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Review Article

miRNA Mediated Post-Transcriptional Gene Regulation in Response to Abiotic Stress in Plants

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Due to their sessile nature, plants are often exposed to harsh environmental conditions. Their livelihood depends on their innate ability to respond to stress and cope with whatever challenges they may face. Phenotypic responses have been characterized to correlate to abiotic stresses, but the molecular mechanisms which underlie these responses are still under investigation. Recently, microRNAs (miRNAs) have been shown to play a key role in post-transcriptional regulation in response to abiotic stress. These small, 21 nt miRNAs, target specific mRNA transcripts and affect their translation which may cause downstream effects leading to abiotic stress response. In the following review, research using *Arabidopsis thaliana* as a model system to investigate post-transcriptional regulation in response to abiotic stressors, such as phosphate depletion and oxidative stress, is discussed. Furthermore, research in more economically important crops, such as corn and rice, is overviewed. Investigation into miRNA function in response to stress could provide a new platform to engineer more resilient crops which may prove crucial as climate change and food insecurity continue to plague our agricultural systems.

Keywords: miRNA, Post-transcriptional Gene Regulation, Abiotic Stress, *Arabidopsis*, Maize, Rice

Introduction

Plants do not have the freedom most animals have to move away from inadequate or dangerous environments. Instead, they are vulnerable to whatever conditions occur in their environment. In order to survive, plants must be able to respond and adapt to the variety of stressful conditions faced during their lifecycle. Economically, these responses are crucial. Our agricultural system relies on crop yield in any and all environmental conditions. Farmers rely on the plant's ability to survive and adapt to the changing soil, weather and climate conditions. With global climate change and environmental degradation becoming increasingly prevalent in the world today, response to abiotic stress has become crucial to plant survival. Environmental conditions are constantly in flux, placing a higher demand on plant response systems. Phenotypic and developmental changes are clearly observed in plants grown under stress conditions, but the molecular mechanisms behind these responses are not fully understood (Sunkar *et al.*, 2012). Further investigation into the molecular basis of plant stress response could provide immense potential for the development of crops adapted to stressful conditions.

MicroRNAs are small regulatory RNA molecules that have been shown to participate in genetic regulation in animals (Cech and Steitz, 2014). Recently, miRNAs have also been discovered to participate in post-transcriptional regulation of gene expression in plants (reviewed by Sunkar *et al.*, 2012). In plants, miRNAs are approximately 21 nucleotides long and are formed after processing of longer miRNA primary transcripts. Much like in animals, plant miRNAs function through complementary binding of the miRNA to target transcripts, although with a much higher specificity than observed in animals. This binding of miRNA to target mRNA will either cause degradation or translational repression. While there are fewer miRNAs identified in plants than animals, most of the targets are transcription factors (TFs) with widespread functions in developmental processes throughout the plant lifecycle. Therefore, plant miRNAs can have dramatic and widespread impact on the development of plants when exposed to stress.

miRNA regulation has been identified in several different response systems in a variety of plant species. Each miRNA, and their effects, has been shown to be widely conserved throughout several species including maize, rice and *Arabidopsis* (Zhu *et al.*, 2011). The evolutionary significance of this response pathway is evidenced by the fact that nearly half of the miRNA families are conserved in a wide variety of species (Chiou, 2007). Therefore, this regulatory response to stress has clearly benefitted the plant kingdom throughout time and must be crucial to plant survival. This mechanism is deeply rooted in the evolutionary history of plants and warrants in depth investigation.

After the discovery of the first stress responsive miRNA, a multitude of miRNAs involved in stress response have emerged from the plant genome. miRNA has been shown to be involved in a vast array of responses including, but not limited to, oxidative stress, UV-irradiation, drought, and nutrient depravation (Zhu *et al.*, 2011). The extensive activity of miRNA post-transcriptional regulation, along with its evolutionary conservation, encourages the study of miRNA as a potential source of crop development. Therefore, if more could be elucidated about their function, composition and existence, a portal of potential could be opened for the scientific community to begin to find solutions to agricultural problems caused by abiotic stress. With the environmental crisis escalating concomitantly with the food security epidemic, solutions must be pursued to help the agricultural community adapt and flourish in the changing environment while withstanding the increased pressure of a growing population. Post-transcriptional gene regulation by miRNAs in response to abiotic stress has established itself as one of the leading areas of study for the scientific community to come alongside the agricultural community and begin to confront these problems.

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***Arabidopsis* as a Model System**

Arabidopsis thaliana has been firmly established as the most prevalent, successful and applicable plant model system. Therefore, initial studies into plant miRNA function in response to abiotic stress have begun in *Arabidopsis*. Information that has been gleaned from *Arabidopsis* can then be translated to other, more economically important, plants. However, it is essential to begin to understand this complex regulatory mechanism in a well-understood plant system. While many abiotic stress responses have been documented in *Arabidopsis* (reviewed by Floris *et al*., 2009 and Mazzucotelli *et al*., 2008), the focus will be on phosphate starvation, nitrogen response and oxidative stress as these factors play a significant role in plant health in today's environmental flux.

Phosphate Starvation

Inorganic phosphate (Pi) is one of many essential nutrients plants mine the soil to acquire and is often a limiting factor of plant growth. As a result, plants have developed adaptations to respond to low Pi levels including modifications of root growth and structure, increased Pi transport, and internal Pi recycling. Plants will secrete organic acids, phosphatases and nucleases from their root cells in an attempt to release insoluble phosphate from the soil. While phenotypic changes in response to Pi starvation have been observed, the molecular signaling by which they are activated is still being elucidated (Bari *et al*., 2006).

An investigation by Fujii and colleagues (2005) into abiotic stress signaling pathways of *Arabidopsis* identified miR399 as a regulatory miRNA involved in Pi starvation response. Northern blot analysis of miRNAs extracted from the roots and shoots of plants grown under several stress response situations including drought, salt, cold, low potassium and low nitrogen confirmed that miR399 is specifically induced only under low Pi conditions. The induction was not immediate; miR399 was not detected until 12 hours after being introduced to low Pi medium, but was detected throughout the remaining time course of 48 hours.

Further experiments by Bari and coworkers (2006) examined the level of miR399 primary transcripts during Pi starvation, as well as during recovery by introducing previously Pi starved plants to medium with sufficient Pi. The primary transcripts of miR399 showed a 1,000 to 10,000-fold increase under low Pi conditions. This dramatic increase has caused miR399 primary transcripts to be considered some of the most Pi responsive transcripts in *Arabidopsis*. The primary transcripts also decreased at the same rate when Pi was reintroduced to the media, showing decreased levels after only 30 minutes. Mature miR399 was shown to decrease within 6 to 12 hours after addition of Pi, suggesting a highly sensitive signaling and response mechanism to soil phosphate levels.

miR399 function in Pi starvation appears to be mediated through a putative ubiquitin-conjugating enzyme (UBC), later confirmed to be *PHO2*, an E2 conjugase (Bari *et al*., 2006 and Fujii *et al*., 2005). Map based cloning located the *PHO2* gene to a 5.5 kb region encoding a 907-amino acid protein. This protein contains a C-terminal domain that is conserved and is the functional ubiquitin-conjugating domain of E2 conjugases. Analysis of the expression profile of *PHO2* revealed that the mRNA is continuously expressed at high levels in all tissues throughout the life of the plant. Initial studies (Fujii *et al*., 2005) demonstrated an inverse relationship between miR399 levels and PHO2 levels. Northern-blot analysis of *PHO2* mRNA from plants grown in Pi-sufficient media revealed considerable levels of expression over a course of 24 hours. However, in low Pi conditions, *PHO2* mRNA levels decreased after 24 hours and were almost completely diminished by 48 hours. The decrease of *PHO2* mRNA corresponded to the observed increase in miR399 levels. These results correlate the decrease of *PHO2* levels as a result of phosphate stress with the presence of miR399 further suggesting the observed decrease was mediated by miR399 activity.

The target of miR399 was confirmed to be *PHO2* through miR399 overexpression experimentation (Bari *et al*., 2006). Overexpression of several miR399 family members under normal Pi conditions showed a reduced level of *PHO2* transcripts when, normally, these levels should not be suppressed. Interestingly, this was also accompanied by an increase in Pi levels in the leaves presenting a possible function of the response mechanism to increase Pi intake when levels are low. As the amount of overexpression increased, the amount of *PHO2* transcript levels decreased and the level of Pi in the leaves increased concomitantly, suggesting miR399 regulates the transcript levels of *PHO2*, in turn, positively affecting the intake of Pi from the environment. The connection between miR399 levels and Pi accumulation was further confirmed through transgenic, overexpression plants (Fujii *et al*., 2005). In plants constitutively expressing miR399, Pi levels were more than double the levels in wildtype plants. The transgenic plants, when subjected to a high-transpiration environment, showed signs of Pi toxicity. Therefore, by altering the expression of miR399, the regulation mechanism for Pi homeostasis was interrupted. The above evidence presents a response mechanism to phosphate stress by post-transcriptional downregulation of *PHO2* mRNA by miR399 to increase the intake of Pi from the environment.

Plant miRNAs are known to target highly specific sequences in the 3'-untranslated region (UTR) of their target transcripts (Chiou, 2007). Interestingly, five miR399 target sites were identified within the 5'-UTR of the *PHO2* mRNA (Fujii *et al*., 2005). Transgenic plants were created in which the wildtype or a 5'-UTR deletion mutant of *PHO2* was placed downstream of the strong constitutive cauliflower mosaic virus 35S promoter. Transgenic plants with the *PHO2* 5'-UTR deletion mutant, in contrast to those with the wildtype sequence, did not show a change of transcript level under low-Pi conditions indicating the essential role this sequence has for recognition and regulation by miR399 during phosphate starvation.

However, the mode of action of the miR399 and *PHO2* 5'-UTR interaction is still unknown. miRNA mediated regulation involves complementary base pairing between target mRNA and the miRNA leading to cleavage and degradation of the target mRNA or repression of translation from the mRNA template. qRT-PCR analysis (Bari *et al*., 2006) of *PHO2* transcript fragments of the sizes that would indicate cleavage found only RNA that is the same size as the original transcript. This indicates the *PHO2* transcripts are intact and any cleaved *PHO2* mRNAs do not persist within the cells. This could either mean the *PHO2* mRNA/mi399RNA complexes are not being directly cleaved

but instead are repressing translation, or there may be a simultaneous regulation of the degradation of the cleavage products explaining why cleavage products are not detected even though *PHO2* is clearly being regulated by miR399.

There are several counterintuitive findings upon further investigation of *PHO2* and miR399 transcript levels (Bari *et al*., 2006). For example, the concentration of *PHO2* mRNA decreased 8-fold during initial Pi starvation reaching a steady state even as miR399 levels continued to increase. The steady state concentration of *PHO2* mRNA persists throughout the course of Pi starvation then decreased when Pi was introduced into the medium even though miR399 levels are also decreasing during this time. These data suggest that, while miR399 clearly plays a role in the regulation of *PHO2* in response to Pi starvation, there may be other, posttranscriptional factors regulating *PHO2* gene expression.

Not only is *PHO2* most likely regulated by several other factors, but miR399 also has been shown to have physiological effects on the plant unrelated to Pi starvation (Kim *et al*., 2011). For example, transgenic miR399 overexpression *Arabidopsis* plants had a greater flowering time variation in response to ambient temperature than wildtype plants. Interestingly enough, *PHO2* also appears to have some role in these effects as well. The miR399 – *PHO2* system is not an isolated system, it is interrelated with other networks and can have broader effects on the life cycle and health of the plant. These complex interactions should not be overlooked because they may provide insight into the importance of miRNA to the overall health of a plant. Clearly, miRNA has a dynamic role not only in homeostasis, but also in other processes and activities such as development and signaling. It is interesting to note that both of the proposed miR399 mediated responses are a result of environmental conditions, suggesting the initial transcription if miR399 relies on the perception of external signals.

miR399 involvement in the regulation of Pi homeostasis has been shown to be conserved throughout several diverse species (Bari *et al*., 2006). In rice plants grown under Pi-depleted conditions, mature miR399 was detected. A broad database search by these authors revealed sequences in wheat, soybean, cotton, orange, apple, and poplar coding for miR399, containing miR399 binding sites, or sequences homologous to *PHO2*. The wide range of species found to contain members of this regulatory mechanism suggests a considerable level of conservation among plants. Therefore, this mechanism must have developed during the evolution of higher plants and has persisted throughout time suggesting its importance to plant health and survival. There is great potential to expand research of miR399 phosphate starvation response in economically important crops. This could help develop plants able to avoid Pi starvation and thus grow in soils not typically arable due to low phosphate levels.

Nitrogen Starvation

Similar to inorganic phosphate, nitrogen is an essential nutrient for plant growth and development. Since it is so crucial, farmers have begun to rely on artificial fertilizers to insure adequate nitrogen levels in their fields. However, large influxes of nitrogen have begun to cause severe, widespread, environmental degradation as excess nitrogen contaminates the water system. Research into the mechanisms by which plants naturally respond to low nitrogen levels may provide insight into how plants with a greater ability to absorb nitrogen from the environment may be developed in order to reduce reliance on artificial fertilizers.

Using real-time RT-PCR, Zhao and coworkers (2011) identified miR169 as a key regulatory miRNA for nitrogen (N) starvation response in *Arabidopsis*. However, the transcriptional response to N starvation was opposite of the response previously discussed in Pi starvation. Under low-N conditions, miR169 is suppressed in both the roots and the shoots of *Arabidopsis.* The decrease was quite substantial, registering an 87% decrease in the roots and an 89% decrease in the shoots. The decrease in miR169 levels was accompanied by an increase in the target mRNAs, the *NF-Y* family of TFs. This suggests that NF-Y TFs are suppressed under normal conditions and relieved under N stress. The authors infer that the NF-Y TFs are important for N homeostasis, perhaps in changing N intake or utilization during stress since it is only activated when N levels are low. Under normal conditions, production of NF-Y TFs is not needed, and therefore their translation is repressed to conserve energy and resources. However, when the plant is stressed due to low N availability, these proteins may play a crucial function in N absorption or utilization and the mRNA is fully expressed.

To further support the importance of miR169 down regulation in response to N starvation, transgenic *Arabidopsis* plants were engineered to constitutively express miR169 (Zhao *et al*., 2011). These plants, when subjected to N starvation, showed typical N-deficient symptoms not seen in the wildtype plants. These plants also had a total N content 7.6% lower than the wildtype, demonstrating the targets of miR169 must play a role in increasing N intake from the environment. This also infers miR169 may play a role in maintaining N levels at a steady state during normal conditions. *NRT1.1* is a member of a gene family involved in nitrate transport and has been shown to play a central role in nutrient transport from the environment and into cells. Accumulation of *NRT1.1* mRNA was 65% lower in transgenic *Arabidopsis* overexpressing miR169, suggesting regulation of this transporter by miR169.

Nitrogen starvation response is not mediated by miR169 alone. Liang, He and Yu (2012) used deep sequencing to identify 15 miRNAs whose expression profile were changed, either by repression or upregulation, as a response to N starvation, opening the door to a broad range of investigation into the function of responsive miRNAs. The variable expression patterns of the miRNAs discovered suggest each may play a different role to produce a unified response to N stress. There may also be cross-talk between different homeostasis pathways, as several miRNAs known to function in other nutrient regulation pathways were found to also respond to N starvation. The absorption of one nutrient often affects the absorption of another vital nutrient, and therefore cross-talk between pathways would be crucial to help maintain overall plant homeostasis (Liang *et al*., 2012). The accumulation of this knowledge provides a very strong jumping off point for further investigation into N regulation in plants. The response relies on complex interactions between multiple pathways and regulatory molecules in nutrient acquisition. The intermingling of pathways, however, may provide a potential source of understanding multiple, vital

nutrient regulation mechanisms which contribute to the overall health of plants.

Oxidative Stress

Not only must plants compensate for nutrient availability in the soil, but they are also exposed to other harsh environmental conditions. Many environmental conditions, such as high salt, copper (Cu^{2+}) and iron ion (Fe^{3+}) levels, environmental ozone, or high light conditions can lead to the accumulation of reactive oxygen species (ROS) causing plants to experience oxidative stress which interferes with important cellular processes. This is a major cause of crop loss in today's agriculture. Response to oxidative stress has also been linked to miRNAs, in particular, miR398 (reviewed by Floris *et al*., 2009 and Mazzucotelli *et al*., 2008).

Based on the complimentarity of miRNA to target mRNAs, two targets with miR398 binding sequences were identified: *CSD1* and *CSD2*, both of which are Cu/Zn-superoxide dismutases (SODs) which act to remove free radicals from the cellular environment (Sunkar *et al*., 2006). *CSD1* is located in the cytoplasm while *CSD2* is in the stroma of plastids. Northern gel blot analysis was used to measure miR398 and target transcripts to determine their expression patterns in *Arabidopsis* tissues. In tissues expressing high levels of miR398, such as the cauline leaves, stem and root, low levels of SOD mRNA were present. Comparatively, low expression of miR398 correlated with high levels of SOD mRNA. Nuclear run-on assays demonstrated that *CSD1* and *CSD2* are constitutively transcribed with translation being regulated by the activity of miR398, confirming posttranscriptional regulation of gene expression. Beauclair, Yu and Bouchè (2010) identified the copper chaperone dismutase, *CCS1*, as another target for miR398. CCS1 shuttles the necessary copper cofactor to the CSD1 and CSD2 apoproteins. These authors used microarray analysis of bulk RNA to identify transcripts having an inverse correlation with miR398 expression. *CCS1* was confirmed as a target of miR398 by the observation that the two RNAs formed a stable duplex, a crucial aspect of miRNA regulation. Furthermore, western blot analysis demonstrated that CCS1 protein abundance was dependent on miR398 levels. Protein levels were lower under copper-limiting conditions when miR398 is upregulated. The presence of several targets of miR398 under oxidative stress demonstrates a multi-facetted response of miR398 to oxidative stress. The first action of miR398 is to act directly upon the super-oxide dismutases, preventing their translation when conditions are suitable and permitting their translation when conditions are stressed. However, this miRNA can also act upon the copper chaperone the SODs rely upon for functionality, preventing the delivery of the essential copper cofactor. Therefore, any SODs that are still present will be inhibited by the lack of cofactor. Multi-level posttranscriptional regulation allows for fine-tuning of stress response, insuring proper protein expression and activity in response to oxidative stress.

As an example of another environmental condition responsible for oxidative stress, one study examined the effect high light conditions may have on miR398 expression (Sunkar *et al*., 2006). When plants are exposed to high light conditions they are not adapted to, plant cells may begin to experience oxidative stress as photosynthesis dramatically increases. When *Arabidopsis* seedlings were exposed to high light for 8 hours, there was an observed decrease in miR398 levels. As the light exposure continued, the miR398 transcript levels continued to decrease. Other oxidative stress inducing conditions, including exposure to the heavy metal ions Cu^{2+} and Fe^{3+}, were tested and further confirmed the relationship between oxidative stress and miR398 down-regulation. It can be inferred from this data that any target transcripts were regulated along with miR398. In addition, Sirè and coworkers (2009) demonstrated a diurnal expression pattern of miR398 in *Arabidopsis* plants that seemed to correspond to light exposure, and therefore potential oxidative stress. This diurnal expression was not correlated with the circadian clock, but instead relied on exposure to light, a likely cause of oxidative stress, even if only from regular sun exposure. Therefore, miR398 may also play a role in daily ROS homeostasis as a result of natural exposure to sunlight or other environmental conditions, not just when these conditions are extreme.

Interestingly, when a miR398 resistant form of *CSD2* (*mCSD2*) was engineered in which the recognition site of miR398 was destroyed, the transgenic plants revealed an increased resistance to oxidative stress, since miR398 could no longer down regulate translation of the SOD (Sunkar *et al*., 2006). Multiple forms of oxidative stress were tested, and the plants with *mCSD2* showed an increase in tolerance against all of them. For example, when the *mCSD2* plants were exposed to high Cu^{2+}, they showed a higher germination rate compared to wildtype plants. Similar results were found when the transgenic *Arabidopsis* plants were subjected to high light. This data suggests there may be potential applications of research into miRNA regulation that could benefit growers in the development of more resilient crops. These studies indicated that further exploration into the possible modification of plants for increased stress tolerance through modifying these gene regulation pathways may have potential.

Other Plant Systems

While *Arabidopsis* is a valuable plant genetic model system for basic studies into the function of plant miRNA response to abiotic stress, it is important to expand research to other plants which may be of higher economic, agricultural or practical value.

Thellungiella halophila

Thelluginella halophila is not an economically important crop, but its similarity to *Arabidopsis* has served as a jumping off point for molecular investigations into more complex plant species. Unlike *Arabidopsis*, *Th. halophila* is highly salt resistant and therefore provides insight into how miRNA response functions in a salt tolerant plant, a desirable quality in many agricultural systems. Halophytes grow in hot, droughty climates. This is the type of climate many farmers are currently facing, and will continue to face as climate change continues. Findings regarding *Th. halophila* may help to further understand plants that naturally grow in high salt environments, or perhaps how other plants may be engineered to grow in these types of soils.

The action of miR398 upon *CSD1* and *CSD2 Th. halophila* orthologues was investigated and found to be responsive to a variety of stressors in the environment (Pashkoviskii *et al*., 2010). In general, *Th. Halophila* reacts

to salinity through the activation of ROS pathways and SODs, particularly *CSD1* and *CSD2*. The presence of *CSD1* transcripts was shown to increase when plants were grown in a high salt environment; similar to the response of *Arabidopsis* plants under oxidative stress. What is interesting to note is that *Th. halophila* experiences differential expression of miR398 in the root and leaf tissue but not in the stems as in *Arabidopsis*. In the roots, miR398 decreased 6 hours after treatment, increased at 12 hours and then experienced a dramatic decrease after 24 hours. In the leaves, expression was steadily enhanced throughout the course of the experiment. This kind of segregated response suggests possible interorgan transport of the miRNA, or its targets, throughout the plant. miR398 may be created in one organ, such as the leaves, and then transported throughout the rest of the plant to achieve holistic response. This experiment shows a clear negative correlation between the presence of miR398 and the abundance of *CSD1* in both the roots and the leaves, demonstrating the possibility of posttranslational regulation of the SOD by miR398 as was observed in *Arabidopsis*. Increased salt tolerance may be granted to *Th. halophila* through the action of miR398 by regulation of important genes, that respond to high salt levels and the resulting physiological consequences, such as SODs.

Due to its native environment, *Th. halophila* is often exposed to high levels of sunlight and thus severe levels of UV-irradiation which can cause oxidative stress, particularly in the plastids. Studies of miR398 under high light conditions uncovered an interesting pattern between expression in leaves and expression in roots. After 1 hour and after 12 hours of exposure to irradiation, there was a marked decrease of miR398 in the roots accompanied by an increase of miR398 in the leaves. This suggests an inverse relationship between tissues above and below ground in their miR398 expression under stressful conditions, perhaps due to the exposure of leaves to the sunlight while root tissue is not exposed to UV-irradiation. The miR398 levels correlated with *CSD1* expression levels as previously observed. In fact, the authors observed a positive correlation between increased regulation of *CSD1* by miR398 as the plant was subjected to more intense irradiation.

The presence of the miR398 response to oxidative stress in *Th. halophila* to both salinity and irradiation confirms a conserved mechanism of abiotic stress response. It is, therefore, highly likely this response is common to all plants and could be a universal adaptation to respond to oxidative stress. The unique responses of *Th. halophila* may help elucidate ways that plants naturally respond to high salt levels which could then be applied to modifying other plants to respond the same way.

Zea mays

Corn (Maize; *Zea mays*) is a vital crop in today's society. A deeper understanding of its miRNA response mechanisms could provide extensive application to increase yield and fight food insecurity. Since maize is such a widely grown agricultural crop, farmers have become accustomed to growing it with current farming practices, which often means intense application of nitrogenous fertilizers. These fertilizers can have dramatic negative consequences on the broader ecological landscape. A study (Xu *et al*., 2011) set out to identify maize miRNAs involved in the low-nitrate response system. Once identified, these miRNAs could possibly lead to the development of low nitrogen tolerant maize and eventually reduce the amount of artificial fertilizer used. Knowledge gained from the research of *Zea mays* could then possibly extend to other cereal crops.

Through microarray analysis, nine miRNAs in the leaves and nine in the roots were found to be sensitive to chronically low nitrate levels. These miRNAs had different expression patterns, some being upregulated while others were down regulated. However, when nitrate availability was only transiently low, five miRNAs were found to be expressed in the leaves and six in the roots. Under these conditions, however, all the root miRNAs were upregulated. Through sequence analysis, the identified miRNAs were used to determine putative roles in posttranscriptional response to low nitrate availability. Some of the miRNAs target transcription factors crucial for development of plant embryos, flowers, tissue and roots. Other roles of these miRNAs include regulating metabolism and response to oxidative stress. Many of the miRNAs expressed under nitrate stress in maize have been identified and characterized in *Arabidopsis*, such as miR169 and miR398, as previously discussed.

Morphological and metabolic adaptation to stress is crucial in the development of seedlings. In maize, the role of miRNA in response to flooding stress has been shown to regulate both morphology and metabolism in developing seedling root cells (Zhang *et al*., 2008). A microarray analysis revealed over 100 miRNAs with differential expression patterns in response to submergence. These miRNAs were shown, as is characteristic, to be conserved over many plant species. The miRNAs had a variety of responses at different points during submergence, suggesting they work together to produce a complete response to flood stress. A phenotypic change as a result of miRNA action is the development of a vascular system with expanded xylem tissue to accommodate the high levels of water. This phenotypic change has been tagged to the accumulation of miR166 during submergence. This data demonstrates that gene regulation by miRNA is at least partially responsible for the phenotypic differences observed during growth in stressful conditions.

The target genes of the identified miRNAs are involved in a wide variety of cellular processes including metabolism, transcription, defense, cell differentiation and signal transduction. The target mRNAs isolated for further study showed characteristic negative regulation expected of translational inhibition by a miRNA. The miRNA loci were then analyzed for upstream *cis*-acting elements potentially involved in activating transcription of the miRNA. Each of the miRNAs was found to have more than one *cis*-acting element, suggesting the miRNA could be activated by several different abiotic stress signals and therefore have a role in several different response pathways. Zhang and coworkers (2008) then examined the hypothesis that each miRNA may not be isolated to only one stress response mechanism. Submergence, for example, seemed to express a unique set of miRNAs. However, many of the miRNAs of that set are also involved in other cellular processes, such as the response to oxygen deprivation. Therefore, it can be proposed that a unique set of miRNAs is expressed for each stress response, but the miRNAs that participate in the response are not confined to only that mechanism.

The interactions and activity of miRNA in *Zea mays* is clearly dynamic. Abiotic stress response results from the combined actions of multiple miRNAs along with other factors. These response pathways have yet to be fully mapped. What is clear, however, is the strong presence of miRNA as a method of regulation and response to abiotic stress. The mechanisms and systems by which these miRNAs function is still not fully understood; therefore, this area of research presents and exciting field for scientists to begin to investigate abiotic stress response.

Nicotina tabacum

The use of nanoparticles to create lightweight, durable materials and as pigments in paints and cosmetics has become common in modern society thanks to their versatility and efficiency. However, these nanoparticles have begun to leech into the environment and may begin to pose problems for farmers by infiltrating the water system and the soil. A recent study (Burklew *et al*., 2012) began to analyze the effects of aluminum nanoparticles on the economically important crop *Nicotina tabacum* (tobacco). This study was able to show a relationship between the presence of aluminum oxide nanoparticles in the soil and the expression of particular miRNAs. Due to the relationship between abiotic stress and miRNAs that has been previously observed, it may be concluded that the presence of aluminum nanoparticles is perceived as stress and thus elicits stress response. These particles will therefore signal stress response in plants resulting in phenotypic and developmental changes which may sacrifice the overall health of the plant.

Preliminarily, the study demonstrated the phenotypic effects aluminum oxide nanoparticles had on plant growth which included decreased root length and leaf number in developing seedlings. The average biomass of seedlings exposed to aluminum oxide also decreased compared to untreated seedlings. These effects were shown to have a direct correlation with the concentration of aluminum in the soil. Therefore, there is a clear phenotypic response of tobacco seedlings to the presence of nanoparticles in the soils. The nanoparticles may initiate phenotypic change as a result of a stress response to high aluminum concentrations.

After establishing the phenotypic consequences of aluminum nanoparticle exposure, the researchers investigated the miRNA expression levels during treatment as an attempt to understand the molecular basis for this response. In plants that were exposed to a low amount of aluminum oxide nanoparticles (0.1%), all examined miRNAs, with the exception of three that were upregulated, experienced down regulation. However, when the tobacco plants were exposed to higher concentrations (0.5% and 1%), all miRNAs were upregulated suggesting a higher concentration of nanoparticles is needed to elicit full response. Nine miRNAs were found to have a statistically significant increase in expression.

This study supports the action of miRNAs as responders to abiotic stress. Furthermore, it shows how human waste of nanoparticles in the environment may affect the development and health of plants by being agents of abiotic stress. If this response is found in *Nicotina tabacum*, a similar response would be expected in other plants due to the highly conserved nature of abiotic stress response systems. Therefore, the use of these particles and their presence in the environment should be carefully monitored. Widespread presence of nanoparticles in soils will begin to affect not only agriculturally important crops, but native ones as well which have never been exposed to this kind of stress.

Phaseolus vulgaris

Phaseolus vulgaris, or the common bean, is the most important legume in the human diet. However, the land it is traditionally grown on often lacks adequate Pi levels and therefore these plants are chronically exposed to Pi depravation. Posttranscriptional regulation under Pi stress had been identified in rice and *Arabidopsis* and therefore the presence of miRNA involved in Pi stress is highly likely in bean crops. Since Pi is often limited in soils, it is important to understand plant response mechanisms to Pi stress. If the entire response pathway is understood, it could provide opportunity to develop a bean crop that can grow in spite of these limiting conditions.

A miRNA library was produced from bean seedlings and compared to miRNAs identified in rice (Valdès-Lopez et al., 2008). miR399, which has been identified in Pi starvation in *Arabidopsis*(*At*), as explored prior, was targeted for further investigation. This miRNA was induced 80-fold under Pi starvation and several target genes were identified, including the common bean orthologue of *At4* designated *Pv4*. *Pv4* and *At4* belong to a gene family that lack a single ORF but instead contain a series of short overlapping ORFs coding for products important in the movement of Pi from roots to shoots (reviewed in Valdès-Lopez et al., 2008). *Pv4* showed a direct correlation with miR399 expression as both increased under Pi-deficient conditions. This suggests that miR399 expression allows for transcription of these gene products, perhaps by regulating a repressor. miR399 was also found to target an orthologous E2 conjugase gene to *AtPHO2*, designated *pvPHO2*. Accumulation of pvPHO2 followed the reverse correlation demonstrated in other plants, decreasing under Pi deficiency as miR399 is induced. This is more indicative of direct translational suppression of the *pvPHO2* transcript by miR399. Furthermore, *pvPHO2* was shown to negatively regulate the expression of other genes involved in Pi response. These genes play a role in altering the root architecture to absorb more Pi from the soil, a common strategy used by plants to adjust to nutrient deficient soil. The presence and role of miR399 suggests a response pathway that does not rely directly on miR399 but is impacted by its expression.

Oryza sativa

Rice is one of the most important crops worldwide and, due to its traditional growing environment, is often affected by several abiotic stress factors. Oxidative stress can be particularly deleterious to a rice crop and can be induced by numerous environmental factors such as drought, the presence of heavy metals in the soil or even biotic infection.

Much like in *Arabidopsis*, rice contains highly conserved *CSD1* and *CSD2* genes (Lu *et al*., 2011). Similarly, the miR398 genes are also highly conserved within the rice genome. The expression patterns of miR398 and *CSD* genes were explored in several different rice tissues in order to gain information on their role in stress response throughout the rice plant. Tissues with high expression of miR398 had low levels of the *CSD* gene products. Interestingly, there was a stronger negative correlation with *CSD1* than *CSD2*. This pattern is

consistent with what has been observed in other plant species, the translation of *CSD* transcripts is dependent on miR398 levels which is dependent on environmental conditions. Expression of miR398 was further shown to be responsive to oxidative stress situations in rice. The levels of miR398 decreased with increased exposure to a variety of oxidative stress inducing conditions allowing the translation of the *CSD* transcripts to facilitate response to the stress.

In this study, Lu and coworkers (2011) engineered rice plants to produce a *CSD2* transcript with an altered miRNA recognition sequence rendering it resistant to miR398 cleavage. These plants showed amplified salt tolerance due to an increased presence of *CSD2*. Wild types rice plants grown in high salt medium for one week showed severe salt toxicity symptoms while transgenic plants showed only mild symptoms to the treatment. While this method has not been widely tested, these results boast great potential for engineering crop plants able to withstand oxidative stress from a variety of sources. With the broad range of stressors that trigger miRNA stress response, this methodology may be applicable to help increase resistance to other abiotic stress factors as well.

Conclusions and Prospectus

While plants may be sessile in nature, their response to environmental stress is quite dynamic. Signals of abiotic stress from the environment are able to trigger responses to the stress factor and therefore allow the plant to continue to grow, despite the poor conditions. It is only recently that scientists have begun to discover the molecular basis for these responses. miRNAs play a major role in facilitating abiotic stress response on the post-transcriptional level through the mediation of translation of gene products which are responsible for responsive phenotypes. Investigation into this mechanism could create many opportunities to advance research in developing more resilient crops as food security becomes more uncertain.

The expansive amount of information that has begun to emerge from research of miRNA response to abiotic stress provides researchers with a new, innovative avenue for research into producing more resilient crops. While research with *Arabidopsis* has been, and continues to be, imperative for foundational understanding of miRNAs, research must move forward to more fully understand the functions and nuances of miRNA response in higher plants, particularly those that are economically and agriculturally important. Of particular interest is *Oryza sativa*, rice, a food staple for over half of the world's population (International Rice Research Institute [IRRI]; irri.org).

Rice evolved from semi-aquatic ancestors, and therefore is highly sensitive to water levels in the soil. Rice fields must always be saturated; if they fall below this level the rice plants will begin to show signs of water stress, affecting the yield of the harvest. The high demand for water can often put strain on farmers when water levels are low, whether it is due to inadequate rainfall or poor irrigation. Drought is the most widespread and devastating of the environmental stresses rice crops face and can lead to yield loss up to 40% according to IRRI. Development of drought-tolerant rice would remove the burden of constant water supply from the farmers and prevent yield loss.

As discussed above, miR398 has been shown to play a role in oxidative stress response in rice. Oxidative stress is often a result of drought and with the highly demanding relationship between rice health and water level, an increased tolerance to oxidative stress in response to low water levels would help rice grow in suboptimal conditions. This not only could help farmers who currently grow rice, but could also expand the areas where this crop could be cultivated.

A comparison analysis between rice varieties that have already been shown to be phenotypically adapted to low water levels, such as *Sahbaghi dhan* in India, *4511* from the Philippines or *Suokha dhan* from Nepal, and rice varieties which are susceptible to drought would expand our understanding regarding the role miRNA plays in this response (IRRI). We propose that further research be done in which several traditionally bred drought-resistance lines, such as those above, are grown alongside susceptible lines and the transcriptomes compared for miRNA expression. Due to the prevalence of miRNA in response to abiotic stress, there should be a measureable level of differential expression of multiple mRNAs between the resistant and susceptible lines. Whether the expression of the miRNA is higher or lower in susceptible lines is dependent on the mode of action for that particular miRNA.

Plants would be grown both in drought conditions as well as normal conditions for comparison. Several tissues (e.g. leaves, roots, stems) from each plant-line would be used to obtain individual transcriptomes to see if there is isolated expression of the miRNAs in different tissues. Transcriptomes would be sequenced by RNA-Seq to obtain a library of all RNAs present within each tissue from each plant-line. These libraries could then be compared to see if there was any differential expression of any miRNAs between the plants which were resistant to drought and those which were not. Any miRNAs which demonstrate a change in expression level would then be used to identify potential targets through bioinformatics.

It is likely that multiple miRNAs will show differential expression in the drought resistant lines. This collection of miRNAs will target a variety of different gene products to facilitate the comprehensive drought response. While it is known the miRNAs work together, full networks of miRNA response have yet to be uncovered. Each of the miRNAs that are discovered to be differentially expressed in the drought-tolerant lines should be further studied. The roles of their targets in response to stress should be fully investigated to understand the link between post-transcriptional regulation and phenotypic response. This work would present a broad idea of the entire response network that is responsible for the drought-resistant phenotype. miRNAs that appear to be tightly regulated or have orthologues with known activities should get particular attention since these may be the underlying, molecular reason for the drought-resistant phenotype. This study would begin to reveal insights into naturally higher response to low water levels, giving researchers an avenue to pursue when attempting to develop more highly resistant lines of rice.

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