microRNA, seeds, and Darwin?: diverse function of miRNA in seed biology and plant responses to stress

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Abstract

microRNAs (miRNAs) are small, single-stranded RNAs that down-regulate target genes at the post-transcriptional level. miRNAs regulate target genes by guiding mRNA cleavage or by repressing translation. miRNAs play crucial roles in a broad range of developmental processes in plants. Multiple miRNAs are present in germinating seeds and seedlings of Arabidopsis, some of which are involved in the regulation of germination and seedling growth by plant hormones such as abscisic acid (ABA) and auxin. The involvement of miRNAs in ABA responses is not limited to the early stages of plant development but seems to be important for general stress responses throughout the plant life cycle. This Darwin review summarizes recent progress in miRNA research focusing on seed and stress biology, two topics which were of interest to Charles Darwin.

Key words: Darwin, germination, microRNA, seeds, stress response.

Introduction: Darwin and seeds

‘Seed’ sounds somewhat off topic in a discussion of Charles Darwin’s life. However, seeds are indeed associated with many of his major discoveries. Darwin’s concept of evolution was originally gained through his analysis of Geospiza or Darwin’s Finches, seed-eating birds which are native to the Galapagos Islands. Darwin’s finches are to evolutionary biology what Newton’s apple is to physics (Pennisi, 2004). Seeds played an important role in Darwin’s observations on the evolution of finches, eventually leading to the concept of natural selection, one of the most important concepts in biology. In Chapter 17, in which Darwin discussed the Galapagos Islands in ‘The voyage of the Adventure and Beagle’, he wrote:

‘The most curious fact is the perfect gradation in the size of the beaks in the different species of Geospiza.’

(Darwin, 1839).

In the book ‘On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life’ Darwin stated,

‘Seeing this gradation and diversity of structure in one small, intimately related group of birds, one might fancy that, from an original paucity of birds in this archipelago, one species had been taken and modified for different ends.’ (Darwin, 1859).

Seed cracking finches have a wide and strong beak, while cactus finches drinking nectar have slender and pointed beaks (Fig. 1). Rosemary and Peter Grant have spent 30 years studying these finches and how their beak shape and size help to determine the foods they eat. They have also studied the effect of long-term drought on the natural selection of finches. During an extended drought, the larger finches with large beaks survive because they can crack the larger, harder seeds that remain after the smaller seeds become more scarce. This results in a predominance of large finches breeding the next year and an increase in their population. When the rains return, smaller seeds will again become more prevalent and this provides an advantage to birds with a smaller beak and the population of these birds will increase (Boag and Grant, 1981; Grant and Grant, 2006). Now, even the gene (BMP4) responsible for the
distribution', of Darwin’s book as below:

**miRNA function in seed germination and seedling development**

miRNAs are small (approximately 21–24 nucleotides), single-stranded RNAs that down-regulate target genes at the post-transcriptional level (Bartel, 2004) through mRNA cleavage (Llave et al., 2002) or translational repression (Aukerman and Sakai, 2003; Chen, 2004; Brodersen et al., 2008). For the microRNA target genes regulated through miRNA cleavage, mutations in the sequence of the target gene that is complementary to a miRNA sequence cause over-accumulation of mRNA. When these mutations occur naturally or are generated artificially in such a way that the amino acid sequences are not altered (silent mutation), the miRNA-resistant mRNA over-accumulates and produces an excessive amount of functional protein, which can cause drastic changes in the phenotype. In this way, multiple developmental events, which are under the control of the miRNA target and miRNA genes, can be identified and characterized. This de-regulation approach has been used for many functional genomic studies including seed biology studies (Palatnik et al., 2003; Mallory et al., 2004, 2005; Liu et al., 2007). Potential involvement of at least several miRNAs such as miR156, miR160, miR169, and miR396 in seed development, dormancy, and germination has been suggested (Martin et al., 2005, 2006). However, only limited numbers of miRNAs and their target genes have been analysed in seed research to date. The *SQUAMOSA PROMOTER-BINDING* (*SPL13*), a miR156 target gene, was characterized using the de-regulation approach. Mutated *SPL13* (termed as *mSPL13*), which is resistant to miR156, over-accumulates at the post-germination stages and causes a delay in vegetative leaf development from the cotyledon-stage seedlings (Martin et al., 2010a, b). Down-regulation of *SPL13* by miR156 appears to be essential for the transition to the vegetative-leaf stages.

The involvement of miRNA regulation of transcription factors in seed germination has been better characterized in the case of *AUXIN RESPONSE FACTOR10*, a target of miR160. The mutant expressing miR160 de-regulated *ARF10* (*mARF10*) exhibited altered hormone sensitivity during seed germination and seedling growth in addition to a serrated leaf phenotype (Fig. 2) (Liu et al., 2007). Germination sensu stricto (before the occurrence of radicle emergence) of *mARF10* is hypersensitive to abscisic acid (ABA) in a dose-dependent manner, suggesting a potential interaction between the auxin and ABA signal transduction pathways. ABA hypersensitivity is mimicked in wild-type seeds germinated in the presence of auxin, supporting the idea of auxin–ABA crosstalk. Overexpression of *MIR160* reduces the sensitivity of germinating seeds to ABA. The ABA hypersensitive phenotype of *mARF10* is more drastic at the post-germination stages (Liu et al., 2007). These observations suggest that the down-regulation of *ARF10* by miR160 is essential to maintain normal auxin–ABA crosstalk during seed germination and seedling development.

Several other miRNAs were reported to be essential for seed germination. The balance between ABA and gibberellin mechanisms of natural selection of beak morphology has been isolated and compared from seed-eating and cactus-eating finches (Abzhanov et al., 2004; Wu et al., 2004).

The book, *On the origin of species* contains many descriptions of seeds including Darwin’s own seed biology experiments. An experiment describing salt tolerance during seed germination, which is currently a hot topic in plant biology, was mentioned in Chapter 11, ‘Geographical distribution’, of Darwin’s book as below:

‘Until I tried, with Mr Berkeley’s aid, a few experiments, it was not even known how far seeds could resist the injurious action of sea-water. To my surprise I found that out of 87 kinds, 64 germinated after an immersion of 28 d, and a few survived an immersion of 137 days.’

(Darwin, 1859).

Darwin even performed seed purity tests to examine gene flow, which is also currently a hot topic in seed and plant biology:

‘I raised 233 seedling cabbages from some plants of different varieties growing near each other, and of these only 78 were true to their kind, and some even of these were not perfectly true,...

How, then, comes it that such a vast number of the seedlings are mongrelised? I suspect that it must arise from the pollen of a distinct variety having a prepotent effect over a flower’s own pollen; and that this is part of the general law of good being derived from the intercrossing of distinct individuals of the same species.’

(Darwin, 1859).

He also had many insights into seed dispersal mechanisms including those on hooked seeds.

Thus, seed and stress biology seem to have played an important role in Darwin’s observation and understanding of nature. This review will focus on the function of microRNA (miRNA) with an emphasis on seed biology and plant responses to stresses.

![Fig. 1. Natural selection of beak shape of Geospiza ('Darwin’s Finches')](image-url)
(GA) is important for determining the dormancy status of seeds. ABA is abundant in dormant seeds and generally decreases during imbibition when seed dormancy is released (Kushiro et al., 2004) whereas GA increases during the transition to germination (Yamaguchi et al., 1998). miR159 is induced by ABA and cleaves MYB101 and MYB33 transcripts which are positive regulators of ABA signalling during germination (Reyes and Chua, 2007). miR159 was also induced during drought conditions suggesting that miRNAs may also play a role in sensing the environment surrounding a seed to ensure plant survival after germination (Reyes and Chua, 2007). Further evidence that miRNAs play a role in sensing stress is provided by the delay in germination and reduced seedling survival of miR417-overexpressing transgenic Arabidopsis plants in the presence of high salt or ABA (Jung and Kang, 2007).

The role of miRNAs in drought and salt stress responses

The involvement of miRNAs in stress responses is not limited to the early stages of plant development such as germination. Studies in Arabidopsis have revealed a general role for miRNAs in drought and salt tolerance (Fig. 3). Several stress-related miRNAs were identified based on the sequencing of a library of small RNAs isolated from Arabidopsis seedlings exposed to various stresses (Sunkar and Zhu, 2004). miR393, miR397b, and miR402 were up-regulated in response to cold, dehydration, NaCl, and ABA, suggesting that these miRNAs may target genes involved in a more general response to stress. The predicted target of miR393 is TIR1, which encodes a ubiquitin conjugating enzyme E2/auxin receptor (Dharmasiri and Estelle, 2002; Dharmasiri et al., 2005), so increased levels of miR393 down-regulate auxin signalling and reduce plant growth during stress. One of the genes, miR397b, is predicted to target a laccase gene which was recently shown to reduce root growth under dehydration in a knockout mutant (Cai et al., 2006). Some miRNAs, such as miR398а, are down-regulated during stress conditions indicating that they may normally repress the expression of positive regulators and/or stress-induced genes (Sunkar and Zhu, 2004).

Early studies in monocots have also confirmed that miRNAs play a role in adaptation to drought and salt stresses. An oligonucleotide microarray was used to examine miRNA expression during drought stress in rice.
The authors found that miR169g was up-regulated in response to drought and that this induction was more prominent in roots than in shoots. They examined the promoter of MIR169g and found two dehydration-responsive elements (DREs) supporting a role for miR169g in drought stress. Later miR169g and another member of the miR169 family, miR169n, were reported to be induced by high salinity (Zhao et al., 2009). The authors found a cis-acting ABA responsive element (ABRE) in the upstream region of MIR169n suggesting that it may be ABA regulated. Both miR169g and miR169n targeted NF-YA which encodes a subunit of the NF-Y complex transcription factor which was previously shown to be down-regulated in drought-affected wheat leaves (Stephenson et al., 2007).

Plants accumulate proline as a protective mechanism in response to drought stress. Proline dehydrogenase (PDH) is involved in the degradation of proline and is down-regulated during dehydration but up-regulated during re-hydration, suggesting that it plays an important role in regulating proline content in plants (Rayapati and Stewart, 1991). miR474, which targets PDH, was recently shown to be up-regulated during drought stress in maize (Wei et al., 2009). With less PDH, proline accumulates in the plant and helps to protect against drought stress.

miRNAs in response to UV-B stress and cold stress

As a result of the decrease in the ozone layer, plants are stressed by an increase in UV-B radiation. To gain an understanding of the role miRNAs play in response to this stress, a computational approach was used to identify Arabidopsis miRNAs induced by UV-B radiation (Zhou et al., 2007). They identified 11 putative UV-B responsive genes, eight of which are predicted to target transcription factors (TF) including: (miR156/157); MYB (miR159/319); ARFs (miR160, miR167); HD-ZIP (miR165/166); CBF HAP2/-NF-YA (miR169); SCARECROW (miR170/171); APETALA2, bZIP, and MYB (miR172). Several of these miRNA-targeted TFs are involved in auxin signalling and would therefore have an effect on plant growth including vascular differentiation/development and lateral root development (Zhou et al., 2007).

In UV-B stress response studies in Populus tremula, some of the same miRNA families (ptc-miR156, ptc-miR160, ptc-miR165/166, ptc-miR167) were shown to be up-regulated in response to UV-B stress using a miRNA filter array (Jia et al., 2009). Interestingly, ptc-miR159, ptc-miR169, and ptc-miR393 families were shown to be down-regulated in Populus tremula but were predicted to be up-regulated in Arabidopsis, suggesting that there may be species-specific adaptations to UV-B stress responses (Jia et al., 2009).

The role of miRNAs in cold stress has been examined in Arabidopsis (Sunkar and Zhu, 2004; Liu et al., 2008), Populus (Lu et al., 2008), and Brachypodium (Zhang et al., 2009). miR397 and miR169 were reported to be up-regulated in all three species and miR172 was up-regulated in Arabidopsis and Brachypodium. Two of these miRNAs, miR169 and miR172 target transcription factors and would therefore affect the expression of genes regulated by these transcription factors. The third miRNA, miR397 is predicted to target laccases which are involved in lignin synthesis and maintaining cell wall structure and integrity. Several other predicted miRNAs were shown to be down-regulated in Brachypodium in response to cold including miRNAs 911/926/927T, 912T, 913/914/915T, 917/922/928T, and 918/919T (Zhang et al., 2009) and it will be interesting to see if the down-regulation of miRNAs in response to cold and other stresses is more common than currently predicted as the field of miRNA research progresses.

miRNAs in response to nutrient and oxidative stress

miRNAs also play an important role in various nutrient stress responses. miR395 is up-regulated during sulphate deficiency and helps in assimilating and allocating sulphur by altering the expression of ATP sulphurylase and a sulphate transporter gene (AtSULTR2;1) (Jones-Rhoades and Bartel, 2004). Phosphate homeostasis is partially controlled through miR399 which targets a gene encoding a putative ubiquitin-conjugating enzyme. miR399 is up-regulated in low phosphate-stressed plants and is not induced by other common stresses. Furthermore, plants overexpressing miR399 display phosphate toxicity, potentially due to increased uptake, translocation, and storage of phosphorus in old leaves, which may be due to miR399-mediated down-regulation of the ubiquitin-conjugating enzyme target gene (Fuji et al., 2005). Rice miR1432 and miR444d which were identified by high throughput sequencing are predicted to target a calmodulin-binding protein and EF-hand proteins suggesting a role for miRNA in calcium signalling (Sunkar et al., 2008).

Plants have specific requirements in terms of micro- and macronutrients. When plants are exposed to high concentrations of some micronutrients, to heavy metals and to other stresses mentioned above, they accumulate reactive oxygen species (ROS) which can be highly toxic to cells. Plants have evolved mechanisms to deal with excess ROS including antioxidant molecules (ascorbate, glutathione, α-tocopherol, and carotenoids) and enzymes (superoxide dismutases, catalases, and peroxidases) (Foyer et al., 1994). Cu/Zn superoxide dismutases (CSDs) can detoxify superoxide molecules. Interestingly, high copper (and cadmium) levels result in increased levels of CSDs. miRNA 398 which targets CSDs is down-regulated by oxidative stress (Sunkar et al., 2006) and high levels of copper and cadmium (Zhou et al., 2008). This results in an increase in CSDs which can then help to detoxify ROS. Sunkar et al. (2006) showed that transgenic Arabidopsis plants overexpressing a miRNA-resistant form of CSD2 were more tolerant to high light, heavy metals, and other oxidative stresses. When copper is limiting, the level of miR398 increases and this reduces the allocation of copper into CSDs allowing it for other essential processes (Yamasaki et al., 2007; Abdel-Ghany and Pilon, 2008).
miRNAs also have an effect on cell specific root growth under nitrogen-limiting conditions (Gifford et al., 2008). They found that miR167a was repressed in the presence of nitrogen and its target, ARF8, is induced in the pericycle in response to nitrogen. This results in the production of many lateral roots when nitrogen is plentiful. When nitrogen is limiting, lateral root outgrowth is stimulated to permit roots to search for nitrogen in the soil further away from the plant (Gifford et al., 2008).

**Perspectives**

This review focused on miRNA involvement in seed germination and stress responses, two topics which were of interest to Charles Darwin. Our preliminary analysis using the ARF10 de-regulation mutants have suggested that miR160 might play a role in circumnutation (plant movement), another topic that piqued Darwin’s curiosity during his science career. Circumnutation may be associated with tropisms, which plants have acquired in the course of evolution. The phenomenon has been known since Darwin’s time, yet the mechanisms are still unknown. Biological significance of the mysterious movement of plants in terms of adaptation might be explained by further analysis of miR160 and ARF10 function.

Darwin commented that

‘Seeing this gradation and diversity of structure in one small, intimately related group of birds, one might fancy that, from an original paucity of birds in this archipelago, one species had been taken and modified for different ends.’

(Darwin, 1859).

One could look at miRNAs in the same way in the sense that miRNAs have evolved different forms within a family which may target different genes involved in different processes. In addition, it appears that within different species, miRNAs may have evolved to perform different functions.

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