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Review

Profiling of the Differential Abundance of Drought and Salt Stress-Responsive MicroRNAs Across Grass Crop and Genetic Model Plant Species

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Abstract: In recent years, it has become readily accepted among interdisciplinary agriculturalists that the current global crop yield to land capability ratio is significantly insufficient to achieve food security for the predicted population of 9.5 billion individuals by the year 2050. This issue is further compounded by the: (1) food versus biofuel debate; (2) decreasing availability of arable land; (3) required reductions to the extensive and ongoing environmental damage caused by either poor agricultural practices or agriculture expansion, and; (4) increasingly unfavorable (duration and severity) crop cultivation conditions that accompany man-made climate change, driven by ever-expanding urbanization and its associated industrial practices. Mounting studies are repeatedly highlighting the critical importance of linking genotypes to agronomically beneficial phenotypes and/or using a molecular approach to help address this global crisis, as "simply" clearing the remaining natural ecosystems of the globe for the cultivation of additional, non-modified crops is not efficient, nor is this practice sustainable. The majority of global food crop production is sourced from a small number of members of the *Poaceae* family of grasses, namely; maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.). It is, therefore, of significant concern that all three of these *Poaceae* grass species are susceptible to a range of abiotic stresses, including drought and salt stress. Highly conserved among monocotyledonous and dicotyledonous plant species, microRNAs (miRNAs) are now well-established master regulators of gene expression, influencing all aspects of plant development, mediating defense responses against pathogens and adaptation to environmental stress. Here we investigate the variation in the abundance profiles of six known abiotic stress-responsive miRNAs, following exposure to salt and drought stress across these three key *Poaceae* grass crop species as well as to compare these profiles to those obtained from the well-established genetic model plant species, *Arabidopsis thaliana* (L.) Heynh. Additionally, we outline the variables that are the most likely primary contributors to instances of differential miRNA abundance across the assessed species following drought or salt stress exposure, specifically; (1) identifying variations in the experimental conditions and/or methodology used to assess miRNA abundance, and; (2) the distribution of regulatory transcription factor binding sites within the putative promoter region of a *MICRORNA* (*MIR*) gene that encodes the highly conserved, stress-responsive miRNA. We also discuss the emerging role that non-conserved, species-specific miRNAs play in mediating a plant's response to drought or salt stress.

Keywords: drought stress; salt stress; *MICRORNA* gene expression; microRNAs; microRNA-directed gene expression regulation; differential microRNA accumulation; *Poaceae* grass species; *Arabidopsis thaliana*

1. Introduction

The global human population relies heavily on the major *Poaceae* cereal grasses, maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.), for their daily calorie intake [1,2]. Covering a large proportion of the global terrestrial land space, *Poaceae* grasses not only act as a primary sustenance source for humans (in the form of calories) but also contribute to agricultural pastures (e.g., rye (*Secale cereal* L.)) used to feed livestock [1,3]. Two other *Poaceae* grass species, Sorghum (*Sorghum bicolor* (L.) Moench) and sugarcane (*Saccharum officinarum* L.), form the primary basis of the plant material source for biofuel production, further highlighting the central importance of *Poaceae* grasses [4,5]. It is no longer debatable that modern society is challenged with the task of trying to address the consequences of climate change, with interdisciplinary research now aiming to provide food crop security for an exponentially growing population during times of increasingly unfavorable conditions, and in unsuitable crop cultivation environments [6]. Alarmingly, numerous studies have demonstrated that drought and/or salinity reduce the yield potential of the major cereal crops maize, wheat and rice, every growing season [7–14]. Moreover, use of regression modeling based on historical data and the predictions based on extrapolated trends of crop yield and climatic trends have highlighted the negative impact climate change associated factors (e.g., reduced precipitation) have had, and are continuing to have, on global *Poaceae* crop yield [15,16]. Having greatly modified the global land cover over the last fifty years, there has been a shift from the historical clearing of depleted grasslands and savannas, to the alarming and current practice of clearing land rich in biodiversity, such as tropical forests, for additional grass crop production [17,18]. As one of the largest contributing factors to greenhouse gas emissions and biodiversity reduction, this practice reinforces the urgent need for an alternate, molecular-based approach that targets crop yield maximization.

In addition to their central role in regulating developmental gene expression, plant microRNAs (miRNAs), and more specifically, miRNA-directed gene expression regulation, have more recently been identified as key regulators of plant metabolism, pathogen defense and for a plant to mount an effective adaptive response to abiotic stress [19–21]. Alterations to; (1) miRNA accumulation, and/or; (2) miRNA-directed target gene expression regulation have been extensively described in a wide range of plant species following exposure of the plant under study to abiotic stimuli such as drought stress, salt stress, extreme temperature (both elevated and reduced temperatures) and nutrient deficiency [19–26]. Such research has aimed to construct a more detailed molecular understanding of the fundamental, abiotic stress induced, miRNA-directed gene expression networks in plants. For example, the goal of many groups now actively researching in this space is use of knowledge gained to develop future plant varieties that have been modified to harbor genetic improvements that will aid in the modified plant's ability to cope with, or adapt to, an altered growth environment. Additional studies have further emphasized the critical importance of using a molecular approach to help address this global crisis, as "simply" clearing even more natural ecosystems for the cultivation of additional, non-modified crop species is not efficient, nor is this practice sustainable [1,27,28].

Considering the high level of conservation of many *MICRORNA* (*MIR*) gene families across the monocotyledonous and dicotyledonous evolutionary divide, in conjunction with the phylogenetic proximity of agronomically significant *Poaceae* crop species [29], investigating and manipulating abiotic stress-responsive miRNA/miRNA target gene expression modules presents a promising new and relatively unexplored avenue for the future development of phenotypically superior cropping species. However, a high level of caution is still required when a traditional genetic model plant species, such as *Arabidopsis thaliana* (L.) Heynh (*Arabidopsis*), is used as the basis of the research platform for knowledge advancement in an unrelated and agronomically important species. Furthermore, even the use of a closely related plant species can be problematic when researching a multilayered molecular mechanism such as miRNA-directed gene expression regulation. Here, we will highlight examples of the degree of variation in the profile of six highly conserved miRNAs across several plant species in response to each species being challenged with either the insult of drought or salt stress. Moreover, the degree of variation in the response of stress-responsive miRNAs to abiotic stress, becomes an even

more pronounced challenge when attempting to translate findings made in the traditional genetic model plant species, *Arabidopsis*, to agronomically important *Poaceae* grass crop species.

2. The Impact of Drought and Salt Stress on American and Australian Agriculture

Plant agricultural yield is heavily dictated by climatic conditions [30–32], and although crops are equipped to cope with year-to-year weather variability, recent research has shown that the increasingly unfavorable conditions that accompany man-made climate change are continuing to have a negative impact on global agricultural yield [19,30,33]. The Food and Agriculture Organization (FAO) defines the four key dimensions of food security as; (1) availability; (2) stability; (3) access, and; (4) use, with each of these key dimensions hindered to differing degrees by climate change events, such as prolonged periods of drought [34–36]. This growing and alarming trend is ultimately reducing the global capability to produce the "viable" crop volume required to provide food security, and to additionally provide the required volume of material to offer an alternate and sustainable biofuel source for an exponentially growing world population [27,37,38]. In recent years, it has become widely accepted among plant biologists that the current yield to land capability ratio is significantly insufficient to meet the needs of the predicted world population of 9.5 billion individuals by the year 2050; a population that will require an additional global agricultural output of 60% to 110% [38–40]. To highlight the negative impacts accompanying the abiotic stresses, drought and salt stress, abiotic stresses that significantly reduce global agricultural output annually, this review focuses on key agricultural crop producing regions of the United States of America (US) and Australia, specifically, the crop producing areas of the west coast of the US that rely on irrigation sourced from the Colorado River, and the Murray-Darling Basin of Australia, respectively.

In the US, during the 2015–2016 financial year, 345 million (M) tons of maize were produced, equating to a projected total dollar value of approximately 49 billion (B) US dollars ($USD; $USD49B) [41]. The total tonnage and dollar value of the 2015/2016 US maize crop is not surprising considering that from 2013 onwards, 70% of the total human calories consumed globally were derived from grasses, and of this 70%, maize comprised 91.7% of the C_4 grass fraction [42]. However, the possibility of drought to devastate crop yield potential is readily apparent with Daryanto and collaegues (2016) demonstrating that a 40% reduction in water availability results in a 39.3% reduction to total maize yield [43]. Furthermore, this alarming finding is in addition to the study published in the journal, *Nature Climate Change* in 2013. Using historical weather records in combination with modern prediction software, Dai (2013) confidently modeled that the US will suffer from severe and widespread incidents of drought throughout the next century as a direct result of reduced precipitation and/or elevated evaporation [15].

Domestically, the terrestrial surface of the Australian mainland consists of approximately 70% (5.5 million km^2) rangeland of mostly arid to semi-arid climate [44,45]. This environment is characterized by; (1) low rainfall; (2) long periods of extreme dryness; (3) infertile soils, and; (4) largely being an inappropriate environment to sustain standard agricultural practices [33,44,45]. The lack of suitability of this environment within the Australian mainland for agricultural use is further shown by the current (March 2018) trend maps obtained from The Australian Government, Bureau of Meteorology (retrieved from http://www.bom.gov.au/). These trend maps clearly display an increase in annual mean temperature from 1970–2015, a decline in total annual rainfall over the same period (1970–2015), and a decline in the Normalized Difference Vegetation Index (NDVI), as of August 2015. This publicly available data clearly emphasizes the alarming trends of a rising mean temperature, a reduction in total rainfall, and the almost complete absence of vegetation across the majority of inland Australia (as shown by the lack of "green" vegetation observed by satellite generated NDVI imagery). Australian agriculture therefore remains heavily reliant on the farming practices of the Murray-Darling Basin, an agricultural region that currently contributes approximately 40% of the nation's agricultural output, equating to $15B Australian dollars ($AUD; $AUD15B) annually [46,47]. This is an impressive production figure considering that the Murray-Darling Basin only represents

approximately 14% of Australia's total land surface area [46,47]. Of considerable concern however, are the reports indicating that by 2030, surface water availability in this region will be drastically reduced, with the utilized data suggesting that this "climatological disaster" has the potential to greatly impede Australia's agricultural commodity production capabilities by up to, and in excess of 27% [48–50]. Furthermore, Australia is the world's sixth largest exporter of agricultural commodities, including; (1) dairy products ($AUD2.5B); (2) wheat ($AUD2.0B); (3) other cereal-derived flours ($AUD1.5B), and; (4) wine ($AUD3B). Together, these commodities contribute significantly to the global food supply and to the national revenue of Australia [47]. It is, therefore, in the nation's best interest to enhance and refine current farming practices to ensure their future stability, efficiency and production capabilities [47].

On a global scale, over 800 million hectares of soil is impacted by salinity, including groundwater-associated salinity, transient salinity, and irrigation-related salinity [51]. Excluding contributing climatic and topographic factors, the severity and prevalence of salinity affected soil is enhanced by the destructive impact of human activities, be it agricultural or industrial practices [52,53]. Similar to the impact of drought stress, increasing salinity is reducing the global capability to meet the ever-increasing demands for ensuring food security while providing an alternate and sustainable source for biofuel production [37,39]. Therefore, the rapidly growing demands for additional cultivatable soil poses a significant issue that also requires urgent attention to achieve sustainable food and energy production [30–32].

In the US, one of the most salt affected rivers, the Colorado River, is also one of the nation's longest rivers, spanning 2330 km across seven US states, and two additional states in neighboring Mexico. The Colorado River is also the main source of agricultural irrigation and domestic water supply for the Southwest coast of North America. Over three decades ago, Holburt (1984) highlighted that up until 1982, salinity was causing $USD113M in damage annually in this region, and further predicted that this dollar figure would at least double in future decades [54]. This prediction has proved accurate with a 2004 study [55] revealing that salinity associated issues within the Colorado basin, were causing $USD150M of annual damage to the entire US agriculture industry, and a total of $USD300M damage to the US economy [55]. Moreover, the United States Department of Agriculture (USDA) estimated that the state of California (one of the seven US states that the Colorado River and its associated tributaries flow through), contributed a total agricultural market value of $USD42.6B in 2012 to the US economy: a figure that represents 10.8% of the total US dollar value for that year (https://www.agcensus.usda.gov/). Further, when the USDA further breaks this dollar value down into individual contributions made by the crops, maize, wheat and rice, the 2012 Californian crop market value of each species in 2012 was $USD419M, $USD341M and $USD782M, respectively (or, equating to 0.62%, 2.17% and 27% of the total US value of each cropping species, respectively) (https://www.agcensus.usda.gov/). Therefore, considering the dollar value that these three *Poaceae* grass crop species contribute to the US economy, in combination with the demonstrated susceptibility of the yield of maize [56], wheat [57,58] and rice [14,59] to salt stress, the immediate requirement for adoption of a molecular approach to generate future phenotypically superior varieties of each of these species, becomes clear.

The Murray-Darling Basin again provides an excellent example of the negative impact salinity has on Australian agriculture, with approximately 71% of the nation's irrigated agricultural production occurring in this region [46]. The process of large-scale commodity production is rapidly exhausting the Murray-Darling Basin's ecological capabilities because of exploitation, drought (see above), and the ever-increasing levels of salinity due to relentless irrigation practices [60,61]. As outlined above, this environmental damage has a direct and negative impact on total crop yield and therefore, Australia's annual agricultural revenue [46,62]. In 2004, the Wilson Report estimated that dryland salinity was costing the Murray-Darling Basin an approximate $AUD305M loss in profit per annum [63]. Further, this dollar value estimate did not include the cost of damage to indigenous heritage sites, nor the natural environment of the Murray-Darling basin as a whole [63]. Moreover, given that the agricultural practices on which the Wilson Report data was generated, have continued largely unchanged since

the release of the Report in 2004, it is reasonable to suggest that this extensive level of damage, and the monetary costs associated with this ongoing damage, have only increased in each of the fourteen years since the Report's findings were released. It is also reasonable to state that if measures are not implemented in the very near future to enhance current crop capabilities, while in parallel adjusting traditional and unfavorable farming practices under the constantly changing environment, rising salinity will continue to have a widespread and negative impact for; (1) landholders; (2) rural communities; (3) countries that import Australian agriculture products, and obviously; (4) the Australian nation and its economy as a whole [46,47,62,64].

3. The Role of Plant microRNAs in Response to Drought and Salt Stress

Abiotic stress, including drought and salt stress, is one of the major contributors to global crop destruction and yield loss. Although plants are evolutionary equipped to employ physiological and phenotypical mechanisms to adapt to, or to at least tolerate abiotic stress, it is becoming increasingly evident that molecular-based approaches offer a new, alternate, and attractive avenue to generate plant lines with enhanced tolerance to this form of stress [65]. Abiotic stress tolerance can be engineered into new plant lines via the molecular modification of hormone signaling or perception pathways, root and/or shoot architecture, osmotic potential, or metabolic pathways [66]. Such a molecular approach primarily requires switching on, or switching off, the expression of a specific gene(s) that encodes for a specific protein product that is functional at a specific stage of plant development. However, a molecular approach may also be used to modulate, or to "fine tune", the expression of a gene to ensure that a key metabolic enzyme or other biologically relevant protein product is; (1) at the correct level; (2) localized to the appropriate cell or tissue type, or cellular compartment, and; (3) functional as a rate limiting step in a complex biochemical pathway [25,67,68].

In the genetic model species *Arabidopsis*, and once processed from the precursor transcript, the mature miRNA is loaded by the endonuclease, ARGONAUTE1 (AGO1), to form the catalytic core of the miRNA-directed, RNA Induced Silencing Complex (RISC), termed miRISC [69]. The activated miRISC uses the loaded miRNA small RNA (sRNA) as a sequence specificity determinant to target highly complementary messenger RNA (mRNA) transcripts for expression repression via either a mRNA cleavage or translational repression mechanism of miRNA-directed RNA silencing [69]. miRNAs are well known regulators of developmental gene expression [70] and have more recently been identified to also act as central regulators of gene expression in plants to effectively mount; (1) a defense response against invading pathogens (including viruses, bacteria, and fungi), or; (2) an adaptive response to environmental challenge, namely to respond to abiotic stress stimuli [20,71]. Taken together, these findings identify the miRNA class of sRNA, an ideal target for molecular modification as part of the future development of plant lines with engineered resistance (or enhanced resistance) to abiotic or biotic stress. The first step in the development of such plant lines is the molecular manipulation of individual miRNA/miRNA target gene expression modules. The most direct route to achieve this goal is the generation of plant lines with altered miRNA abundance. miRNA overexpression is a very straightforward procedure and is achieved via fusion of the DNA sequence encoding the miRNA precursor transcript to a constitutively, and frequently ubiquitously expressed, promoter such as the 35S promoter from the *Cauliflower mosaic virus* (CaMV), a widely used promoter in *Arabidopsis* transformation approaches [72–75]. Such an approach essentially generates a knockout mutation for each gene transcript that harbors a target site sequence complementary to the miRNA sRNA being over-expressed (see studies; [76–78], respectively for *Arabidopsis*, rice, and wheat-specific examples). miRNA knockdowns, or complete knockouts, have been generated *in planta* via the use of a range of molecular technologies, including the miRNA mimicry [79,80], *MIR* gene promoter methylation [81], artificial miRNA [82], short tandem target mimicry [83], and miRNA sponge [84] technologies. Each approach differs in the degree of efficacy it offers for the suppression of miRNA abundance (which also differs for each targeted miRNA, per technology). However, each technology allows for the generation of a plant line with elevated miRNA target gene expression, and therefore,

enabling use of the generated plant line to study the biological consequence of miRNA target gene overexpression. The parallel generation of both a miRNA overexpression (a miRNA target gene knockdown plant line) and knockdown (a miRNA target gene overexpression plant line) plant line is highly recommended for the accurate assignment of biological function to the miRNA target gene whose expression is altered in the resulting engineered plant lines: modified plant lines that would be expected to display reciprocal phenotypes when applying the miRNA overexpression and knockdown approaches in parallel.

Numerous studies across the key grass crop species, maize, wheat, and rice, have identified both conserved (found across numerous plant species within the plant kingdom) and non-conserved (found only in a single species, or a group of closely related species within the plant kingdom) miRNAs responsive to either drought or salt stress [85–90]. For example, the studies of [85–88], identified 34, 13, 30 and 5 miRNAs, respectively in maize, wheat, rice, and *Arabidopsis*, responsive to drought stress. Drought-responsive miRNAs were identified in all four of these studies via the application of miRNA microarray hybridization technology. This approach enables direct comparison of the miRNA abundance profiles of "stressed" versus "non-stressed" plants [85–88]. Similarly, 98, 44 and 10 miRNAs were determined responsive to salt stress in the microarray hybridization assays performed in maize [89], wheat [90] and *Arabidopsis* [88], respectively. Additionally, Shen and colleagues (2010) used a modified high throughput assessment, a one-tube, stem-loop primer-based, reverse transcriptase approach to quantify miRNA abundance via subsequent RT-qPCR assessment [91]. This approach identified 18 salt responsive miRNAs in rice [91]. More recent miRNA detection studies, primarily rely on the use of next-generation RNA sequencing (of the sRNA fraction) to identify known and novel miRNAs responsive to either drought or salt stress [23,92,93]. Next-generation sequencing is a high throughput approach that allows for the identification and quantification of transcriptome-wide stress-responsive miRNAs (or other RNA transcripts), compared to a more traditional technology, such as miRNA microarrays. For example, a next-generation sequencing approach was used in rice [93] and wheat [23], to identify 18 and 66 drought-responsive miRNAs, respectively.

4. The Varying Responses of Six Highly Conserved microRNAs to Drought and Salt Stress

Curiously, despite instances of high conservation of miRNA sequence, and miRNA target gene function, across diverse plant species, in combination with the close phylogenetic proximity of key agronomical *Poaceae* family members, numerous examples of differential miRNA accumulation responses to either drought or salt stress have been reported. This is a major issue that requires consideration when comparing the profile of *Arabidopsis* abiotic stress-responsive miRNAs, to those obtained from agronomically important crop species. For example, Zhou and colleges (2010) revealed that nine miRNAs (including miRNAs, miR156, miR168, miR170, miR171, miR172, miR319, miR396, miR397, and miR408) in drought-stressed rice, returned an opposing accumulation profile comparative to the miRNA profile of drought-stressed *Arabidopsis* [87,88]. Such differences in the response of individual miRNAs to the same abiotic stress treatment (as determined by sRNA abundance fold changes) across *Arabidopsis*, maize, wheat, and rice, are highlighted in Figure 1. Figure 1 clearly shows that the accumulation trend of six highly conserved miRNAs, including miR159, miR164, miR167, miR168, miR396 and miR397, can differ following either drought or salt stress treatment of these four plant species. The selection of the six miRNAs listed in Figure 1 was based on each miRNA being; (1) firmly classified as a highly conserved miRNA; (2) reported in each of the four plant species discussed here, and; (3) demonstrated to direct a regulatory role in a plant's response to abiotic stress in at least one of the four plant species focused on in this study. We, the authors, readily acknowledge that several recent review articles have detailed the abundance trends of much larger cohorts of miRNAs, and across additional plant species to those reported on here (see the following recent reviews [68,94–97]). However, the primary focus of this article is to identify the experimental and molecular variables that when taken together, potentially account for the reported accumulation differences in the same miRNA

sRNA following exposure to abiotic stresses, drought, and salt stress, across the four plant species under analysis.

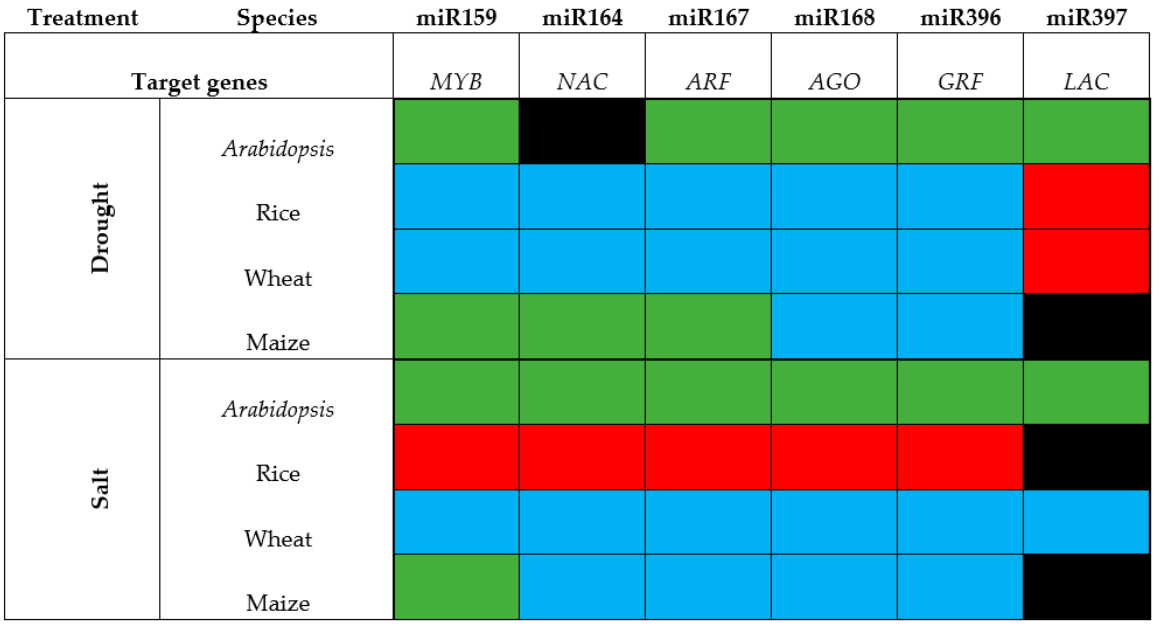

Figure 1. MicroRNA accumulation trends in Arabidopsis, rice, wheat, and maize in response to drought and salt stress. The accumulation of miRNAs, miR159, miR164, miR167, miR168, miR396 and miR397, in response to drought and salt stress in *Arabidopsis*, rice, wheat and maize. Green shaded boxes indicate elevated miRNA abundance in response to the applied stress. Red shaded boxes indicate reduced miRNA abundance in response to the applied stress. Blue shaded boxes indicate that miRNA abundance has been reported by different studies to have an opposing abundance trend post exposure to the same stress. Black shaded boxes identify miRNAs for which data is currently lacking in the assessed species. The gene family to which the target gene(s) of each of the 6 selected miRNAs belongs is indicated in the line immediately below the name of the targeting miRNA at the top of each column, more specifically *MYB* (*MYELOBLASTOSIS*), *NAC* (*NAM/ATAF/CUC2*), *ARF* (*AUXIN RESPONSE FACTOR*), *AGO* (*ARGONAUTE*), *GRF* (*GROWTH REGULATING FACTOR*) and *LAC* (*LACCASE*). The data used to construct Figure 1 was sourced from studies [23,68,85–103].

Taking a single example, miR396, from the six presented in Figure 1, differential accumulation trends have been reported for this miRNA in drought-stressed *Arabidopsis* and maize. Namely, in *Arabidopsis*, miR396 abundance was elevated 2.6-fold by drought stress treatment (200 mM mannitol) however, miR396 levels were only mildly upregulated by 0.7-fold in drought-stressed maize (16% *w*/*v* polyethylene glycol (PEG)-6000) [88,101]. Furthermore, this "positive" drought-induced accumulation profile for the miR396 sRNA is not universal across plant species. For example, microarray assays of "drought-shocked" Emmer wheat (*Triticum dicoccoides* (Körn.) Thell), demonstrated a negative response for miR396 to this stress with miR396 abundance reduced 3.0-fold [86]. Differential abundance trends are also observed for miR396 to salt stress across individual plant species. For example, Liu and colleagues (2008) showed that in *Arabidopsis*, miR396 abundance was upregulated 3.0-fold in response to a 300 mM salt (sodium chloride (NaCl)) stress growth regime, while an opposing and negative accumulation profile was reported for the miR396 sRNA in maize post exposure to salt stress [88,89]. It is important to note here however, that the accumulation profile for miR396 was determined via microarray analysis in *Arabidopsis* [88], whereas a PCR-based approach was used to quantify miR396 abundance in the salt-stressed maize samples [89]. The variation in miRNA abundance profiles across the four assessed plant species in response to drought and salt stress exposure extends beyond miR396, as readily demonstrated for the other five miRNAs also profiled in Figure 1. Figure 1 also clearly

highlights the degree of caution that needs to be exercised by a researcher when assessing miRNA accumulation profiles in response to abiotic stress across individual plant species.

5. Investigation of the Transcription Factor Binding Site Landscapes of *MICRORNA* Gene Promoters

To attempt to account, at least partially, for the reported variability in miRNA accumulation profiles across plant species exposed to the same abiotic stress, the promoter regions of the *MIR* gene loci of maize, rice and *Arabidopsis* that encode miRNAs, miR159, miR164, miR167, miR168, miR396 and miR397, were assessed for the presence of known plant-specific *cis*-regulatory elements (*CRE*s). Utilizing PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), an online database that houses 435 known, plant-specific *CRE*s, the three kilobase (kb; 3000 base-pairs) region immediately upstream of the *MIR* gene sequence encoding the precursor-miRNA (pre-miRNA) transcript (*pre-miRNA*; the region of the larger sized non-protein-coding transcript, the primary miRNA (pri-miRNA) that folds back onto itself to form the stem-loop structure of miRNA precursor transcript) of each assessed miRNA was retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/) database for this analysis [104]. To reduce the number of *CRE*s returned for this analysis, search parameters within the PlantCARE database were limited to only *CRE*s previously associated with responses to plant hormones, circadian rhythm, or abiotic stress (see Table 1).

Table 1. The number of transcriptional *cis*-regulatory sites in *MICRORNA* gene promoter regions. The presence of known plant-specific *cis*-regulatory elements (*CREs*) were identified within the 3 kb region immediately upstream of the pre-miRNA encoding sequence (the putative promoter region of each assessed *MIR* gene). Only plant hormone, circadian rhythm, and abiotic stress-related *CREs*, were reported for the putative promoter regions of the 70 *MIR* genes that encode the mature miRNAs, miR159, miR164, miR167, miR168, miR396 and miR397, of maize, rice, and *Arabidopsis*, were included in this analysis.

Mature miRNA	Number of Pre-miRNAs	Number of *cis*-Regulatory Elements in Promoter Region of *PRE-MIRNA* Encoding Sequence			Total
		Hormone Related	Circadian Rhythm-Related	Abiotic Stress Related	
Ath-miR159	3 (A-C)	20	4	16	40
Osa-miR159	6 (A-F)	40	8	47	95
Zma-miR159	8 (A-H)	66	15	56	137
Ath-miR164	3 (A-C)	25	4	25	54
Osa-miR164	6 (A-F)	54	5	65	124
Zma-miR164	8 (A-H)	88	11	76	175
Ath-miR167	4 (A-D)	20	4	31	55
Osa-miR167	10 (A-J)	95	28	82	205
Zma-miR167	4 (A-D)	33	6	31	43
Ath-miR168	2 (A-B)	11	2	10	23
Osa-miR168	1 (A)	6	1	14	21
Zma-miR168	2 (A-B)	16	6	11	33
Ath-miR396	2 (A-B)	18	2	25	45
Osa-miR396	3 (A-C)	26	5	16	47
Zma-miR396	2 (A-B)	12	2	20	34
Ath-miR397	2 (A-B)	10	3	17	30
Osa-miR397	2 (A-B)	22	4	9	35
Zma-miR397	2 (A-B)	17	0	9	26

The online miRNA Repository, the miRBase database (http://www.mirbase.org), was initially used to identify the pre-miRNA transcript sequences from which the six mature miRNA sRNAs under analysis are liberated. This approach identified 70 unique pre-miRNA transcripts from maize, rice and *Arabidopsis*, and subsequent use of these 70 pre-miRNA transcript sequences in NCBI, further revealed

that each is derived from a distinct chromosomal position (a unique *MIR* gene locus) within the three searched plant genomes. Upon screening the 3 kb putative "promoter region" upstream of each of the 70 *MIR* genes, a total of 1209 *CREs* relating to plant hormones (n = 579 *CRE*s), circadian rhythm (n = 110 *CRE*s) and abiotic stress (n = 560 *CRE*s) were identified using PlantCARE (Table 1). The abiotic stress-related *CREs* included in this analysis have been demonstrated responsive to, extreme temperature (heat or chilling), drought, anoxic response, aerobic response, and abscisic acid (ABA) signaling. Although abiotic stresses such as extreme temperatures or flooding (driving an anaerobic response) are not the focus of this review, *CREs* responsive to such stimuli were included nonetheless. Their inclusion was to attempt to document the considerable overlap in complex gene networks that the protein products encoded by these genes function in, in a plant that is mounting an adaptive response to an array of abiotic stresses [105,106]. Similarly, given the high degree of documented crosstalk between the plant hormone directed gene expression pathways throughout development, and/or in response to either abiotic and biotic stress [106–109], all plant hormone related *CREs* responsive to, ethylene, salicylic acid, auxin, ABA, gibberellin, and methyl jasmonate, were also included in the PlantCARE analyses (Table 1). Table 1 clearly shows that there is a distinct occurrence of *CRE*s harbored within the putative promoter region of each *MIR* gene family assessed (the *MIR159*, *MIR164*, *MIR167*, *MIR168*, *MIR396* and *MIR397* gene families), and further, that the number, and class of *CRE*, differs widely per *MIR* gene family, and per plant species (*Arabidopsis*, rice, and maize). This wide variability in *CREs* presence/absence, and frequency per *MIR* gene locus/gene family, could explain in part, the documented differences in response of *MIR* gene expression (and subsequent mature miRNA accumulation) to either drought or salt stress across *Arabidopsis*, rice, and maize. Table 1 also clearly indicates that when studying miRNA-directed responses to either drought or salt stress, all experimental analyses should be performed in the specific species of interest, in parallel to the functional characterization of the miRNA/miRNA target gene expression module in *Arabidopsis* (if such functional studies cannot also be performed in the specific plant species of interest).

6. Timing, Treatment, Tissue and "Tolerance" to Drought and Salt Stress

To further account for the variability in miRNA accumulation profiles in response to drought or salt stress stimuli, the experimental methodology of the stress treatment must also be considered. It is readily apparent from investigation of the large body of work stemming from either drought or salt stress treatment of plants, that although the "same" abiotic stress is under investigation, there are distinct differences arising from variations in the treatment or preparation of tissues being sampled for subsequent molecular analyses. Specifically; (1) the time of day the tissue is sampled (morning sampling versus sampling in either the afternoon, evening, or night); (2) the developmental phase of the plant (e.g. is the plant being stressed during, vegetative phase, reproductive phase or grain/seed development?); (3) the tissue type selected for analysis (whole plant or seedling versus sampling of only the root tissue, shoot tissue, or reproductive tissue); (4) the form of stress treatment applied (withholding water from soil cultivated plants versus the use of various osmotica in growth media for tissue culture cultivated plants); (5) the severity, timing and length of stress application (mild stress application over an extended treatment period versus a short and intense burst of stress application), and; (6) the degree of stress tolerance across cultivars of an investigated species, across subspecies, or even across closely related plant species. Each of these listed parameters will add to the overall degree of observed variance in miRNA accumulation, and therefore, miRNA-directed target gene expression regulation, in response to either drought or salt stress.

Assessment of the *CRE* landscape of the promoter regions of the six assessed *MIR* gene families, including the *MIR159*, *MIR164*, *MIR167*, *MIR168*, *MIR396* and *MIR397* gene families, across maize, rice and *Arabidopsis*, identified 110 *CRE*s related to circadian rhythm harbored within these putative promoter sequences (Table 1). The frequency at which circadian rhythm-related *CRE*s were identified, suggests that the transcription of these *MIR* genes is likely already influenced by environmental cues, even in the absence of abiotic stress stimuli. Similar findings have already been reported for *Arabidopsis*

miRNAs, miR167, miR168, miR171 and miR398, with the abundance of each sRNA demonstrated to oscillate between night and day [110]. Further, a tiling array of 114 *Arabidopsis* miRNAs [111] identified multiple circadian rhythm-related miRNAs. These two *Arabidopsis* focused studies, together with the *CRE* data presented here in Table 1, clearly identify the importance of considering temporal dynamics when a researcher is deciding on the appropriate time of day to harvest their tissue(s) of interest for subsequent molecular assessment of miRNA accumulation and miRNA-regulated gene expression responses to drought or salt stress treatment. The diurnal cycle has also been shown to influence the stability of the key machinery protein, DOUBLE-STRANDED RNA BINDING1 (DRB1). In the plant cell nucleus, DRB1 together with functional partners, DICER-LIKE1 (DCL1; an endonuclease), and SERRATE (SE; a zinc-finger protein with binding affinity for double-stranded RNA (dsRNA)), are an absolute requirement for the accurate and efficient processing of miRNA precursor transcripts as part of the production of the mature miRNA sRNA [112–115]. Cho and colleagues (2014) demonstrated that in the cytoplasm of *Arabidopsis* cells, the E3 ubiquitin ligase, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), functions to prevent the protease-mediated degradation of DRB1 [116]. At night however, COP1 is imported into the nucleus and this relocation allows for protease-mediated cleavage of DRB1 (in the absence of COP1 in the cytoplasm) [116]. Given the vital role DRB1 plays in accurate and efficient DCL1-catalyzed miRNA production, this elegant study further identifies the importance of considering the time of day that samples are to be harvested post drought or salt stress treatment of *Arabidopsis*.

Another concern in relation to "timing" is selection of the developmental stage for the application of drought or salt stress to the plant. As a plant transitions between developmental stages, such as the transition from vegetative to reproductive development (floral transition), or the subsequent transition from reproductive to grain and/or seed development, there are pronounced variations to both the physiological and phenotypic characteristics of the plant, both of which are underpinned by intricate, yet distinct genetic networks [117,118]. Moreover, the gene networks controlling these transitions in development, have been shown to be themselves, regulated by miRNAs [119–121]. It is, therefore, highly probable that if an abiotic stress such as drought is encountered by a cropping species such as rice during vegetative development, that the molecular responses underpinning the physiological and phenotypic alterations at this stage of development, would vary greatly compared to those of a rice plant during the reproductive phase of development if an identical stress was encountered. For example, He and colleagues (2012) showed that drought stress during reproductive development in rice resulted in reduced fertility and therefore, overall yield [122]. However, if rice (as well as most other plant species) encounters drought stress during vegetative development, the stressed plant will induce ABA regulatory pathways to ensure that its developmental processes are maintained [123–125]. Therefore, these two vastly distinct physiological responses to the same stress, when encountered at different stages of plant development, would be directed by highly distinct molecular pathways, including unique miRNA-directed gene expression regulation profiles [123,124].

Stress severity is a very important consideration when designing an experiment. A plant will employ specific molecular and physiological networks depending upon the severity, and the duration, of the encountered stress (i.e. is the stress application mild, over an extended treatment period requiring the plant to adapt with adjustments in photosynthetic rates and stomatal conductance or is the stress application intense, for a brief treatment period, requiring the plant to circumvent irreversible damage with heavily reduced transpiration rates and water retention?). In addition to the severity of the applied stress, one must consider the known limitations that exist when using non-ionic stress osmotica, such as mannitol (a penetrating osmotica), or PEG (a semi-penetrating osmotica), as a substitute for drought stress. Osmotica are frequently used to simulate "drought stress" in the genetic model plant *Arabidopsis*, as a desired concentration (and therefore stress severity) is easily included into standard plant cultivation media, allowing for straightforward monitoring and maintenance of environmental variables. With these points taken into consideration, not only is there variation in the severity of the stress based on the selected osmotica, unlike "real" drought stress, molecular

data has the potential to be skewed when osmotica are used: the molecular and/or physiological response of the plant may be accounting for the reduced water status proximal to the root structure, and/or to the *in planta* accumulation of absorbed mannitol or PEG. When investigating miRNA accumulation studies across key grasses and *Arabidopsis*, differences in the treatment used to stimulate drought stress exposure are readily apparent. Of the five papers that investigated miRNA responses to drought stress in either maize, wheat, rice, or *Arabidopsis*; one study used mannitol [88], a second study used PEG [101], 2 studies withheld water to soil cultivated plants (but to differing degrees) [85,87], and Kantar and colleagues (2011) placed plants on paper toweling to induce "drought-shock" [86]. Obviously, each of these different approaches to stimulate drought stress would yield different miRNA responses, even if each approach was being applied to the same species, and at the same stage of development. Furthermore, such a degree of caution should be extended when considering the tissue type to be sampled for subsequent molecular profiling. The division of higher plant organs into source and sink tissues is well documented. More specifically, source tissues include those organs that are photosynthetically active, primarily mature leaves, while sink tissues broadly encompass the photosynthetically inert tissues such as immature leaves, seeds, and roots [126,127]. Given the vastly different roles played by these tissues types, in conjunction with the known crosstalk between the activity of these tissues and plant hormones during periods of abiotic stress, it can be assumed that there would be variance (potentially considerable variance) in miRNA levels between these tissue/organ types communicating each tissue's changed physiological requirements during abiotic stress [128,129]. Again, when considering the same five papers as above, Liu and colleagues (2008) used whole *Arabidopsis* seedlings 14 days post germination [88], while studies [85–87,101] sampled a variety of young, mature, or whole leaf tissue samples for each plant species under investigation. Such sampling differences will also add further variance in the results generated, namely the abundance of individual miRNA sRNAs under investigation.

It is also important to note that, given the demonstrated regulation of the abundance of the key miRNA pathway machinery protein, DRB1, to external cues such as circadian rhythm (see above), we next determined whether the encoding genes of other key miRNA pathway machinery proteins, including the *DCL1*, *SE*, *DRB1*, *DRB2* and *AGO1* loci, are responsive to drought or salt stress. To address this, the online tool "Expression Angler" was utilized on The Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca/ExpressionAngler/) [130]. The gene identification numbers for *DCL1*, *SE*, *DRB1*, *DRB2* and *AGO1* (*AT1G01040*, *AT2G27100*, *AT1G09700*, *AT2G28380* and *AT1G01040*, respectively) were retrieved from The *Arabidopsis* Information Resource (TAIR, https://www.arabidopsis.org/). This analysis revealed that there were no significant expression changes for the *DCL1*, *SE*, *DRB1*, *DRB2* or *AGO1* genes when *Arabidopsis* was exposed to a salt or drought stress growth regime. This finding was unsurprising given that in response to exposure to either stress, the abundance of some *Arabidopsis* miRNAs is elevated while that abundance of a different set of *Arabidopsis* miRNAs is reduced.

It is important to note that all plant species, cultivars, or genotypes of a specific species, respond differently to either drought or salt stress due to the respective baseline tolerance of each to either stress stimulus. Within the *Poaceae* family of grasses for example, maize, wheat, and rice, are all deemed sensitive to reduced water availability or salinity, with each displaying severe yield reductions in response to either stress. However, barley (*Hordeum vulgare* L.), a closely related member within the *Poaceae* family, appears largely unaffected when exposed to either stress [131]. Moreover, it is common within abiotic responsive miRNA studies to profile the miRNA landscape of a "tolerant" versus a "sensitive" cultivar. Frequently, such studies elegantly demonstrate considerable differences in miRNA accumulation profiles for these almost genetically identical plant lines. For example, studies comparing maize [89], or wheat [90] cultivars, identified reciprocal miRNA abundance profiles for 8 (an additional ten miRNAs were only detected in one cultivar and not the other) and nine miRNAs, respectively in response to salt stress. Similarly, a contrast in stress-responsive miRNA, or transcriptome profiles, is noted for genotypes of the same crop species [23,132,133]. Many contemporary research groups

are utilizing genotype-specific molecular stress responses to compare transcriptomes and/or miRNA profiles between genotypes classed as "stress-sensitive" or "stress-tolerant" for the development of superior phenotypes for incorporation into future cereal crop breeding programs [23,132,133]. More specifically, [23] revealed that the significant difference in stress (water-deficiency) tolerance between four closely related genotypes of durum wheat was underpinned by notable differences in their respective miRNA profiles. Most notably, 5 novel, and 16 conserved miRNAs, were demonstrated to have reciprocal abundance profiles in the two "stress-sensitive" and "stress-tolerant" genotypes. Each of the above outlined variables, including the; (1) timing of stress application; (2) specific form of stress treatment applied; (3) tissue sampled for subsequent miRNA profiling, and; (4) degree of stress "tolerance" of the assessed species, all require careful consideration when designing a study to identify either drought or salt stress-responsive miRNAs in the plant species under investigation, or when a researcher is considering translating miRNA findings made in one species, to a second species, regardless of the degree of relatedness of these two species.

7. Non-Conserved microRNAs Responsive to Drought or Salt Stress

A further significant limitation to the use of *Arabidopsis* as a model species for stress-responsive miRNA studies is that many of the miRNAs determined "stress responsive" in the species under investigation, are not present in *Arabidopsis*. The advent of high throughput sequencing technologies has repeatedly highlighted that each plant species produces a population of miRNA sRNAs specific to that species (or across a small clade of closely related species). Such miRNAs are termed, "non-conserved" or "species-specific" miRNAs, a discovery that further questions the use of *Arabidopsis* as an appropriate model for researchers interested in functionally characterizing miRNA-directed stress responses in species such as maize, wheat and rice. For example, Sunkar and colleges (2008) conducted RNA sequencing to produce control, drought-stressed and salt-stressed sRNA libraries. This approach resulted in the identification of 23 lowly abundant, previously unidentified miRNAs, and an additional, 40 candidate novel miRNAs. Furthermore, each of these newly identified miRNAs were also shown to have differing abundance across the three generated libraries [102]. Similarly, studies by Jiao et al. (2011) and Wei et al. (2009) identified 66 and 23 novel miRNAs in maize and wheat, respectively [85,134]. Although these two studies did not investigate the responsiveness of the identified species-specific miRNAs to drought or salt stress, these two studies in conjunction with the findings of Sunkar et al. (2008), readily highlight the shortcomings of using *Arabidopsis* as a model to study miRNA-directed responses to drought or salt stress application in agronomically important cropping species [85,102,134]. Further, given the high prevalence of contemporary research to employ high throughput sequencing technologies, one can safely hypothesize that the continued identification of species-specific miRNAs, also demonstrated responsive to abiotic stress stimuli, will only further highlight this class of miRNA as potential central players in the future development of modified plant lines with resistance, or enhanced tolerance, to either drought or salt stress. This is evidenced with several recent next-generation sequencing studies identifying novel miRNAs that differentially accumulate in response to abiotic stress, such as drought, in the key cereal crops, rice and wheat [23,92,93]. Interestingly, of the three novel miRNAs (*Osa*-cand027, *Osa*-cand052 and *Osa*-cand056) identified as drought responsive by Berrera and colleagues (2012), published degradome analysis failed to identify a putative target gene(s), for any of these three novel miRNAs [93]. Given that target genes, such as phosphate transporters, amino acid transporters, and ATP-dependent RNA helicases, were identified as target genes for other novel rice miRNAs also identified by Berrera et al. (2012), future studies where target genes of these novel species-specific miRNAs are identified, would form an additional and interesting avenue of further research.

8. Conclusions and Future Perspectives

While not always the case, the accumulation profile of an abiotic stress-responsive miRNA can vary considerably across different plant species following exposure to drought or salt stress. This

variation is particularly prevalent when attempting to translate research findings made in the classic genetic model plant species, *Arabidopsis*, to agronomically significant crops, such as maize, wheat or rice. Although *Arabidopsis* has long served as an exceptional model to functionally characterize the plant miRNA pathway, including the characterization of miRNA-directed gene expression regulatory responses to abiotic stress, findings made in *Arabidopsis* may have little, to no, biological relevance in an agronomically important crop species. Therefore, miRNA-directed responses to drought or salt stress need to be experimentally validated in the crop species under assessment prior to the researcher undertaking molecular modification of a specific miRNA/miRNA target gene expression module. Such an approach will ensure that a similar biological response is elicited in the modified species, while also ensuring that other agronomically important parameters, such as yield, are not adversely affected by this modification.

Many researchers now regard plant phenotyping as the bottleneck when attempting to link genotype to phenotype for crop improvement [135,136]. Implementation of a high throughput phenotyping platform is therefore ideal to overcome this bottleneck as such an approach allows for a highly controlled environment, including; watering capabilities in combination with non-destructive imagery techniques that can monitor a plants response to stress at regular intervals across the course of plant development. Further, the parallel application of high throughput sRNA sequencing technologies to complement the high throughput phenotyping platform will allow researchers to identify abiotic stress-responsive, and potentially species-specific miRNAs, that underpin a specific crop plant's ability to mount an effective response against the imposed stress; miRNAs that would otherwise remain elusive if the same miRNA sRNA exploration study was conducted in the long-standing genetic model species, *Arabidopsis*.

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