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Abiotic Stress Responsive microRNome and Proteome: How Correlated Are They?

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Highlights

- -Plant response to stress requires a programmed change in gene/protein expression
- miRNAs play a central role in fine-tuning of gene expression
- -The correlation between stress-induced proteome and miRNome are discussed

Abstract

Environmental stresses, notably drought, soil salinity, extreme temperatures, nutrient deficiency and UV radiation, act both singly and in combination to reduce crop productivity. Plant survival depends on an ability to either tolerate or to avoid stress, so a variety of means have evolved to adjust the growth and development of plants in response to perturbations in their external

environment. Plant response to environmental fluctuations requires a programmed change in gene/protein expression which necessitates a tight regulation. The required fine-tuning of gene expression is achieved at multiple levels, of which post-transcriptional regulation represents as one of the main regulatory routes in which microRNAs (miRNAs) play a central role. These short RNA sequences act to suppress their target genes either by inhibiting their translation or by degrading their transcript. The genes encoding miRNAs can themselves be responsive to external stresses. The extent to which stress-induced changes in the plant proteome are influenced by miRNA activity is uncertain, but the implementation of high throughput analytical technologies is beginning to reveal that miRNAs do contribute to the proteomic response to stress. The present review focuses on the impact on the crop plant proteome of stress-induced alterations to miRNome. A particular emphasis is placed on the need to experimentally validate predictions based on bioinformatic data.

Keywords: proteomics; miRNA; miRNA target prediction; environmental stresses; degradome sequencing

Introduction

The rising global demand for food, feed and fiber, stoked by the continuing growth in the world's population, can only be met by increasing the land area used for cropping and/or by breeding more productive crop varieties (Myers et al., 2017). The situation is exacerbated by anthropogenic climate change, which in many current crop production regions, is expected to result in a greater exposure to drought, soil salinity, extreme temperatures and excessive photoirradiation (Munns and Gilliham, 2015; Reynolds et al., 2016). In the light of this major challenge, a research-based effort directed at ensuring global food security will require a systems biology approach to understand the molecular and physiological determinants of crop yield and tolerance to abiotic stress (Shaar-Moshe et al., 2017).

The growth, development and metabolism of a healthy plant depends upon a well-regulated program of gene expression, which can be perturbed by unfavorable changes in its external environment. The changes induced in gene expression reflect the ability of the plant to respond to

the stress. In plants which are sensitive to stress, exposure can induce major changes in gene expression, while those which are more tolerant are better able to control their transcriptome, specifically up-regulating genes encoding protective products and down-regulating those encoding proteins inimical to stress adaptation (Ghaffari et al., 2014). The regulatory machinery operates at the transcriptional, the post-transcriptional, the translational and the post-translational level (Akdogan et al., 2016; Alptekin et al., 2017; Chiou, 2006; Deng et al., 2015; Ferdous et al., 2015; Jones-Rhoades et al., 2006; Megha et al., 2018b; Wang et al., 2014a; Xie et al., 2014; Zhang, 2015; Zhou et al., 2010a), but the most critical of these occurs at the level of transcription. A class of short (20-24 nt) non-coding RNAs (Fig. 1) (Axtell and Meyers, 2018; Jones-Rhoades et al., 2006; Voinnet, 2009), termed microRNAs (miRNAs), has emerged as contributing critically to the post-transcriptional regulation of gene expression (Sunkar et al., 2012). They act by binding to a target mRNA(s) via complementary base pairing, thereby either inducing the cleavage of the target (the primary route) or inhibiting the process of its translation (Voinnet, 2009). miRNAs can also induce suppression of their target genes transcription through an RNA-directed DNA methylation mechanism (Fig. 1), adding further complexity to miRNA-based gene expression regulation (Bao et al., 2004; Wu et al., 2010).

In contrast to the set of miRNAs generated in animals, those generated in plants appear to target a limited subset of mRNAs, amounting only to around 1% of the complement of protein-encoding genes; the majority of these encode a transcription factor (TF) (German et al., 2008; Jones-Rhoades et al., 2006; Sunkar et al., 2012; Zhou et al., 2010b). Given that TFs are an integral component of gene regulation, the impact of miRNAs on the growth, development and stress response of plants is likely to be substantial, so that even a minor change in the array of miRNAs present may well have a major impact on a plant's physiology (Sunkar et al., 2012). A deal of evidence has been provided to show that the abundance of miRNAs which target stress-related TF genes responds strongly to the presence of abiotic stress (Sunkar et al., 2012). Of particular interest is that the speed of the interaction between an miRNA and its target allows for the rapid and reversible modulation of gene expression (Mittal et al., 2016). In addition, gene regulation by miRNAs can spread systematically throughout the plant via an interconnected network of phloem (Kehr and Buhtz, 2008; Pant et al., 2008). This will enable plants to sense a specific stress in one organ and respond systematically to that stress in their distal parts using miRNAs movements. miRNAs have

also been identified in the xylem sap of maize and *Populus* (Puzey et al., 2012; Wang et al., 2019), but there is no direct evidence for their movements through the xylem.

The array of miRNAs present varies from species to species and is also dependent on the individual's genotype and developmental stage, the tissue/organ sampled and the severity of the stress (Zhang, 2015). Even subtle differences in the growing conditions can affect the miRNA profile (Ferdous et al., 2015). The targets of a given miRNA can also differ from species to species, so it is important to experimentally validate an miRNA's target(s), rather than to assume that these are conserved across species (Ferdous et al., 2015). Since miRNAs bind to their targets on the basis of base pairing, the targets of variants within an miRNA family can differ substantially (Alptekin et al., 2017). Sequence complementarity is assumed in order to make initial predictions of targets from *in silico* data, but experimental validation is desirable (Fei et al., 2018). This validation is conventionally achieved using standard molecular biological technologies e.g. qRT-PCR, Western blotting, and reporter assay (Chou et al., 2018; Sun et al., 2014), but ongoing advances in nucleotide sequencing technology have generated some more effective methods, such as the parallel analysis of RNA ends (PARE) or degradome sequencing (Fig. 1) (Addo-Quaye et al., 2008). Degradome sequencing combines a modified version of RNA ligase mediated-5´rapid amplification of cDNA ends (5´RLM-RACE) with high-throughput deep sequencing for genome-wide amplification and sequencing of 3´-mRNA cleavage products (Addo-Quaye et al., 2008; German et al., 2008; Thomson et al., 2011). In 5´RLM-RACE, an RNA adaptor is ligated to the 5′-end of the uncapped 3′-fragment of a cleaved miRNA target and the ligation product is reverse transcribed using a gene-specific oligonucleotide primer. The resulting cDNA is subsequently PCR amplified, cloned and sequenced to identify the target gene. Measuring the overall mRNA translation is another route by which one can gain insight into the mechanism of miRNA-mediated translational repression. Identification of mRNAs associated with ribosome enables to identify translationally active mRNAs. This can be particularly adapted to miRNA target identification by ribosome profiling approach (Ingolia et al., 2009; Li et al., 2013). In this method, cells are treated with cycloheximide and lysed and subsequently treated with RNAse I to degrade mRNAs not protected by ribosomes. The mRNA fragments associated with ribosomes are then purified and identified by high-throughput sequencing.

Since most plant miRNAs target protein-encoding genes (exceptionally, long non-coding RNAs can be targeted (Axtell and Meyers, 2018)), changes in their relative abundance are likely to induce

alterations in the proteome (Thomson et al., 2011). The ability to successfully identify and quantify the components of the proteome present in a biological sample has benefited from improvements in mass spectrometry (MS) technology, which has been incorporated into a number of proteomics-based studies of the plant stress response (Barkla et al., 2013b; Chakraborty et al., 2015; Gharechahi et al., 2016; Kerry et al., 2018; Kosova et al., 2011; Kosová et al., 2013; Zhang et al., 2012). Since nearly all physiological and molecular processes in living tissue are orchestrated and executed by proteins, proteomic changes will generally have phenotypic consequences. The extent to which any such change induced by exposure to abiotic stress is caused by an altered miRNA profile is not as yet well understood, although it is thought likely that the contribution towards stress tolerance made by miRNAs is mainly related to their interaction with stress-responsive proteins on which a plant relies for its ability to adapt to a non-favorable growing condition. Although the mRNAs for many stress responsive proteins have not been predicted to be targeted by miRNAs, but they might be indirectly regulated by stress-responsive miRNAs. This indirect regulation could be explained, for example, when the primary miRNA target is a gene regulatory protein which influences the expression of secondary targets (Shin et al., 2010). Therefore, most stress-responsive proteins might be the potential secondary targets of stress-responsive miRNAs. The extent to which miRNAs exert control over genome-wide translation in animal cell lines has been elegantly demonstrated (Baek et al., 2008; Jovanovic et al., 2010; Selbach et al., 2008), but equivalent studies in plant cells are lacking. This may reflect the problem that most plant miRNAs target TFs and signaling proteins, most of which are present in concentrations below the experimental limits of detection. Furthermore, changes in the abundance of these molecules are typically highly dynamic, which complicates the choice of sampling strategy. As a result, these key proteins are seldom represented in plant proteomic profiles. An indirect means of addressing this knowledge gap could be to infer proteomic variation from that of the microRNome, thereby helping to form a molecular view of the adaptive response to stress. This review seeks to summarize the current state of knowledge with respect to the impact of miRNA activity on the proteome, specifically in the context of the response of crop plants to abiotic stress.

Drought stress

Drought stress is responsible for a significant loss to crop production, exceeding the losses caused by all other stresses combined. The success of taking a molecular approach to breeding for greater drought stress tolerance will depend on a prior understanding of the physiological basis of

tolerance (Gharechahi et al., 2015), which is challenge given that the response to drought stress involves the coordinated action of many gene products (Fang and Xiong, 2015; Shinozaki and Yamaguchi-Shinozaki, 2007). Experiments have demonstrated that drought stress induces changes in the abundance of large numbers of proteins (Chakraborty et al., 2015; Mousavi et al., 2016). Few of the genes encoding these proteins are known to be regulated by miRNAs, although it may well be that the mode of regulation in some cases is indirect (such as through the action of a TF). However, several examples have been described where a drought-responsive protein-encoding gene has been shown to interact directly with an miRNA: some of these are discussed in detail below.

miRNAs probably regulate plant adaptation to drought condition by targeting the expression of key stress-related TFs. These TFs might have the ability to regulate the expression of genes belonging to different metabolic and regulatory pathways. Genes encoding a TF mediated by the phytohormone abscisic acid (ABA) are prominent among the targets of drought-responsive miRNAs. The drought stress-induced miRNA miR159, for example, targets the wheat (*Triticum aestivum*) genes encoding the MYB family TFs MYB33 and MYB65 (Fig. 2) (Giusti et al., 2017). The effect of miR159 activity is to enhance the accumulation of ABA, which in turn encourages the synthesis of reactive oxygen species (ROS). A second example in wheat is miR160, the abundance of which is promoted by exposure to drought stress (Akdogan et al., 2016). Through its targeting of genes involved in auxin and ABA signaling pathways (such as auxin response factors ARF10, ARF16, and ARF17), it is believed to make a significant contribution to the crosstalk between these pathways (Wang et al., 2005). Cucumber (*Cucumis sativus*) plants experiencing hypoxic stress have been shown to be deficient with respect to an auxin response factor (ARF) (Li et al., 2012), which might suggest an miRNA-based regulation. Transgenic Arabidopsis thaliana plants engineered to over-produce miR160 develop a greater abundance of lateral roots and the growth of their roots is strongly gravitropic under drought condition (Wang et al., 2005). In both chickpea (*Cicer arietinum*) and switchgrass (*Panicum virgatum*), most of the miRNAs which noticeably changed in droughted plants appear to target genes encoding TFs: these include members of the SPL (promoter-binding protein-like), MYB, NAC (NAM/ATAF/CUC), HD-ZIP (homeodomain-leucine zipper) and GRAS (GAI/RGA/SCR) families (Jatan et al., 2019; Xie et al., 2014). A proteomic analysis of switchgrass seedlings was able to establish that drought stress reduced the abundance of the MYB family TFs TRY and APL, while also promoting that of

a basic leucine zipper domain (bZIP) TF (Ye et al., 2016), suggesting a potential link for miRNA based regulation. Similarly, miRNA-directed regulation also has been suggested to raise the abundance of an HD-ZIP TF in the nuclear proteome of chickpea plants subjected to drought stress (Pandey et al., 2008). Drought-stressed A. thaliana plants under-accumulate miR169, known to target the TF-encoding gene NFYA5 (Fig. 2) (Li et al., 2008), thereby lifting the suppression of several drought-responsive genes. Plants engineered to over-accumulate miR169, by contrast, experience a high rate of water loss from their leaf surface, and become in consequence more sensitive than wild type to drought (Li et al., 2008). Exposing a drought-tolerant tomato (*Lycopersicum esculentum*) accession to drought stress results in a partial suppression of the production of the miRNA sly_miN_716 (Liu et al., 2018a), predicted to target a gene encoding the production of annexin, a phospholipid binding protein known to be involved in stress tolerance. An increased abundance of annexin protein is frequently observed in diverse plant species under various stress conditions (Mousavi et al., 2016), suggesting a potential link for miRNA-based regulation.

A common consequence of drought stress is the accumulation of ROS, compounds which, when present in excess, are highly damaging to membrane lipids, DNA and proteins. Their function is to act as signaling molecules during the plant's adaptation to stress (Choudhury et al., 2017). A number of enzymes are produced by plants in order to prevent the over-accumulation of ROS: of these the most prominent are superoxide dismutase (SOD), various peroxidases and catalase. In both maize (*Zea mays*) and wheat, the gene encoding Cu/Zn-SOD (a form of SOD which requires both copper and zinc) is targeted by miR398, a drought-repressed miRNA (Bakhshi et al., 2017; Wei et al., 2009). A deficiency in miR398 results in the increased abundance of Cu/Zn-SOD transcript, which is beneficial with respect to the plants' tolerance of oxidative stress (Fig. 2). This result has been supported at the proteomic level in maize, rice (*Oryza sativa*) and wheat (Ali and Komatsu, 2006; Faghani et al., 2015; Salekdeh et al., 2002; Zhu et al., 2007a). SOD transcript has been reported to also be targeted by miR528 in moisture-stressed rice, wheat and maize plants (Bakhshi et al., 2016; Kantar et al., 2011; Wei et al., 2009). In rice, for example, proteomic analysis of the same set of samples (those used for miRNA analysis (Bakhshi et al., 2016)), revealed an increased abundance of SOD following drought stress, suggesting a strong link for miRNA-based regulation (Mirzaei et al., 2012). In *T. turgidum*, the gene encoding Mn-SOD (a form of SOD requiring manganese) has been predicted to be targeted by the drought-responsive miRNA

miR1450 (Kantar et al., 2011). Mn-SOD is concentrated in the mitochondria, one of the major sites of ROS production. An increased abundance of Mn-SOD in response to drought stress has been reported in wheat, cotton (*Gossypium hirsutum*), rice and alfalfa (Medicago sativa) plants, thought to result from induced changes in the abundance of miRNAs targeting Mn-SOD transcript (Aranjuelo et al., 2011; Deeba et al., 2012; Ge et al., 2012; Mirzaei et al., 2014; Peng et al., 2009). Transgenic alfalfa plants over-expressing Mn-SOD have been shown to suffer less severely from drought stress than do wild type plants (McKersie et al., 1996), confirming the importance of SOD activity in the adaptation to drought stress. The gene encoding cytochrome C oxidase subunit V (COX5b) is an additional known target of miR398. In both the model legume barrel medic (*M. truncatula*) (Trindade et al., 2010) and wild emmer (*T. dicoccoides*) (Kantar et al., 2011), the abundance of miR398 rises in response to drought stress, thereby suppressing COX5b with negative consequences on the plants' levels of respiration.

The genes encoding certain enzymes involved in cellulose synthesis, sugar metabolism and starch metabolism have also been identified as targets of drought-responsive miRNAs (Xie et al., 2014). Examples are the switchgrass genes encoding starch synthase, the activity of which is compromised by drought stress (Ye et al., 2016), as well as those encoding a number of cellulose synthesis (Xie et al., 2014). The exposure of a drought-sensitive accession of wheat to drought stress has the effect of enhancing the abundance of miR4402, an miRNA predicted to target genes encoding enzymes required for cellulose synthesis (Bakhshi et al., 2017). Meanwhile, the effect of the stress on a tolerant accession results in a decreased abundance of miR13541 and miR18611, miRNAs which both target genes encoding the chitinases required for cell wall degradation. A proteomic analysis of these wheats was able to confirm the effect of the stress on the tissue concentration of the various enzymes (Faghani et al., 2015). The miRNA miR408 is believed to target a gene encoding NADH dehydrogenase (a component of the mitochondrial respiratory complex (Bakhshi et al., 2017)), since the effect of drought stress is both to promote the presence of this enzyme, while reducing that of miR408. The same miRNA in chickpea appears to target the transcription of a gene encoding plantacyanin, so that plants engineered to accumulate miR408 exhibit an enhanced tolerance to drought stress (Hajyzadeh et al., 2015). The analysis of wheat plants exposed to drought stress has suggested that a gene encoding phenylalanine tRNA synthetase-like is targeted by miR1432 (Kantar et al., 2011; Liu et al., 2015; Ma et al., 2015b), a finding that need to be further experimentally validated.

Photosynthesis is strongly compromised by drought stress, which at the same time increases a plant's level of respiration (Shinozaki and Yamaguchi-Shinozaki, 2007). A number of genes encoding enzymes associated with starch synthesis are likely targeted by miRNAs in plants exposed to drought stress. A soybean (*Glycine max*) example relates to miR397, which is less abundant in drought-tolerant than in drought-sensitive plants subjected to drought stress (Kulcheski et al., 2011); the target of this miRNA is a gene encoding the enzyme β-fructofuranosidase, a component of starch and sucrose synthesis (Fig. 2). The same miRNA in rice responds to drought stress treatment by a reduction in abundance (Zhou et al., 2010a). A drought stress-induced increase in the abundance of the product of its target gene (β-fructofuranosidase, INVA) has been noted in both rice and wheat (Liu and Bennett, 2011; Qin et al., 2014).

The heat shock proteins (HSPs) represent a further class of stress-related proteins potentially regulated by miRNAs in response to drought stress. In wheat plants, for example, the miRNAs miR444b, miR396b and miR395b target the genes encoding, respectively HSP83, HSP90 and an HSP-binding protein (Akdogan et al., 2016; Liu et al., 2017). Numerous proteomic studies have demonstrated the responsiveness to various abiotic stresses of HSPs (for review see PlantPReS database (Mousavi et al., 2016), which act to maintain the normal folding of cellular proteins (Wang et al., 2004). The response of wheat to high temperature stress, for example, includes a rise in the abundance of HSP70, the encoding gene for which is targeted by miR160 (Kumar et al., 2015). Some genes encoding enzymes involved in carbohydrate and energy metabolism are known to be the target of drought-responsive miRNAs. Thus, in a drought stress tolerant wheat accession, the content of both miR528 and miR1120, miRNAs predicted to target a gene encoding glyceraldehyde-3-phosphate dehydrogenase, is promoted by drought stress in the leaf, but suppressed in the root (Akdogan et al., 2016). Note that this enzyme is a component of the glycolysis pathway and has been frequently identified as responsive to both drought and salinity stress (Mousavi et al., 2016). The content of the metabolic enzyme NADP-dependent malic enzyme (NADP-ME) has also been documented as being regulated by miRNAs in stressed plants. A change in the abundance of this enzyme has been repeatedly noted in the tissue of both crop and non-crop plants subjected to a variety of stresses (Mousavi et al., 2016). Its abundance is promoted by drought stress in both maize (Dworak et al., 2016) and barley (*Hordeum vulgare*) (Ashoub et al., 2013) but is decreased in soybean (Mohammadi et al., 2012). The gene encoding for the NADP-ME is predicted to be targeted by miR395 (Ding et al., 2009); in wheat, members of this

miRNA family increase in abundance as a result of drought stress (Akdogan et al., 2016), and plants treated with the oxidizing agent hydrogen peroxide experience a fall in the content of the enzyme (Ge et al., 2013), suggestive of the encoding gene's miRNA-based regulation. Although the connection between miR395 expression and sulfur metabolism has been well established (Jones-Rhoades and Bartel, 2004), its contribution to the regulation of NADP-ME needs further experimental validation.

In both maize and rice, the content of miR474 responds positively to drought stress (Wei et al., 2009; Zhou et al., 2010a). As this miRNA targets a gene encoding proline dehydrogenase, the effect of drought stress is to promote the accumulation of proline. This helps to maintain cellular osmotic potential, while also contributing to the removal of ROS (Szabados and Savoure, 2010). A reduction in the abundance of this enzyme in response to salinity stress has been reported in A. thaliana (Taji et al., 2004), suggesting a potential link for miRNA-based regulation.

Salinity stress

Soil salinity is another environmental factor limiting agricultural production worldwide. Plants have evolved a number of ways to limit the entry of salt into roots, to restrict its loading into the xylem, to sequester it into vacuoles and to remove it via the transpiration stream (Deinlein et al., 2014; Munns and Gilliham, 2015). 'Omics-based approaches have provided a deal of information regarding the molecular and biochemical impact of exposure to salinity stress (Deng et al., 2015; Hashemi et al., 2016; Hosseini et al., 2015; Kosová et al., 2013; Mittal et al., 2016; Munns et al., 2006; Sarhadi et al., 2012; Zhang et al., 2012). The strong indication from these studies is that the plant response to salinity stress is regulated at many levels. Salinity has a profound effect on the plant proteome, with proteins functioning in photosynthesis, respiration, carbohydrate and lipid metabolism, protein synthesis and processing, ROS scavenging, osmotic adjustment, membrane and cell-wall related proteins and metabolism of secondary metabolites and osmolytes all being affected (Mousavi et al., 2016). Many of these proteins are also responsive to other environmental stresses, suggesting a general stress response (Barkla et al., 2013a; Barkla et al., 2013b). Some are highly abundant, but others are low abundant regulatory proteins, such as TFs and signaling proteins, which represent the major targets of miRNAs.

miRNAs are likely critical for the regulation of genes that are involved in the upstream pathways of salt stress response. In both barley and wheat, salinity stress raises the abundance of miR171 (Deng et al., 2015; Wang et al., 2014a), an miRNA which targets genes encoding various MYB

type TFs (Alptekin et al., 2017). The abundance of the conserved miRNA miR396 is reduced by salinity stress in rice plants, even though its encoding gene includes an ABRE element in its promoter region (Gao et al., 2010). Rice plants engineered to over-accumulate miR396 display an enhanced level of sensitivity to both salinity and alkaline stress, which implies that this miRNA probably targets genes encoding regulatory proteins involved in salinity tolerance (Gao et al., 2010). The over-accumulation of miR393 in rice has a similar phenotypic consequence (Gao et al., 2011); this miRNA targets both an auxin transporter gene (AUX1) and a rice tiller inhibitor gene (TIR1). As a result, miR393 over-accumulators exhibit enhanced tillering and early flowering, in addition to their hypersensitivity to auxin and salinity stress (Fig. 2) (Xia et al., 2012). The barley gene encoding enolase, a key enzyme involved in glycolysis, is thought to be targeted during an episode of salinity stress by miR-n05 (Deng et al., 2015). Supporting this notion is the observation that a tolerant barley accession responds to the stress by an increased accumulation of the enzyme (Mostek et al., 2015), which also occurs in salinity-challenged rice (Abbasi and Komatsu, 2004; Yan et al., 2005), wheat (Caruso et al., 2008) and *A. thaliana* (Ndimba et al., 2005). However, it is clearly unknown whether this upregulation is the consequence of the regulation by miRNAs or it is exerted at the transcriptional level. A negative correlation has been suggested to exist between the abundance of miR398 and that of its targets CSD1 and CSD2 in the presence of salinity stress (Jagadeeswaran et al., 2009). Stressed rice, wheat and canola (*Brassica napus*) plants accumulate Cu/Zn-SOD (Abbasi and Komatsu, 2004; Bandehagh et al., 2011; Caruso et al., 2008; Hosseini et al., 2015), the product of these genes (Fig. 2), suggestive of a potential link for miRNA-directed regulation. These data suggest a critical role for miRNA-mediated regulation of oxidative stresses under salinity condition.

Low temperature stress

Many temperate zone plant species can withstand exposure to sub-zero temperatures, provided that they have beforehand experienced a period of low (but above freezing point) temperature, a process referred to as cold acclimation (Thomashow, 1999; Xin and Browse, 2000). The process requires an extensive reprogramming of gene expression and cellular metabolism, featuring inter alia the accumulation of protective proteins and metabolites (Guy, 1990; Zhu et al., 2007b), such as late embryogenesis abundant proteins (LEAs), HSPs, cold-regulated proteins (CORs), antioxidant enzymes and antifreeze proteins (Gharechahi et al., 2014; Gharechahi et al., 2016; Kawamura and Uemura, 2003; Kosova et al., 2011; Sarhadi et al., 2010). The overall response

involves the up-regulation of genes encoding proteins participating in catabolism and the down-regulation of those encoding proteins participating in anabolism (Kosova et al., 2011; Mousavi et al., 2016). The contribution of miRNAs to cold acclimation was first recognized in A. thaliana (Sunkar and Zhu, 2004), where it was recognized that in seedlings exposed for 24 h to 0°C, the stress induces an increase in the abundance of miR393 and a decrease in that of both miR319c and miR398a. The contrasting pattern of the low temperature stress-induced accumulation of miR393 shown by two accessions of sugarcane (*Saccharum officinarum*) differing with respect to their tolerance of low temperature was taken to imply that this miRNA functions in the process of adaptation to low temperature (Yang et al., 2018). When A. thaliana was engineered to over-accumulate sugarcane miR393, it was found that the transgenic plants synthesize higher amounts of both anthocyanin and proline than do wild type plants, that they generate a higher transcript abundance of certain low cold-regulated genes (including CBF TFs) and that they exhibit an enhanced level of tolerance to prolonged low temperature stress (Yang et al., 2018).

Since low temperatures reduce the rate of enzyme-catalyzed reactions, cellular metabolism is slowed down, thereby increasing the likelihood of oxidative stress. As mentioned above, oxidative stress can discourage the synthesis of miR398, freeing up the level of expression of CSD1 and CSD2, and thereby increasing the plants' capacity to detoxify ROS (Fig. 2), a process which has been noted in both wheat and tomato plants subjected to low temperature stress (Gharechahi et al., 2014; Wang et al., 2014a). The over-expression in alfalfa of Cu/Zn-SOD has been associated with an enhanced level of tolerance to low temperature stress (McKersie et al., 1999). This data suggests that miRNA directed regulation of SOD abundance appears to be critical for cold acclimation and low-temperature tolerance.

In rice plants exposed to low temperature, the up-regulation of a gene encoding a pentatricopeptide repeat (PPR) protein has been correlated with a reduced presence of the miRNA miR1425 (Jeong et al., 2011). Members of this RNA binding family are known to involve in processing RNA in organelles as well as the nucleus and have been characterized as contributing to the defensive response to low temperature stress (Komori and Imaseki, 2005; Manna, 2015), while the rice pentatricopeptide repeat WSL5, a chloroplast targeted PPR protein, appears to be required for the development of chloroplasts in plants suffering from low temperature stress (Liu et al., 2018b). Other stress-responsive pentatricopeptide repeats have been identified from the proteomic profiling of low temperature-stressed rice seedlings (Yan et al., 2006), suggestive for a possible

miRNA-based regulation. A number of low temperature stress-responsive miRNAs have been identified: these include miR164, which targets transcripts generated from genes encoding LEAs; miR166, which targets homeobox-leucine zipper TF transcripts; miR156 which targets a gene encoding a squamosa promoter-binding like protein; miR171 which targets a gene encoding a scarecrow-like protein; miR394 which targets a gene encoding a F-box protein; and miR167 which targets genes encoding various ARFs (Megha et al., 2018b). Many of these proteins act as TFs mediating plant growth, development and the stress response. An altered abundance of the TFs bZIP, MYB34, bHLH and MYB2 has been documented in the nuclear proteome of low temperature-treated *A. thaliana* seedlings (Bae et al., 2003), consistent with their interaction with low temperature-responsive miRNAs. The rice miRNA miR156 regulates the gene SPL3, the product of which influences the expression of MYB2 (Zhou and Tang, 2018). The MYB2 product exerts control over the expression of a number of stress-responsive genes, including LEA3, DREB2A and RAB16A, and thus contributes to the tolerance of low temperature stress in rice. An increased abundance of LEA3 has been noted in both rice and wheat plants subjected to either low temperature or drought stress, a further potential example of the contribution of miRNAs to the stress response (Ke et al., 2009; Sarhadi et al., 2010).

The abundance of the conserved miRNA miR408 responds to several environmental stress agents, including drought, salinity, low temperature and heavy metal pollution (Ma et al., 2015a; Megha et al., 2018b; Sunkar and Zhu, 2004; Trindade et al., 2010). It has been predicted to target genes encoding members of the phytocyanin protein family, including cupredoxin, plantacyanin and uclacyanin, as well as the laccases LAC3, LAC12 and LAC13 (Fig. 2) (Abdel-Ghany and Pilon, 2008; Megha et al., 2018b). An analysis of A. thaliana plants transformed to produce an elevated content of miR408 showed that the genes encoding cupredoxin and LAC3 are both down-regulated, while CSD1 and CSD2 are both up-regulated; overall, the transgenic plants exhibit an improved degree of tolerance to low temperature (Ma et al., 2015a). The reduced presence of Cu-containing proteins such as cupredoxin likely increases the availability of this micronutrient for other Cu-containing proteins, such as Cu/Zn-SOD. On the other hand, a reduction in LAC3 activity imposes a downward pressure on the deposition of lignin in the cell wall, thereby increasing cell wall elasticity and limiting the damage caused by ice crystallization to both the cell wall and cell membranes (Megha et al., 2018b). Genes encoding laccase are also targeted by miR397, an miRNA positively associated with low temperature stress tolerance in A. thaliana (Dong and Pei, 2014).

A contribution of miR319 to low temperature stress tolerance has been noted in both rice and sugarcane (Megha et al., 2018b; Thiebaut et al., 2012; Yang et al., 2013). At low temperatures, miR319a/b is repressed and consequently its target genes are up-regulated, resulting in a limited ability to tolerate low temperatures (Thiebaut et al., 2012; Yang et al., 2013). However, over-expressing miR319 in rice plants results in an enhanced accumulation of proline, increases the expression of low temperature tolerance genes and decreases that of its target genes OsPCF6 and OsTCP21; the overall impact is an improved level of tolerance to low temperature (Wang et al., 2014b). These data suggest that miR319 contributes to the low temperature stress tolerance response, likely via the down-regulation of its target genes and the induction of CBF genes and those encoding ROS scavenging enzymes (Megha et al., 2018b). In A. thaliana, low temperature stress has also been shown to affect the cellular content of miR417 (Jung and Kang, 2007), an miRNA which likely targets genes encoding C2-domain containing and SNF7 family proteins, both of which are associated with cold acclimation. HSPs have also been well documented to contribute to cold acclimation (Gharechahi et al., 2016): in canola, for example, a novel cold responsive miRNA bna-N_miR12 has been shown to target HSP70 in plants subjected to low temperature stress (Megha et al., 2018a). The existence of an inverse correlation with its target and the detection of HSP70's mRNA cleavage product using RLM-RACE further demonstrated that it is targeted by this miRNA under the condition of cold stress.

Nutritional stress

Deficiency with respect to both macro- and micronutrients leads to restricted growth and defective development, so plants have evolved a variety of regulatory mechanisms to ensure a sufficiency in the supply of nutrients (Alptekin et al., 2017). The uptake of micronutrients needs to be precisely controlled, because when over-accumulated, they can become phytotoxic (Kerry et al., 2018): for example, an excessive uptake of a number of metals, including Cu, Fe, Mn, Mo (molybdenum), Zn and Ni (nickel), into plant cells results in oxidative damage to DNA, proteins and membrane lipids. A wide range of proteins, involved in energy metabolism, photosynthesis, amino acid metabolism, protein synthesis and processing, chaperoning and antioxidation, are known respond negatively to macronutrient (nitrogen, potassium and phosphorus) deficiency (Mousavi et al., 2016). The proteomic changes induced by nutrient deficiency are similar to those classified as belonging to the general stress response. Consideration of the miRNome has led to predictions that miRNAs are intimately involved in the plant response to mineral deficiency and toxicity. The

miRNAs miR395 and miR399 have been shown to target genes encoding, respectively, the sulfate transporter SULTR and the phosphate transporter PHO1 (Fig. 2) (Chiou, 2006; Hsieh et al., 2009; Jones-Rhoades and Bartel, 2004). In plants starved of phosphorus, the PHR1 transcription factor is activated, which induces the synthesis of miR399. In A. thaliana, the engineering of an increased abundance of miR399 results in the down-regulation of PHO2, a gene which encodes a ubiquitin-conjugating E2 enzyme (Bari et al., 2006; Hackenberg et al., 2013). The constitutive expression of the gene encoding A. thaliana miR399d in tomato results in the accumulation of inorganic phosphate in the leaf and an increase in the secretion of protons from the root, thereby facilitating the conversion of soil organic to inorganic phosphate, the latter being the form of phosphorus most readily taken up by the plant (Gao et al., 2009). This is an example that clearly shows how miRNAs and proteins cooperate to shape the response of the plant to a stress condition. Plants engineered to over-accumulate miR399 respond to phosphate starvation by suppressing the gene encoding for PHO2 (known as UBC24), known to regulate Pi uptake and translocation under conditions of limited phosphate availability (Bari et al., 2006; Fujii et al., 2005). Downregulation of UBC24 is required for maintaining Pi homeostasis under Pi starved condition, likely via the induction of high-affinity Pi transporters and the attenuation of primary root elongation (Chiou, 2006; Fujii et al., 2005). Sulfur starvation particularly affects the expression of genes encoding proteins involved in sulfate metabolism. This process has been shown to be regulated by miR395, a conserved miRNA induced by sulfur starvation to reduce in abundance; the miRNA targets genes encoding ATP sulfurylases and sulfate transporter 2 (Liang et al., 2010). ATP sulfurylases catalyze the first step of sulfur assimilation, while sulfate transporter 2 is involved in translocation of sulfate from roots to shoots (Takahashi et al., 1997).

Certain aspects of Cu metabolism are known to be mediated by miRNAs. Cu deficiency has been shown to enhance the abundance of miR398, which targets not just CSD1 and CSD2 but also CCS1, a gene encoding a Cu chaperone for SOD1 (Fig. 2) (Beauclair et al., 2010; Sunkar et al., 2006). A reduced presence of these proteins could enable the plant to allocate scarce Cu to other essential enzymes, such as Cu/Zn-SOD, which, as discussed earlier, acts to limit the accumulation of ROS. In addition to miR398, the abundance of both miR397 and miR408 is enhanced by Cu deficiency likely by the action of SPL7 transcription factor (Yamasaki et al., 2009). These miRNAs target a number of genes encoding Cu-containing proteins, such as cupredoxin, plantacyanin,

uclacyanin and laccase; the repression of these proteins likely improves the availability of scarce Cu required for other essential Cu-contacting enzymes.

Some miRNAs are known to regulate the uptake and metabolism of nitrogen, largely through their targeting of genes encoding key growth regulating TFs. For example, the abundance of miR393 is raised in response to the greater availability of nitrogen; this miRNA targets a gene encoding an auxin signaling F-Box protein (AFB3) known to influence root architecture (Vidal et al., 2010). A second nitrogen-responsive miRNA, which is reduced in abundance by nitrogen starvation, is miR167; this miRNA interacts with ARF8 (Gifford et al., 2008), the product of which promotes lateral root formation and hence influences the plant's efficiency in acquiring nitrogen from the soil. Wheat roots and leaves accumulate higher levels of miR444a in response to nitrogen deficiency (Gao et al., 2016), and the constitutive expression of the gene encoding this miRNA in tobacco results in an enhancement to nitrogen uptake under nitrogen deficient conditions. Nitrogen uptake is regulated by miR169 via a rather different regulatory circuit: under conditions of nitrogen starvation, the miRNA's abundance is reduced, resulting in an increase in the production of NFYA5 transcript and the up-regulation of genes such as NRT2.1 and NRT1.1 which encode proteins acting to promote the uptake and transport of nitrogen (Zhao et al., 2011). Suppressing the synthesis of nitrogen-rich secondary metabolites represents yet another mechanism by which miRNAs can exert control over nitrogen metabolism during an episode of nitrogen starvation. For instance, miR826 and miR5090 target AOP2, a gene encoding the enzyme 2-oxoglutarate-dependent dioxygenase, involved in the synthesis of glucosinolates (He et al., 2014). As a result, scarce nitrogen is freed up to support other vital processes.

Certain miRNAs are also involved in the plant response to heavy metal toxicity. In M. truncatula, for example, an increased abundance of miR393, miR171, miR319 and miR529 has been reported in leaves following the plants' exposure to a high concentration of cadmium, mercury or aluminium (Zhou et al., 2008), while in the roots, the abundance of both miR319 and miR390 is reduced in the presence of toxic levels of aluminium, and that of miR390 by toxic levels of either cadmium or mercury (Chen et al., 2012). Members of the miR319 family target genes encoding TCP/PCF TFs. In rice, the products of two such genes have been implicated as influencing the expression of PCNA, a gene encoding a proliferating cell nuclear antigen (Kosugi and Ohashi, 1997). The abundance of PCNA in barley responds to exposure to aluminium stress (Dai et al., 2013), suggestive for an indirect miRNA based regulation. TAS3 transcript is degraded by miR390,

leading to the production of trans-acting small interference RNAs which target the genes ARF2, ARF3 and ARF4 (Marin et al., 2010). The abundance of miR398 is reduced as a result of exposure to excessive Fe and Cu, thereby promoting Cu/Zn-SOD activity and consequently strengthening the plant's defense against oxidative stress (Fig. 2) (Cuypers et al., 2011; Sunkar et al., 2006). However, in the presence of excessive levels of cadmium, the abundance of this miRNA is reduced, resulting in a decline in SOD activity (Cuypers et al., 2011). A number of miRNAs have been recently reported in maize to respond positively to the presence of arsenate in the growing medium (Ghosh et al., 2017); these include miR319b, prompting the down-regulation of its target genes GAMYB and PCF 6/8-like.

Combinations of abiotic stress

Field-grown crop plants need to be capable of adjusting their transcriptome, proteome and metabolome to confront combinations of stress factors, since these often occur in tandem. Drought, low temperature and salinity stress all induce tissue dehydration and osmotic stress, and so require some commonality in terms of the cellular response. Coping with different stresses occurring simultaneously requires, however, a degree of cross-talk between different regulatory networks. Genome-wide transcriptomic analyses carried out in both tobacco and poplar plants subjected to both high temperature and drought stress have revealed little evidence of any overlap between their responses to the two stresses applied separately (Jia et al., 2017; Rizhsky et al., 2002). A similar analysis of the A. thaliana response to high temperature, low temperature, salinity, high light and pathogen infection, applied either individually or in combination, has revealed that although almost 25% of transcripts display a similar response to the various stressors when imposed singly, the response to combined stresses is quite distinct (Rasmussen et al., 2013). At the level of the proteome, in contrast, extensive overlap has been demonstrated between the responses to combined stressors and those to single stresses. In rice, for example, the imposition of both high temperature and drought stress results in the accumulation of the same set of HSPs (Jagadish et al., 2011). Similarly, in barley and the conkerberry (*Carissa spinarum*), the combined imposition of high temperature and drought stress significantly affects the profile of HSPs, as well as that of proteins involved in photosynthesis, detoxification, energy metabolism and protein synthesis (Ashoub et al., 2015; Rollins et al., 2013; Zhang et al., 2010), while in A. thaliana, the same combination resulted in the promotion of enzymes taking part in malate metabolism, the Calvin cycle and ROS detoxification (Koussevitzky et al., 2008). The increased abundance of several

HSPs, a characteristic feature of these combined stress experiments, may serve to protect photosynthetic and other metabolic enzymes from inactivation and aggregation (Ashoub et al., 2015). Changes in the cellular content of proteins involved in photosynthesis, protein synthesis and processing, antioxidation, carbohydrate and energy metabolism, amino acid metabolism, among others, have been frequently reported as forming part of the response to exposure to multiple abiotic and biotic stresses (for review see (Mousavi et al., 2016)). To the best of our knowledge, however, the only attempt published to date which has addressed the identity of miRNAs responding to multiple stresses has focused on bermuda grass (*Cynodon dactylon*) plants exposed to both low temperature and salinity stress (Hu et al., 2018). When subjected to just salinity stress, 43 miRNAs were found to be responsive, but only 18 of these also responded to the combined low temperature and salinity treatment. Represented among the latter were miR159, miR160, miR166, miR169, miR172, miR395 and miR398, which target genes encoding products involved in metabolism, phytohormone synthesis, growth and development, and tolerance to either low temperature or salinity stress (Ghosh et al., 2017; Hu et al., 2018; Sunkar and Zhu, 2004).

The miRNAs miR398, miR408 and miR169, which exhibit either similar or contrasting responses to various stresses, are featured in Fig. 2. A relatively large number of miRNAs are induced by both drought and salinity stress – these include miR156, miR159, miR165, miR167, miR168, miR319, miR393, miR395, miR396, miR399 and miR402; the implication is that these all regulate common aspects of the responses to the two stresses. Other miRNAs, namely miR156, miR169, miR394 and miR828, are known to respond to low temperature, drought stress and high light intensity. An analysis conducted in alfalfa plants over-expressing the gene responsible for the synthesis of miR156 has revealed a deficit in the abundance of WD40-2 transcript, an observation which was taken to suggest that this miRNA influences drought stress tolerance by down-regulating WD40-2 (Arshad et al., 2018).

Conclusions and future perspectives

The functional significance of an miRNA depends on that of its target gene(s), so the identification of these targets is central to gaining an understanding of their biological role. As most predictions made to date of plant miRNA targets has relied on a bioinformatics approach, it is likely that some proportion of them will be prove to be false positives; distinguishing these from genuine targets will require experimental data (Thomson et al., 2011). A number of experimental platforms have been developed for target validation: 5′ RLM-RACE and degradome sequencing can be used to

identify miRNA-directed cleavage products, immunoprecipitation to characterize the mRNAs associated with the RNA-induced silencing complex, ribosome profiling to measure the translation rate of the target gene and MS-based proteomics to profile the proteome (Fig. 1) (Baek et al., 2008; Fei et al., 2018; Selbach et al., 2008; Thomson et al., 2011). The reliability of proteomic analysis to quantify proteins targeted by miRNAs has been well demonstrated in animal cell lines engineered to either over-accumulate or fail to synthesize specific miRNAs (Baek et al., 2008; Vinther et al., 2006; Yang et al., 2010), but this approach has not as yet been successfully extended to plant cells because of practical difficulties encountered in the construction and/or maintenance of the appropriate transgenic cell lines. While cell suspension cultures can be used as an *in vitro* model system for the characterization of miRNA targets, their informativeness is limited by their failure to represent the events occurring in a whole plant. Furthermore, many miRNA targets are expressed in an organ- or even in a tissue-specific manner, so may not be transcribed at all in a cell suspension culture. The generally poor correlations pertaining between miRNA expression data with protein abundance data emphasize the need to develop more sophisticated and targeted methods such as those based on selected reaction monitoring (SRM) to enable accurate miRNA target quantification and validation using proteomics approaches.

Current proteomics methodologies still suffer from a lack of sensitivity and inadequate reproducibility, making it hard to draw reliable conclusions regarding correlations between miRNA and protein abundances. The identification and validation of the genes targeted by plant stress-responsive miRNAs will, however, make a significant contribution to revealing the pathways and regulatory mechanisms involved in the stress response, paving the way for the implementation of genetic engineering approaches to improve the stress tolerance of important crop species.

Conflict of interest

The authors declare no conflict of interest.

Author Statement

JG, GS, MZ, MM, and GHS contributed in writing and revising the manuscripts.

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Figure legends

Fig. 1. The synthesis and function of miRNAs and the approaches used for the identification and validation of their target genes. The gene encoding a given miRNA is initially transcribed by RNA polymerase II; the resulting transcript subsequently forms a stem-loop structure referred to as a pri-miRNA. Pri-miRNAs are processed into pre-miRNAs through the action of the enzyme DCL. The nuclear export protein HST1 transfers miRNA/miRNA* duplexes to the cytosol. The miRNA strand (guide strand) is loaded into an RNA silencing complex which enables the miRNA to bind to its target mRNA. Successful binding leads to either the cleavage and degradation of the target transcript, (B) to the inhibition of its translation, or (C) to the repression of its transcription through miRNA-directed DNA methylation (Bao et al., 2004; Voinnet, 2009; Wu et al., 2010). Targets are typically predicted (1) in silico, and can be subsequently validated using a variety of approaches, including (2) the identification and quantification of miRNA cleavage products using 5′ RLM-RACE, (3) degradome sequencing, (4) measurement of the translation rate of the target gene using ribosome profiling, or (5) quantification of the target gene product using a proteome profiling (Fei et al., 2018; Thomson et al., 2011).

Fig. 2. The contribution of miRNAs to plant adaptation/tolerance to environmental abiotic stress. The abundance of certain miRNAs is altered following the imposition of stress, which in turn leads to changes in the abundance of their target genes' products and hence an altered phenotype. Here, only the direct impact of the miRNome on the proteome has been depicted, but miRNAs can also influence the expression of non-target genes where the primary miRNA target is a regulatory protein, such as a TF, which controls the transcription of one or more downstream genes (Tu et al., 2009). The abundance of specific miRNAs is regulated by proteins at multiple levels, for example, by proteins which control the transcription of their encoding gene, by RNA processing enzymes which affect their synthesis, maturation and/or transport, as well as by proteins involved in their degradation and turnover (Xie et al., 2015). In certain miRNAs, a feedback loop forms between an miRNA and its target, particularly in the situation where the target encodes a TF which regulates the transcription of the miRNA's encoding gene (Meng et al., 2011). The regulation by miR160 and miR167 of the genes encoding the ARF transcription factors ARF6, ARF8 and ARF17 provides an example of such a feedback loop (Gutierrez et al., 2009). ABA: abscisic acid, ROS: reactive oxygen species, ARF: auxin response factor, INVA: β-fructofuranosidase, CSD1/2:

Cu/Zn-superoxide dismutase, PDH: proline dehydrogenase, NFYA5: nuclear factor YA5, LAC: laccase, AFB3: F-Box protein 3, APS: ATP sulfurylase, SULTR: sulfate transporter 2, PHO1: phosphate transporter, UBC24: ubiquitin-conjugating enzyme.

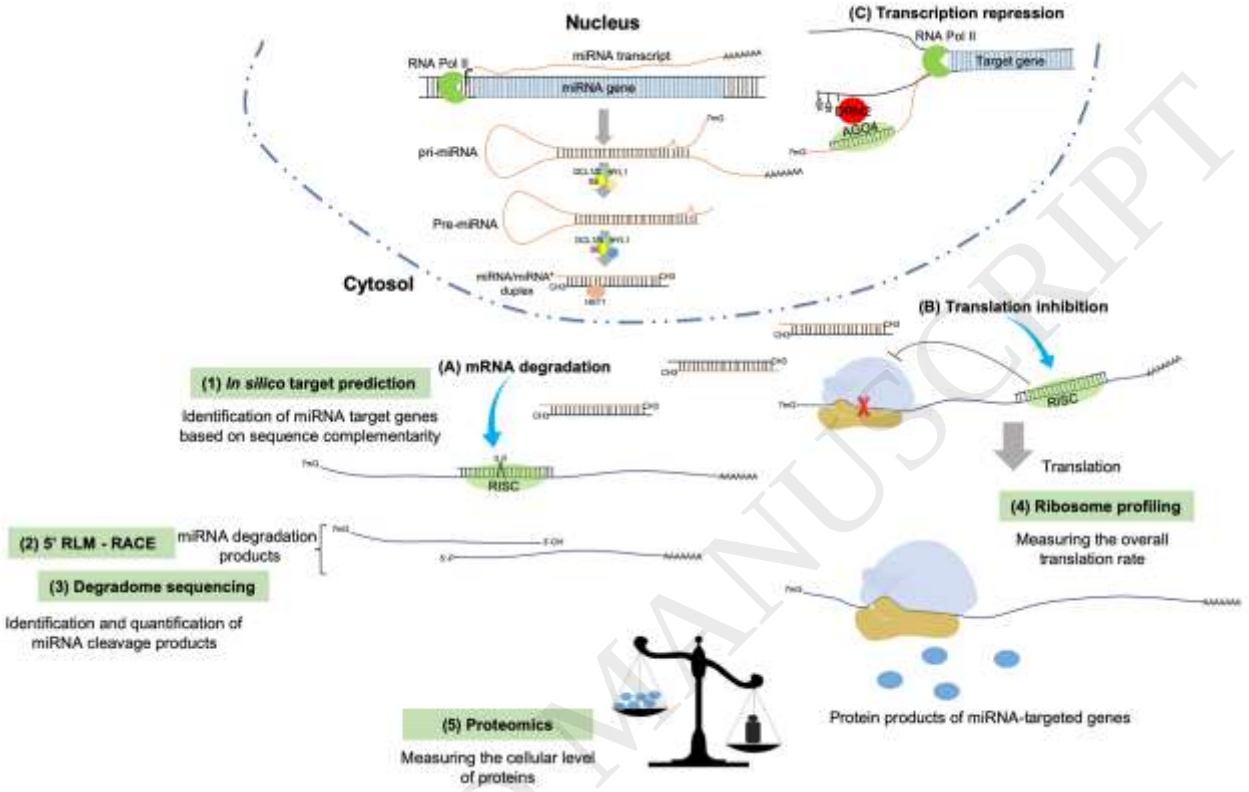

Fig. 1

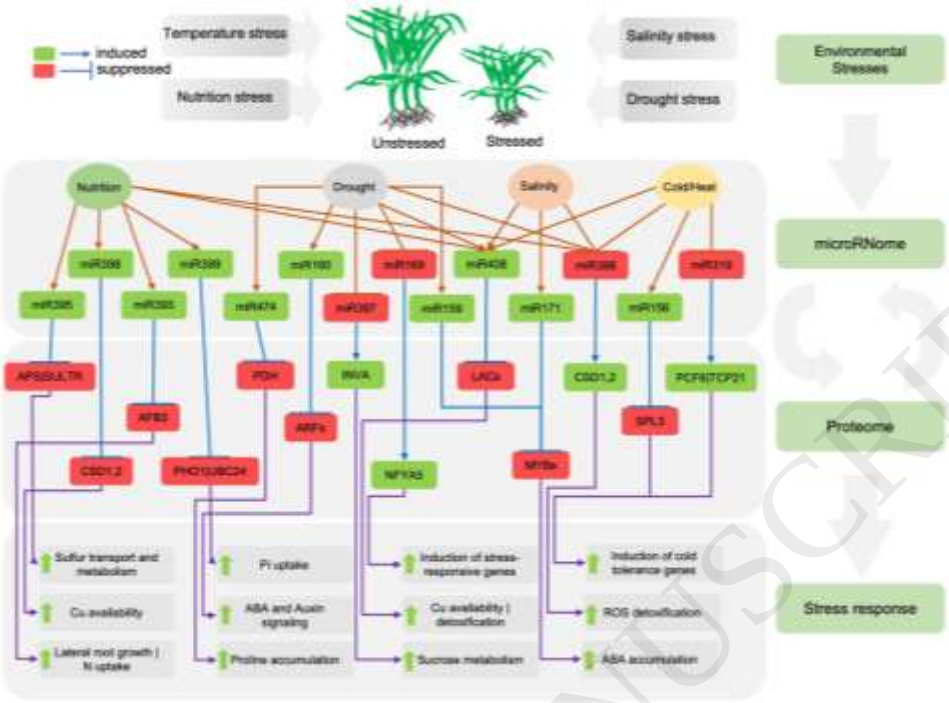

Fig. 2