleotide sequence processed from the 5' end of tRNA<sup>Gly</sup><sub>TCC</sub> represented over 80% of all tRNA-derived small RNAs in the roots and accounted for up to 18.44% and 27.70% of total sequence reads in the +Pi and -Pi root libraries, respectively, compared with only 1.00% to 1.79% in the shoot libraries. These results revealed a spatial and temporal expression pattern of small RNAs derived from the specific cleavage on tRNA molecules. The authors speculated that such differential accumulation of tRNA fragments between roots and shoots may represent the consequence of long-distance movement. Similar movement phenomenon of tRNA halves was observed in the phloem sap of *Curbita maxima* [100]. In this study, the presence of all tRNA anticodon families was inspected by northern blot hybridiza-

tion assays. In 12 out of 20 probed tRNA cases, stable processing products were detected in the phloem sap but not in leaf tissue extracts. The size of tRNA fragments ranged from 31 to ~60 nt and they derived from both 3' or 5' ends of particular tRNA molecule. The authors proposed that these phloem-delivered tRNA fragments may be a long-distance signal to coordinate the metabolic status between source and sink tissues.

A comprehensive expression profile of non-coding small RNAs was also performed in barley (*Hordeum vulgare* L.) under phosphorous-deficient and -sufficient conditions [101]. The deficiency in phosphorus decreases the plant growth and grain yields. Hackenberg et al., using high-throughput sequencing techniques, found that 56 out of the total 61 tRNAs were generating stable fragments in both P-deficient and P-sufficient shoots. Six tRNA-derived fragments were significantly upregulated, whereas four were significantly downregulated in P-deficient shoots. Notably, the read count of tRNA fragments was two-fold greater in P-deficient shoots than in P-sufficient shoots. The most abundant of these small RNAs was tRF derived from tRNA<sup>Gly</sup><sub>TCC</sub> (58.6% of the total tRFs in P-deficient shoots and 58.2% in P-sufficient shoots). tRNA<sup>Ala</sup><sub>AGC</sub>-derived sRNAs (previously reported as the most abundant in rice meristems) were the second most abundant species in P-sufficient shoots, but the third in P-deficient shoots. Such differential accumulation of tRFs between the two P treatments indicate that P has a great impact on the tRNA processing to small RNAs.

Deep sequencing technologies also provided evidence that novel subset of small RNAs are derived from the chloroplast genome (csRNAs) of Chinese cabbage [102]. The chloroplast small RNAs (csRNAs) included, among others, those that derive from tRNAs. Wang et al. found that the csRNAs derived from most of the chloroplast tRNA sequences constituted 5' parts of the molecules. Moreover, the first nucleotide of these csRNAs were predominantly located at the first nucleotide of the mature chloroplast tRNA sequences, revealing that the biogenesis of csRNA in tRNA molecules was specific to the 5' end. The tRNA-derived csRNAs only slightly declined in the heat-treated seedlings. However, the length of these csRNAs was related to heat stress response. In the heat-treated seedlings, the abundance of longer csRNAs (29–32 nt) decreased, but the of the shorter ones (16–25 nt) increased. csR-trnA-1 and csR-trnA-2 were the two most predominant tRNA-derived csRNA families and originated from chloroplast tRNA<sup>Ala</sup>. Such reduced abundance of tRNA-derived fragments 1 h after the heat treatment (the time period before the appearance of leaf etiolation) may suggest that the heat-responsive csRNAs play roles in the maintenance of subcellular structures and photosynthetic capacity of chloroplasts. The csRNAs derived from tRNAs may, therefore, play a role similar to the reported tRNA halves under various stress conditions.

In 2013, Loss-Morais et al. summarized all existing sequencing data aiming at the characterization of plant tRNA-derived fragments, their accumulation patterns under abiotic and biotic stresses, the identification of their putative targets, as well as possible association with Argonaute (AGO) proteins [103]. They have inspected 34 Arabidopsis deep sequencing libraries, including 25 AGO-IP libraries and found tRFs in the AGO1, 2, 4, and 7 IP libraries. Both, 5' and 3' tRFs were associated with AGO, mirroring previous results in mammalian systems [104–105]. Interestingly, tRFs from the central part of tRNAs were also detected, although 5' tRFs formed the most abundant class and showed the highest sequence diversity.

The AGO-associated 5' tRFs were predominantly 19-mers. Then, to investigate if the 5' tRFs associated with AGOs act in the RNAi pathway in plants, as has been suggested in animals [104], the authors searched for tRF targets in Arabidopsis and identified four possible target genes. They have found that drought conditions enhanced the expression of the four tRFs, including the 5'-tRF<sup>Gly</sup><sub>TCC</sub>, which was already known to be up-regulated in response to phosphate deprivation [78].

The existence of transgenerationally transmitted, heat-responsive tRNA-derived fragments in plants was revealed for the first time by Bilichak et al. in *Brassica rapa* [106]. In the Bilichak study, small RNA sequencing was performed to compare alterations in RNAs in somatic and reproductive tissues of *B. rapa* plants and in their progeny in response to heat treatment. The authors have demonstrated high tissue-specific alterations in the small RNA accumulation profiles in tissues that were not directly exposed to stress, namely, in the endosperm and pollen. Importantly, they have revealed that the progeny of stressed plants exhibited the highest fluctuations in the small RNA accumulation levels (tRNA fragments among them). It has been speculated that perturbations in the expression of small RNAs in somatic tissues caused by environmental fluctuations would eventually be imprinted in the transcriptome patterns in gametes and progeny.

The presence of tRNA-derived fragments has also been reported in industrially important budding yeast *S. cerevisiae* [107, 108]. Thompson et al. were the first to demonstrate (already in 2008) that *S. cerevisiae* contain a small RNA population consisting primarily of tRNA halves and rRNA fragments [106]. tRNA fragmentation to stable shorter pieces occur in yeast cells in the absence and in the presence of stress conditions. However, the accumulation of tRNA fragments in yeast was most pronounced during oxidative stress conditions, especially during entry into stationary growth phase. Both 5' and 3' fragments of tRNAs were detectable suggesting the occurrence of endonucleolytic cleavage. The nuclease involved in this cleavage was characterized one year later [109]. It has been shown in yeast that tRNAs are cleaved by RNase T2 family member Rny1p, which is released from the vacuole into the cytosol during oxidative stress. Rny1p modulates yeast cell survival during oxidative stress independent of its catalytic ability. This suggests that upon release to the cytosol, Rny1p promotes cell death by direct interactions with downstream components.

The possible function of tRNA fragments in yeast has been described in 2012 [108]. Although RNA interference (RNAi) is conserved in diverse eukaryotic species, including budding yeast *Saccharomyces castellii* and *Candida albicans* [110], it has been lost in *S. cerevisiae*. Thus, this organism provides an ideal system for studying the RNAi-independent mechanisms of tRF-based gene expression regulation. The potential novel function of tRNA fragments is supposed to appear as a response to specific environmental conditions and includes tRNA processing and association with the ribosomes. As a result of high throughput sequencing of ribosome-associated small RNAs, it appeared that one of the most abundant classes of processed RNAs were tRNAs. In addition to previously reported cleavage in the anticodon loop in yeast tRNAs [107], other breakage points were also detected (e.g., in the D- and T-loop regions), reminiscent to those observed previously in higher eukaryotes [111]. Moreover, an obvious differential stability of tRNA halves was recognized. Northern blot analysis confirmed the presence of two



stable processing products derived from tRNA<sup>His</sup> and revealed that cleavage is stress-dependent. Similar to previous findings tRNA processing was mainly detected during amino acid and sugar starvation conditions. On the contrary, experimental results obtained for tRNA<sup>Ser</sup> suggested that only the 3' part of this tRNA is stable. The association of tRNA fragments with yeast ribosomes suggest its potential regulatory function in protein biosynthesis, as already reported for *Archea* [97] and humans [98].

### 3.2. snoRNA-derived small RNAs

Small nucleolar RNAs are a highly evolutionarily conserved class of RNAs, which are present throughout the eukaryotes and whose origin lies in the Archaea. There are two classes of snoRNAs (C/D and H/ACA box) that function as ribonucleoprotein (RNP) complexes to guide enzymatic modification of target RNAs at sites determined by RNA:RNA antisense interactions. Generally, C/D box snoRNAs are ~70–120 nucleotides (nt) long and guide the methylation of target RNAs, while H/ACA box snoRNAs are ~100–200 nt long and guide the pseudouridulation of their targets. These RNAs were initially discovered in the nucleolus and thought to exclusively target ribosomal RNAs inside this sub-nuclear compartment. However, numerous snoRNAs do not possess target RNAs—therefore, they are called “orphan snoRNAs”.

Small nucleolar RNAs are localized in the nucleoli and Cajal bodies in eukaryotic cells. Components of the RNA silencing pathway associate with these structures, and two recent reports have revealed that a human and a protozoan snoRNA can be processed into microRNA-like RNAs [112–113]. By systematic analyses of deep-sequencing libraries from diverse eukaryotic organisms, Taft et al. revealed that small RNAs with evolutionary conservation of size and position are derived from the vast majority of snoRNA loci in animals (human, mouse, chicken, fruit fly), *Arabidopsis*, and fission yeast [114]. These small RNAs derived from snoRNAs (sdRNAs) in *Arabidopsis* were strongly associated with AGO7 proteins. *Arabidopsis* Argonaute proteins preferentially load small RNAs with specific 5' nucleotides and AGO7 is selective for 5' uracil (U) and 5' adenine (A). Intriguingly, H/ACA sdRNAs are dominantly 5'A, while C/D box are dominantly 5'U. Therefore, it is likely that sdRNAs play roles in the regulation of gene expression and transcriptional silencing. In particular, the fact that both miRNAs and sdRNAs are 5'U biased strengthens the link between them and suggests that some snoRNAs, including “orphan” snoRNAs whose targets are unknown, may function solely as intermediates in the sdRNA pathway.

However, snoRNA-mediated change in gene expression should be treated not as an example but as an addition to siRNA-based mechanisms. The reason for this statement is that canonical snoRNAs interact with their target RNAs through their 10–20 nt long antisense-box elements. In contrast, snoRNA-derived small RNAs can interact with other sequence elements with their targets, as they form a different ribonucleoprotein complexes.

The presence of snoRNA processing products was experimentally verified by high throughput sequencing, as well as northern blot hybridization in *S. cerevisiae* [108]. The results confirmed the presence of a shortened version of both types of snoRNAs. The processing events were most prominent under most tested yeast growth conditions, which included UV radiation,



anaerobic growth, high or low pH treatment, amino acid starvation and sugar starvation. Like in the case of tRNA-derived fragments, the association of sdRNAs with the ribosomes implicates their possible regulatory role in protein biosynthesis in *S. cerevisiae*. In this organism, RNAi silencing pathway has been lost during evolution, therefore the mechanism of sdRNAs action in *S. cerevisiae* is likely to be different from microRNA-like sdRNA action in plants.

Despite the differences in possible mechanism of action of sdRNAs, for both plants and yeast, the presence of small regulatory RNAs in the nucleolus allows us to speculate that the nucleolus is involved in the regulation of expression, possibly in response to cellular conditions.

### 3.3. mRNA-derived RNAs

Small mRNA fragments have been reported in diverse eukaryotes and a portion of them is stress-related [reviewed in 115]. A part of them is annotated as antisense RNAs (asRNAs), overlapping protein coding transcripts on the opposite strand. Some asRNAs were found to repress sense RNAs and lead to inverse expression between asRNAs and their corresponding sense RNAs in respect to growth phases, stress conditions, or environmental changes. Therefore, the corresponding protein-coding transcripts (mRNAs) represent a mixture of cell-cycle factors, chromatin remodellers, and metabolism related proteins. It has been shown recently that about one-fifth of the ORF genes in *S. cerevisiae* are coupling with asRNAs; however, the ratio of genes coupling with asRNAs shows a negative association with gene regulatory complexity [116]. This observation supports previous hypothesis that some asRNAs belong simply to a transcriptional noise. Nevertheless, asRNAs evolve more slowly when their sense genes are under more complex regulation. Older genes coupling with asRNAs are more likely to demonstrate inverse expression, reflecting the role of these asRNAs as repressors, especially under heat stress stimuli.

Recent tiling assays in plants (mostly in *Arabidopsis*) also revealed the majority of stress-responsive novel antisense transcripts [117]. Despite a linear correlation between the expression ratios of selected sense and antisense transcripts, biological functions of most antisense RNAs remains unclear. In plants, it has been postulated that antisense transcripts might have the potential to produce endogenous siRNAs (nat-siRNAs) as mentioned in Paragraph 2.2. Recent genome-wide analysis reported an accumulation of sRNAs in their overlapping region, suggesting the occurrence of an RNA interference event [82]. However, the biological processes of generating nat-siRNAs is not completely understood at this time.

The power of mRNA-derived ncRNAs for rapid global translation attenuator in stress was recently demonstrated in *S. cerevisiae* [118]. mRNA exon-derived 18-residue-long ncRNA (picked up in previous genomic screen for ribosome-bound small RNAs in *S. cerevisiae*) has been functionally characterized [108]. This ribosome-bound ncRNA, originating as a short sense fragment of TRM10 open reading frame, is needed for the rapid shutdown of global translation and is capable of adjusting translation rates by interacting with polysomes under hyperosmotic growth conditions. Therefore, the existence of a largely unexplored mechanism of translation control has been clearly demonstrated.

## 4. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are a wide group of molecules identified in yeast, plants, and mammals. In general, lncRNAs can be defined as polyadenylated or nonpolyadenylated, more than 200 nt long transcripts with low protein coding potential (coding for less than 100 amino acids). Acceleration in transcriptome research, achieved thanks to the development of high-throughput technologies such as microarrays or next-generation sequencing methods, allow us now to fully appreciate complicated interactions that lead to precise gene expression regulation. It turned out that apart from genes encoding proteins, transposons, genes for housekeeping RNAs (like ribosomal RNAs), intergenic regions, which lie between protein-coding sequences, are also being expressed. Intriguingly, similarly to mRNA, those transcripts, namely lncRNAs, are capped at the 5' end and many of them are targets of the splicing process [119]. However, in contrast to mRNA, their expression level is very low and they do not have long open reading frames, which are evolutionary conserved [120]. LncRNA also differ from protein-coding transcripts in ribosome occupancy [121]. Large-scale genomics projects, such as Encyclopedia of DNA Elements (ENCODE), proved that lncRNAs are not only transcriptome noise, but indeed transcripts with biological functions. As a result of the ENCODE project, it has been shown that 75% of human genome is transcribed and about 80% of those RNA molecules have some biochemical function [122]. Most of the intergenic regions of Arabidopsis, rice or corn are shown to be transcribed as well as human and constitute a source of lncRNAs that are polyadenylated. Such polyadenylated stable lncRNAs are transcribed by RNA polymerase II and can be divided into four groups based on their genomic origin and relationship with adjacent protein-coding genes:

1. intergenic lncRNAs (lincRNAs) that are transcribed from sequences between two genes
2. intronic ncRNAs (incRNAs) that overlap with intronic sequences within another transcript
3. natural antisense transcripts (NATs) derived from complementary DNA strand of their associated genes
4. sense lncRNAs overlapping with one or more exon sequences of the transcript on the same strand.

Recently in Arabidopsis and rice [123, 124], another category of lncRNAs has been described — these are nonpolyadenylated transcripts, 50-300 nt in length with low coding potential, but without any sequence similarity to known ncRNAs. This novel group is referred to as intermediate-sized ncRNAs (im-ncRNAs).

Despite the effort of 20 years of investigation [125], the elusive role of lncRNAs is still not fully described nor understood. Thus far functions of only few such molecules are characterized. We know that lncRNAs are engaged mainly in transcriptional gene expression regulation by acting as scaffolds for transcriptional factors and genetic modifiers, molecular signals, decoys or guides. Moreover lncRNAs can also encode for miRNA and target specific mRNAs for decay or function as miRNA sponges. Most studies has been performed on animal systems, but

although research on plants are limited, the emerging picture is that the regulatory functions of plant lncRNAs are similar to animal ones [126]. Till today, almost 40,000 putative lncRNAs have been identified in *A. thaliana* [127] and thousands of them in *Oryza sativa* [128], *Zea mays* [129], *Medicago truncatula* [130], *Populus trichocarpa* [131], and other plant species [132, 133]. With rapid development of bioinformatics tools and transcriptome analysis methodologies, genome-wide identifications of plant lncRNAs have been conducted. In maize implementation of SVM tools (support vector machines), together with Python pipeline on cDNA dataset resulted in the identification of 2,492 potential ncRNAs, which represent 13.3% of initial sequences. In total, 237 ncRNAs were classified as shRNA precursors, and 1,225 as siRNA precursors, which constituted 59.4% of predicted ncRNAs particles. The remaining 1,011 was considered to be potential long non-coding transcripts [134]. Recently, new gold standard to study the complexity of eukaryotic transcriptomes emerged – the RNA-sequencing technology (RNA-seq). It allows an accurate quantification of expression levels of transcripts and also reveals transcripts that are missing or incomplete from the reference genome. Computational prediction based on RNA-seq data from rice anthers, pistils, seeds, and shoots, together with 40 available rice RNA-seq libraries led to the identification of 2,224 reliably expressed lncRNAs, including 1,624 lincRNAs and 600 long non-coding natural antisense transcripts (NATs). Further verification of rice insertional mutants allowed to set a pool of lncRNAs that are preferentially expressed at the reproductive stage. Several lncRNAs were identified as competing endogenous RNAs (ceRNAs), which sequester miR160 or miR164 in a type of target mimicry [135].

Another feature that complicates the retrieval of true lncRNAs is their weak sequence conservation. It is estimated that only from 2% to 5.5% of lncRNAs are conserved in their primary sequence and only some of them may be associated with short conserved elements. Most likely, it is a result of rapid evolution – lncRNAs are frequent targets of positive selection [136]. Some lncRNAs and their target genes can be distinguished by their conserved synteny across species – those lncRNAs play roles in cis-functions [136]. Other lncRNAs may be recognized by conserved secondary structures, which allow them to interact with RNA-binding proteins [124]. Genome-wide analyses carried out so far determined that expression of different groups of lncRNAs is highly tissue-specific and many of them are responsive to biotic and abiotic stress conditions.

In conjunction with the climatic changes, drought is the condition that has been recently extensively studied, and thus many drought-responsive lncRNAs were identified. In *Populus trichocarpa*, a model tree species, RNA-seq experiments conducted on control- and drought-treated plants revealed 504 drought-responsive lincRNAs and allowed for basic annotation set of 2,542 of them. Mutual interaction of miRNA and lncRNAs was also reported; a total of 30 miRNAs were predicted to target the sense strand of lincRNAs, 21 were found to target the antisense strand, and 20 target mimicry events was predicted of known *Populus* miRNA [130]. A potential new model organism of the family Poaceae, *Foxtail millet*, was also subjected to water deficient conditions. Deep transcriptome sequencing revealed 585 lncRNAs responding to PEG-induced drought stress. Those stress conditions induced the expression of 17 lincRNAs and 2 NATs at different expression levels. Qi et al. [138] identified one lncRNA, whose

sequence was shared with its counterpart in sorghum. In maize, one of most important crop species, genome-wide identification of differentially expressed lncRNAs during drought conditions led to the identification of 567 upregulated and 97 downregulated lncRNAs, among them 538 particles were considered to be novel. Moreover, 8 lncRNAs molecules were homologous to the miRNA precursors, 62 were classified as both shRNA and siRNA precursors, and 279 were classified as siRNA precursors [139].

In the best known model plant, *Arabidopsis thaliana*, genome-wide characterization of lncRNAs was performed as well. A correlated expression of lncRNAs with its epigenetic and structural features in response to four stresses (heat, cold, drought, and salt) has been described [140]. The authors identified 245 polyadenylated and 58 nonpolyadenylated lncRNAs that are differentially expressed under stress stimuli, and most of the selected candidates were further validated by qRT-PCR. From experiments on *Arabidopsis* came best studied cases of plant lncRNAs functions such as: *COLDAIR*, *COOLAIR*, *At4/IPS1*, *npc48*, and *npc536* [141–145].

One of best described mechanism of lncRNAs action is lncRNA transcript IPS1 (Induced by Phosphate Starvation 1). IPS1 can interact with miRNA as a competitor and function as miRNA target mimics, which resembles the miRNA sponges from animal systems. Maintaining the phosphate balance is a complicated mechanism in plants, regulated, among others, by miR399 as described in Paragraph 2.1. Low activity of PHO2, ruled by mRNA cleavage mediated by miR399, causes the elevation of phosphate uptake by increasing the expression of two root phosphate transporters. Phosphate starvation also increases the level of IPS1 transcript that has a 23-nt conserved domain, partially complementary to miR399 with 3-nt mismatch overlapping with the miR399-mediated cleavage site. As a non-cleavable product, IPS1 competes with PHO2 and can therefore weaken the miR399-mediated repression of PHO2 [142]. The miRNA sponge strategy is used in the therapy of human diseases and similar processes in plants (target mimic) and can be a very useful tool in plant research as well as in agricultural applications. As mentioned before, in *Arabidopsis* to date about 20 putative target mimicry events were predicted, which suggest the potential role of this mechanism in other pathways than the maintenance of phosphate homeostasis [146].

Another model organism, *Saccharomyces cerevisiae*, allows researchers to define and clarify a large number of new and unexpected roles of lncRNAs, such as promoting the timing of gene expression [147], cell cycle regulation during stress conditions [148], or local reduction of histone density and chromatin remodeling in response to glucose starvation [149]. Upon osmostress in yeast, hundreds of stress-responsive genes are induced by the stress-activated protein kinase (SAPK) p38/Hog1. Whole-genome tiling arrays were used to identify a set of Hog1-induced lncRNAs. One of the genes expressing a Hog1-dependent lncRNA in antisense orientation is *CDC28*, the cyclin-dependent kinase 1 (CDK1) that controls the cell cycle in yeast. *Cdc28* lncRNA mediates the establishment of gene looping and the relocalization of Hog1 and RSC from the 3' UTR to the +1 nucleosome to induce *CDC28* expression. The increase in expression level of *Cdc28* makes cells able to re-enter the cell cycle more efficiently after stress conditions occur. This may represent a more general mechanism to prime the expression of genes needed after stresses [148].



## 5. Conclusions

The rapidly growing human population is constantly stressing agro-ecosystems. In 2007, a FAO (Food and Agriculture Organization of the United Nations) report stated that only about 3.5% of the global agricultural area is not affected by any of the environmental stresses. Since recent advances in biotechnology and molecular biology have dramatically changed our understanding of gene expression regulation and responses of plants to abiotic stresses, we are now armed in new tools that could help us fight various stress conditions and improve the growth and yield of crops and other industrially important species. Our journey in the small RNA world has begun in the past century, in the 90s, with the discovery of RNA interference pathway, followed by siRNAs and their role in DNA methylation and chromatin modifications. Consequently, new and relatively surprising small RNA groups (tRNA-derived small RNAs, snoRNA-derived small RNAs, mRNA-derived small RNAs) have been discovered and slowly but surely their role in cells and in the adaptation to various environmental conditions is being established. Concurrently, thousands of lncRNAs have already been identified and thousands of them are still waiting to be discovered in different organisms, tissues, or in response to different stimuli. With the advent of new high-throughput techniques, it became easier and much faster to collect a vast amount of different types of molecular data. The analysis of such a high amount of data and understanding of mutual correlations and relationships between genes, their roles, and small RNAs is now a challenge that researchers and scientists must face. Nevertheless, we believe that by combining new methodologies with the help of bioinformatic approaches and wet lab experiments, we can shed light on a genomic "dark matter" and start to appreciate important physiological roles of various types of non-protein coding RNAs, as well as their potential applications.

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