Characterisation of miRNA during drought stress in wheat plants

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Abstract

Climate change is one of the main global themes that impact human food security. Wheat is a staple food for more than two billion of the world's population, whose production will need to increase by 60% in the coming decades, under the backdrop of climate change. Drought has been the most important limiting factor for wheat productivity. Many studies on model plants have demonstrated that miRNAs play a major role in drought stress responses, however, very little is known about their function in wheat. To gain insights into the role of miRNAs in drought stress in wheat we used two wheat varieties (Pavon 76 and Yecora Rojo) with different responses to drought. Small RNA sequence data were obtained from 48 leaf and root samples for both varieties under well-watered and water deficit conditions. Principal Component Analysis clustering showed that miRNA expression in leaf samples was very different from root samples. Under drought stress, Pavon 76 and Yecora Rojo samples were clustered in two distinguished groups, while under well-watered conditions no clear separation was observed.

As a result, a total of 44 different miRNA sequences were found differentially expressed that can be derived from 90 loci. The miRNA sequences were compared to all plant miRNAs deposited in miRbase (https://www.mirbase.org). We found that 27 of the differentially expressed miRNAs belong to 21 miRNA families and 8 of these are conserved (*miR160, miR166, miR169, miR319, miR396, miR398, miR528* and *miR9657*) while the other 13 are non-conserved miRNA families. While, the family *miR164* have 4 family members and the families *miR172, miR390* and *miR9772* have 3 members, each. The other 9 miRNA families *(miR171, miR530, miR827, miR874, miR1120, miR5067, miR5070, miR9674* and *miR9776*) have less than three family members, each. In addition to the 27 (21 families) known miRNAs, the 44 differentially expressed miRNAs also included 17 miRNAs that could not be assigned to any of the known miRNA families.

MiR319, miR528, and *miR7714* were selected for further validation. Northern blot analysis showed fairly good signals for the three miRNAs, but the extent of the signal was different for each one, with clear changes in the expression pattern between well-watered and water deficit treatments confirming the results obtained

from the sequencing analysis. Target genes of the selected miRNAs were predicted and their cleaved mRNA fragments were successfully amplified using 5'RACE. However, only the *miR319* cleavage site was confirmed by sequencing. The current research has important implications for future studies to develop drought-resistant wheat that can cope with the unpredictable stresses associated with climate change and ensure food security for the future.

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Chapter 1. Introduction

1.1 Food security and climate change

It is generally accepted that food security is a major issue globally, as well as at a national level, for communities, households and individuals. Worldwide, there are numerous populations who suffer from some level of food insecurity and this is exacerbated by increased demand for food and by climate change (CC). The following sections consider in more detail the issue of food security and CC.

1.1.1 Food Security

Some 200 definitions of food security have been suggested (Smith, Pointing and Maxwell, 1996; IFPRI, 1999). The most commonly used of these is: 'Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life' (http://www.fao.org). Thus, food security is defined in a multidimensional manner, taking four 'pillars' as its foundation. These are whether there are physically adequate quantities of food available, the accessibility of food, both economically and physically, the use of food and how stable these three aspects of security are (Trudel, 2006). Food insecurity occurs when "the availability of nutritional adequate and safe foods or ability to acquire acceptable foods in socially acceptable ways is limited or uncertain" (Anderson, 1990). The Sustainable Development Goals (SDGs) 2030's second goal acknowledges that it is essential to tackle the issues of food insecurity (*The State of Food Security and Nutrition in the World 2020*, 2020).

Although food production worldwide had been stable in 2019, the projection made by the Food and Agriculture Organization (FAO) projected that 687.8 million people (8.9%) globally are inadequately nourished. In addition, 750 million people (9.7%) across the world were undergoing severe food insecurity (*The State of Food Security and Nutrition in the World 2020*, 2020). In a number of countries, especially those with high incomes, there is regular monitoring of food insecurity by means of a system of national nutritional surveillance which includes surveys (Tarasuk, 2005; Nord *et al.*, 2010). In the U.S., it was reported that food insecurity affects 12.3% of

households (Nord *et al.*, 2010). In Canada, 6.0% of households experience food insecurity (Tarasuk, 2005). Food security has also been investigated using a method which concentrates on vulnerable populations, especially those from low socioeconomic groups, those who live in poverty and people in certain regions or isolated areas. Populations which are vulnerable are more likely to suffer from food insecurity. In Puerto Rico, for example, 40.0% of households on low incomes were placed in the food insecure category (Dhokarh *et al.*, 2011). In Brazil, Alagoas is amongst the most deprived of the country's regions. Here, 58.3% of households suffered from food insecurity. In 33.1% of cases the risk was considered mild, in 17.9% it was moderate, and in 7.3% of cases the risk was severe (Costa *et al.*, 2017). In South Africa, in the rural province of Limpopol, 53.0% of households were considered to suffer from severe food insecurity (De Cock *et al.*, 2013). In urban areas, levels of household food insecurity were also widespread. In native American households including young children, food insecurity was identified as a problem for 44.6% of those in rural areas and 79.5% in an urban setting (Tomayko *et al.*, 2017). In Nigeria, at Ibadan metropolis in Oyo state, a study found that food insecurity affected 29.3% of households (Adepoju and Oyegoke, 2018). These examples from across the world demonstrate how prevalent the problem of food insecurity is and clearly indicate the need to address the problem immediately, especially amongst those populations which are disadvantaged.

Food insecurity negatively impacts on the health of populations, mainly as a result of inadequate food intake and poor dietary variety amongst food-insecure groups (Rosas *et al.*, 2009; Almeida *et al.*, 2017). This may result in behaviours which are detrimental to health, including high fat intake (Mello *et al.*, 2010), high consumption of sugary drinks, sweet cakes and desserts, red or processed meats, nuts, seeds and legumes but lower vegetable consumption (Leung *et al.*, 2014; Araújo *et al.*, 2018).

Amongst indigenous people, the level of food security depended upon their environment and lifestyles. Major issues were environmental degradation, such as deforestation, concerns about the contamination of the water supplies and traditional foods, the move away from traditional practices as hunting of traditional food as a cultural activity, sometime as a result of the time constraints of employment. Traditional knowledge is also being lost, in the process known as acculturation, with

changes in farming methods, greater access to western food and harvesting tools becoming prohibitively expensive (Kuhnlein, Erasmus and Spigelski, 2009; Schuster *et al.*, 2011). According to article 25 of the Universal Declaration of Human Rights, food is a basic human right (http://www.un.org). Thus, it is essential that everyone worldwide, including people who are vulnerable or in poverty, are able to eat an adequate quantity of safe, nutritious food, in accordance with the above-mentioned second aim of the Sustainable Development Goals (SDGs), which is to end hunger by 2030 (*The State of Food Security and Nutrition in the World 2020*, 2020). Food insecurity must therefore be eradicated across the globe. It is the responsibility of governments not to hinder the attempts of their peoples to obtain food, to protect them against violations of food rights and to help them source the food they need (http://www.fao.org). However, food security is becoming increasingly vulnerable in the face of global warming and changes to the climate.

1.1.2 Climate Change

Over the past 65 years significant global climate changes have been noted and, with global warming, more change is anticipated during the twenty-first century, representing a significant and complex challenge to governments across the world. Climate change (CC) affects many aspects of the disciplines of ecology, environmental sciences, socio-political sciences, and socioeconomics (Neil Adger, Arnell and Tompkins, 2005; Leal Filho *et al.*, 2021; Feliciano *et al.*, 2022), due to raised temperatures across the world (Weisheimer and Palmer, 2005; Battisti and Naylor, 2009; Yadav *et al.*, 2015). The industrial revolution has exacerbated the problems of the global climate (Zargar *et al.*, 2017). It has been suggested that immediate and appropriate action could mitigate some of CCs devastating results, although it is impossible to pinpoint precisely all the consequences of CC for every sector (Jurgilevich *et al.*, 2017; Izaguirre *et al.*, 2021), which is apparent from the way in which policymakers nationally and locally are taking climate uncertainties into account (Ayers *et al.*, 2014).

CC is described according to patterns of long-term temperature and precipitation, alongside such features as pressure and humidity levels, and its most well-recognised effects are irregular weather, the shrinking of ice sheets, and the associated sea level rises (Lipczynska-Kochany, 2018; Murshed, 2020; Michel, Eriksson and Klimes,

2021). Prior to the industrial revolution, natural occurrences such as forest fires, volcanoes and seismic activities, were producers of greenhouse gases (GHGs) such as Carbon dioxide(CO_2), Nitrous oxide (N_2O) and water vapor (Shahbaz, Balsalobre-Lorente and Sinha, 2019; Hussain *et al.*, 2020; Murshed, 2020, 2022; Sovacool *et al.*, 2021; Usman, Makhdum and Kousar, 2021). A significant agreement was made by United Nations Framework Convention on Climate Change (UNFCCC) at Conference of the Parties (COP-21), on December 12, 2015 in Paris, about addressing CC and hastening and intensifying the necessary activities and investments to move towards a sustainable low-carbon future. This Paris Agreement developed the Convention because, for the first time, it brought together all nations for the single purpose of instigating ambitious plans to avert climate change and adapt to its effects. Funding was boosted to help developing countries in this respect. This marked a turning point in the global fight against CC. In essence, the Paris Agreement aimed to enhance the response to the dangers of CC by maintaining temperature increases across the world during the 21st century at less than 2°C above pre-industrial levels and to endeavor to restrict temperature rise to 1.5° C (Sharif *et al.*, 2020; Sharma, Sinha and Kautish, 2020; Chien *et al.*, 2021). In addition, the agreement sought to enhance countries' capacity to manage the impacts of CC and to keep financing flows in line with low GHG emissions and approaches to enhance climate-resilience (Shahbaz et al, 2019; Chien *et al.*, 2021). The changes in temperature and precipitation associated with climate change will have a profound impact on agriculture in the future. Wheat is one of the key crops worldwide and is essential to food security. However, it is a plant which is sensitive to drought and therefore vital wheat production is under serious threat from CC and the resulting increased incidences of drought. The following sections will consider the importance of wheat to the human diet, wheat and the experimental system, the need to increase production and the challenges facing producers.

1.2 Wheat

Humans are dependent on a very small number of plants to fulfil their food requirements, despite the existence of some 350,000 documented species. It is therefore essential that we understand what affects their productivity and how they adapt to the constraints of the environment if the future good of humanity is to be

ensured (Chaves *et al.*, 2013). Wheat (*Triticum aestivum*) is one of the plant species upon which we most rely (Austin, 2000). Wheat is one of the crops which have formed the foundation of human civilization. In the so-called Fertile Crescent, some 8 to 10 thousand years ago, farmers who developed the very first permanent agricultural settlements took emmer and einkorn grasses and cultivated wheat from them (Smith, 1998). The emmer wheat is tetraploid (AABB, *T. dicoccoides*) and the grass with which it was hybridized was diploid (DD, *Aegilops tauschii*) (Brenchley *et al.*, 2012).

Wheat growing worldwide now exceeds the production of any other cereal. Over 220 million hectares are now used for wheat production annually, covering a wide range of geographic areas and a considerable variety of climatic conditions. Annual production is in the region of 670 million tons, although this figure depends on agricultural and weather conditions. Both the developed and the developing world produce wheat with an approximately equal split between the two, although methods of production vary considerably. The developing regions, which include Central Asia and China, produce 50% of overall harvests from 53% of the land under production. The developed world has yields which are 14% higher, largely due to the fact that Central Asian production relies on rainfall, which produces lower yields. If Central Asia is not included in the calculation, production in the two areas is comparable at approximately 3 tons per hectare. The main reason for this is that wheat grown in developing countries is largely rain-fed, while production in developed countries relies on irrigation (Shiferaw *et al.*, 2013).

Thus, without question, wheat is hugely significant economically and to meet the nutritional needs of people and livestock. According to recent figures, an average of approximately 763 million tonnes (mt) of wheat have been produced annual across the globe over a period of 5 years from 2018 to 2022, (https://www.fao.org). Thus, wheat is the third most important crop when considering global production, the other two major cereals being rice and maize. However, wheat has a far greater range of cultivation. It's most northerly point of growth, in Scandinavia and Russia, is at 67°N, while its most southerly point is 45°S in Argentina. The range includes various elevated tropical and subtropical regions (Feldman 1995). Demand for wheat is also expanding into markets which are outside its climatic adaptation range, due to the

unique nature of the types of food which can be produced from it, foodstuff which are being increasingly consumed as industrialization and westernization continue. Of particular appeal are the characteristics of the gluten protein fraction which makes it possible to produce wheaten bread, other baked products, pasta and noodles. Such wheat products are regarded as an aspect of a western lifestyle and are often easier and more convenient to produce and to eat than traditional foods. Given the importance of wheat, it has attracted the attention of scientists and researchers worldwide. The following section considers wheat as an experimental system.

1.2.1 Wheat as an Experimental System

Since the three cereal crops grown most commonly worldwide, rice, maize and wheat, make up approximately 50% of humanity's calorie consumption (Alexandratos and Bruinsma, 2012), understanding the biology of these cereal crops is essential if rising demands for food, feed and fuel are to be met, especially in the face of population growth and the issues connected to climate change. However, these cereals are challenging to research because of their large genomes, the length of time required for production and their large sizes.

There has been extensive research into model species in an effort to understand specific biological processes, and it has been anticipated that such work will also generate insights about other species. Certain practical features are associated with model plant species. They are small, ease to grow, highly productive, require only a short time for generation, have a small genome and can easily be manipulated genetically, including by means of such processes as crossing, gene modification and mutagenesis. Chang, Bowman and Meyerowitz, 2016, posited that there were other reasons in addition to these practical characteristics which have influenced the spread of model species. With increasing numbers of researcher using model species, more straightforward and reliable methods for lab protocols have become available, including those for the extraction of DNA and for the purification and extraction of proteins. In addition, as the numbers of researchers using these model species expands, available resources also increase, including genetic stocks kept by centres for germplasm, databases and annotated genomes which leads to further adoption.

Arabidopsis has been commonly employed as model species since the 1980s, as a means to enhance what we know about plant biology. Research into Arabidopsis has provided researchers with a basic understanding of numerous plant processes and has informed studies of these processes in cereal crops. Between 1965 and 2015, 41,682 papers on Arabidopsis have been published and received one or more citations. Of these citations, 37% were by papers which concentrated mainly on another species, not Arabidopsis itself (Provart *et al.*, 2016), which is a clear indication that research on Arabidopsis is being applied by scientists studying other species. Nevertheless, there are significant crop generation processes, such as mycorrhization, which do not occur in Arabidopsis. Furthermore, knowledge about Arabidopsis cannot be applied to certain processes, such as starch metabolism (Smith, 2012). Similar gene families have been demonstrated to regulate characteristics in Arabidopsis and cereals, but the individual members of the relevant families may have a different network of connections, as has been demonstrated for the case of both flowering (Hill and Li, 2016) and senescence (Borrill *et al.*, 2019). If the biology of cereal is to be completely understood then cereal species themselves must be studied. A number of monocot models for crop species have been suggested as a way of to dealing with some of the restrictions of using Arabidopsis to research cereal biology. It has been suggested that *Setaria viridis* could be used as a model for maize and *Brachypodium distachyon* for wheat because their evolutionary relationships are clear and the model plant features are appropriate (Brutnell, 2015). At the time when these potential model species were suggested, there was already molecular research in progress on rice, thus highlighting the need for direct research into crop species, whose findings can then be applied in the field. It became possible for molecular studies of wheat and maize to be conducted, thanks to technological developments which were partly founded on the techniques applied in rice research. Using other monocot model species as a means of understanding processes in maize and wheat did not therefore become particularly popular.

The availability of genome sequences is key to research in molecular biology. The introduction of techniques such as inexpensive next generation sequencing, enhanced methods for assembly and scaffolding and long-read technology, have meant that large genome size need not be a barrier to producing a reference genome sequence. For the past decade a high-quality genome sequence for rice (International Rice

Genome Sequencing Project, 2005) and a draft genome for maize (Schnable *et al.*, 2009) have been available. The maize genome sequence has recently been enhanced by long-read technologies and optical mapping (Jiao *et al.*, 2017). Furthermore, 2018 saw the publication of a chromosome-level assembly for wheat's 16 Gb hexaploid genome (IWGSC et al., 2018), demonstrating that high-quality genome sequences can be produced regardless of genome size, although it remains expensive to sequence large genomes. The potential for researching gene function in various plant species has increased with the development of gene editing. In the past, researchers investigating model species such as rice and Arabidopsis were at an advantage when characterising gene function, due to the extent of the collections of mutant or insertion lines for established model species (reviewed in Holland and Jez, 2018; Hong *et al.*, 2019). However, it has been demonstrated that CRISPR-Cas9 functions for all three major cereals and it is possible to apply it to generate transgene-free genome-edited plants with the potential for commercialization (reviewed in Zhu *et al.*, 2017). Generally, CRISPR-Cas9 has been applied to stimulate small deletions within genes to trigger frame-shift or knock-outs. However, it can be applied much more widely for more complex editing, for example epigenetic modification or specific base editing (reviewed in Adli, 2018). Application of these techniques to cereals is now becoming more common (Li *et al.*, 2018).

1.2.2 The need to increase wheat production

Wheat represents a major source of calories for the global population and any threat to its production risks serious damage to food security. The population of the world is predicted to rise to nine billion by the middle of the 21st century, which raises considerable challenges with regard to feeding such large numbers of people. It is therefore essential that modern agricultural practices develop along with the requirement to intensify sustainable food production. Current wheat breeding research is now ever more oriented towards these future needs and makes use of both old established technologies and those that are novel and pioneering. The aim of wheat breeding is to achieve the United Nations Millennium Development Goals of ending hunger and extreme poverty (MDG1).

1.2.3 Historical increases in wheat production

Many developing regions, such as West Asia, South Asia, South East Asia, Latin America and North Africa have benefited from the so called "Green Revolution" of the 1960s and 1970s with wheat growth and productivity rising to unprecedented levels. Famines have been avoided with the result that millions of lives have been saved. As a result, wheat has become known as the 'miracle crop' of the 21st century. Such a remarkable increase in productivity was largely due to the development of semi-dwarf or short-strewed varieties of wheat which respond well to fertilizers and have high yields. The support of various institutions and policy changes to maximize the benefits from these technological developments have made it possible for farmers to use the new seeds and to have access to the fertilizer and irrigation technology needed to grow them. These have often been subsidized. In addition, markets have been made available to ensure that farmers can sell their crops and higher wheat prices have been guaranteed (Shiferaw *et al.*, 2013). Such public investments in wheat saw huge yield increases, but initially the areas targeted were in Asia and already using irrigation, with marginal and rain-fed areas being largely ignored. However the use of high yield varieties spread progressively to areas such as South Asia, South Africa, Brazil, Turkey and Iran, which relied on rain-fed production (Pal and Byerlee, 2005). Between 1966-79 average wheat yields for developing countries rose by 3.6 % per annum. As a result of the Green Revolution, poverty and hunger have been considerably reduced (Datt, Gaurav, Ravallion, 1998; Rosegrant, Fan and Hazell, 2001; Renkow and Byerlee, 2010).

1.2.4 Challenges to further increasing wheat production

Between 1984–94 wheat productivity fell by 2.8 % per annum and during the decade 1995–2005 (Dixon *et al.*, 2009) it decreased to 1.1%. By the 1990's it was becoming apparent that technical changes were patchy in their effects and that not all areas were experiencing the advantages of production increases. It was recognized, partly through the "farming systems" movement, that the standard practice packages were not benefitting farmers in poor areas with higher risks of crop failure (Howie, 2008). Even those farmers in wealthier areas with the benefits of irrigation were suffering

from problems in the environment, such as waterlogging and increased levels of salinity, and productivity failed to increase further (Byerlee and Siddiq, 1994).

Food security has now become a major worry, especially in developing countries which rely on wheat as a staple food, and these concerns have been exacerbated by climate change and what this may mean for wheat production. Since the 1980s, wheat production has fallen by 5.5% as a result of temperature increases (Lobell, Schlenker and Costa-Roberts, 2011) and wheat is particularly vulnerable to high temperatures, especially at night at low latitudes (Ortiz *et al.*, 2008; Lobell, Schlenker and Costa-Roberts, 2011). It may be worth considering that in high latitude areas, wheat production could be threatened by high temperatures during the day, as suggested by Semenov *et al.*, (2011). A particular issue of higher temperatures is that yields are reduced due to shorter periods of grain filling because increased warmth leads to more rapid plant development (Fahad *et al.*, 2018). Overall projections suggest that in developed countries, yields will drop by 0.1%, while in developing countries, even where irrigated systems are used, the fall will be 5.3 to 7.4 % (Nelson., 2010).

A reduction in supplies of water for irrigation will also impact negatively on wheat production, alongside the risks from newly emerging diseases and loss of soil fertility (Shiferaw *et al.*, 2013). The heat stress and water shortages resulting from climate change are predicted to have the most serious impact in North African and West Asian systems of farming. If there is inadequate water available for irrigation, rain-fed growing of staples will become the norm, and wheat is the most water-efficient and drought tolerant of the major staple crops (Ortiz *et al.*, 2008). However, this will increase the risk for farmers and prices for consumers are likely to fluctuate considerably. The price of fertilizers is likely to rise simultaneously, due to increasing prices of fossil fuels and falling potassium and phosphorus reserves (Cordell, Drangert and White, 2009).

In addition to the problems of stagnating productivity, demand for wheat is increasing as patterns of food consumption worldwide are changing and both the directions and the amounts of trade are impacted by economic integration and changes in markets. It is predicted that greater amounts of wheat will be required for the developing world, with projections for Africa suggesting that demand will rise by

60% by the middle of the 21st century (Nelson *et al.*, 2010). Already wheat is the most imported foodstuff in most developing countries (Dixon *et al.*, 2009).

With populations growing rapidly and many areas which are densely populated having become reliant on imported wheat, it is imperative that the risks posed by climate change and other factors affecting the productivity of wheat be addressed. If food security is to be ensured something greater and more far-reaching than the Green Revolution will be required because conditions are very different now compared to the 1960s. It will be essential not to focus on short-term solutions but to espouse sustainable principles to intensify production and ensure that agro-ecosystems are resilient and that their benefits are widespread. While new varieties will be needed, attention must also be paid to sustainable management practices to avoid soil degradation and water shortages. Research is required to develop new cultivars to replace those which have become outdated and to address the challenges of the current situation (Shiferaw *et al.*, 2013). However, one major problem affecting the growth and yields of wheat is drought stress and, since this is such a significant challenge for the increase in wheat production, the following section will consider the issue in depth.

1.3 Drought stress and tolerance

1.3.1 What is drought stress?

Drought limits plant growth, having a detrimental impact on both growth and crop production. Drought stress is exacerbated by water depletion in the rooting zone and raised atmospheric vapor pressure deficit (Ahanger *et al.*, 2014). Losses in crop yields resulting from drought conditions are thought to be greater than those from any other types of abiotic stress. Thus, crop productivity is seriously affected by the severity and length of drought stress events, as well as by the interaction between drought and other environmental factors. The level of reduction in productivity depends on such factors as plant species, the stage of growth when drought occurs, how long the drought lasts and how severe it is. Drought has a damaging effect at every stage of development (Valliyodan and Nguyen, 2006), although severity and duration of drought as well as growth stage were key to the plants' response to water

stress, and how ion exchange is disrupted when the cell membrane is affected by drought. Furthermore, imbalances may occur in the composition of nutrients within the plant cell, and transpiration is hindered by drought, although nutrient uptake may be affected differently (Peuke *et al.*, 2002). Plants employ a number of mechanisms to adapt to drought, to minimize its adverse impact on growth and reproduction (Krasensky and Jonak, 2012). These include morphological adaptations such as reduced leaf size, decreased stem elongation and root proliferation; physiological mechanisms such as enhanced water-use efficiency, gas exchange controls, closing of the stomata and a reduction in turgor pressure; and molecular mechanisms such as specific protein synthesis and a rise in osmolyte production to protect the bio-chemicals from becoming deformed.

1.3.2 How drought affects wheat development

Progress has already been achieved by researchers seeking to understand in more detail the effects of drought on wheat plants and their yields. Drought stress timing has been shown to be an important factor affecting phenological development, affecting both grain size and numbers of individual grains. Reduction in the number of grains may be the result of fewer spikes and spikelets being produced during tillering and while differentiation of the floral meristems is taking place. If drought happens when flowers are developing, both spikelets and florets may be aborted (Ji *et al.*, 2010). Abortion of grain development under stress conditions is a serious problem in wheat. When subjected to drought stress, wheat crops show more out-crossing, which is thought to indicate that pollen sterility is a factor contributing to a reduction in grain set during drought (Bingham, 1966). This supports early work which noted that it is during the development of the pollen that wheat is especially vulnerable to drought (Ashton 1948; Salte *et al* 1967).

1.3.3 Breeding for drought tolerance in wheat

Drought is a major risk factor for global food security, particularly with the on-going increase in global population and diminishing water resources for irrigation, which dictate a need to increase yields dramatically and achieve harvests from hitherto marginal, dry land (Mohammadi, 2018). Drought reduces yields significantly, even drastically, but is a complex phenomenon in terms of its impact on plant growth. The

development of drought tolerance in crop plants is limited by the fact that it depends upon a number of different genes with small individual effects (Gahlaut *et al.*, 2017), as well as micro-RNAs, transcription factors, proteins, hormones, metabolites, ions and co-factors (Mohammadi, 2018) .There are further complications in the breeding of drought-tolerant varieties, as plants can experience a variety of environmental stresses at the same time, particularly excessive heat and solar irradiance, together with reduced nutrients, or nutrient toxicity (Mohammadi, 2018).

In the case of wheat (Triticum aestivum), breeding for drought tolerance in challenging environments has generally been as a result of selection according to phenotype, with no significant contribution from marker-assisted selection (MAS) (Mohammadi, 2018). As well as drought, food production in dry environments is vulnerable to temperature extremes, rainfall variability, new pests and novel diseases. If it is to become more efficient, therefore, it is important that plant breeding is improved and wheat cultivars can be targeted according to local environmental factors and agricultural practices.

The genetic improvements that contribute to increased yield in wheat crops have been studied by comparing different cultivars grown together. However, in-field trials are limited by the fact that genotypic expression in terms of yield can be masked by the variability of environmental factors, but some success has been achieved in developing genetic modifications which offer increased yield potential coupled with improved yield stability, a greater tolerance of environmental variability (Mohammadi, 2018).

Breeders have become interested in high-throughput phenotyping (HTP) and genomic selection (GS) in order to be able to predict such complex outcomes as yield, growth rates and stress responses. There is still an issue with coupling genetic variation to phenotypes and predicting the phenotypes arising from novel genetic combinations. HTP combines remote viewing, robotics (e.g. using drones) and cutting edge computing to analyse a variety of phenotypic measurements accurately and rapidly, allowing accelerated phenotypic experimentation. GS is a more efficient form of MAS, using markers across the genome rather than concentrating on a small selection of high-effect genes, so allowing genetic gain to be assessed for biotic and environmental factors simultaneously (Mohammadi, 2018).

HTP and GS appear to offer great potential, but the greatest issue with identifying factors relevant to drought tolerance is due to the fact that the climatic conditions under which wheat crops are grown vary so widely, so that the contexts for drought globally are diverse. Approaches which bring together plant breeders, gene banks, plant physiologists and biotechnologists would accelerate the process of exploiting the diversity found in the wheat genome (Mohammadi, 2018) .Quantitative trait loci (QTL) are sections of the genomes which give rise to particularly phenotypic traits. Identification of QTLs is therefore commonly seen as an important way of defining molecular markers for phenotypic expression related to drought tolerance (Gahlaut *et al.*, 2017; Appels *et al.*, 2018). Whilst there is as yet no complete understanding of the genetics of drought resistance in wheat, research is on-going to determine genetic regions in the wheat genome relevant to drought tolerance (Gahlaut *et al.*, 2017).

1.4 MicroRNAs (miRNAs)

One of the striking discoveries of RNA biology was 20 to 30 nucleotides long non-coding RNAs that function as genetic regulators, known as microRNAs (miRNAs). MiRNAs help in post-transcriptional repression to control expression and evolution (Bartel, 2009). MiRNAs were first characterised in *Caenorhabditis elegans (C. elegans)* when 2 genes called *lin-4* and *let-7* were seen to be regulating gene expression involved in larval development. *Lin-4* gene coded for 2 small RNAs one being 22 nucleotide long and the other 61 nucleotide long, and it was shown that the longer one formed a stem loop and acted as the precursor of the smaller one (Lee, Feinbaum and Ambros, 1993). The *lin-4* RNAs were then shown to have multiple sites of complementarity within the 3' UTR region of the *lin-14* gene that decreased expression of *lin-14* proteins without changing its mRNA levels. The *lin-14* gene was therefore repressed as a part of the regulatory pathway in cell division that occurs during the change from the 1st to the 2nd larval stage (Wightman, Ha and Ruvkun, 1993). The shorter *lin-4* RNA then came to be recognised as the founding member of the group called miRNAs.

Gene expression in eukaryotes is regulated, protected and stabilised by small, non-coding RNA molecules which recognise complementary RNA targets. They do this by means of diverse processes known as RNA interference (RNAi). However, all

RNAi processes have the same complex at their core: a guide RNA bound to an Argonaute (Ago) family protein. This is known as the RISC (RNA-induced silencing complex). Diverse structures based on Argonaute proteins are also present in prokaryotic cells. These prokaryotic Argonautes (pAgo) proteins are also involved in protecting cells from invasive genetic material, but respond to DNA targets instead of RNA ones (Lisitskaya, Aravin and Kulbachinskiy, 2018).

Both eukaryotic and prokaryotic Argonaute (eAgo and pAgo) proteins perform similar slicing and binding actions on nucleic acid. However, eAgo proteins employ RNA guides (miRNA and siRNA) in gene expression regulation and some can repress transcription by binding to newly formed RNA molecules in the nucleus, whereas eAgo proteins mostly employ DNA guides to locate DNA targets. Some do however employ RNA guides, although the function of this has yet to be confirmed. No accessory proteins appear to be needed for the actions of pAgo proteins on DNA or RNA (Lisitskaya, Aravin and Kulbachinskiy, 2018).

The general consensus has been that pAgo proteins operate principally by nucleolytic cleavage to eliminate invasive genetic material, but they also appear to be involved in other regulatory functions that do not depend upon DNA cleavage and it is possible that pAgo proteins could be used for artificial gene expression regulation, including suicide systems which terminate bacterial cells under stress and could be used to stop phage multiplication (Dy *et al.*, 2014; Lisitskaya, Aravin and Kulbachinskiy, 2018). It is also possible that pAgo proteins have a role in DNA repair, causing cleavage where there are DNA errors (Lisitskaya, Aravin and Kulbachinskiy, 2018).

1.4.1 MiRNA biogenesis

Plant miRNAs consist of 20–24 nucleotides. These are small RNAs which are responsible for the regulation of a number of processes including metabolism, development, and the way in which the plant responds to stressors, both biotic and abiotic (Chen, 2009; Sunkar, Li and Jagadeeswaran, 2012). MiRNAs differ from other kinds of small RNAs, in that they derive from mRNA precursors and their transcription is from miRNA genes (Yu, Jia and Chen, 2017), by means of RNA polymerase II (Pol II). The primary miRNA transcript is also known as the pri-

miRNA, and this develops into a secondary structure with a hairpin formation. Firstly, the pri-miRNA is cut into pre-miRNA, which is the miRNA precursor which is comprised of just the hairpin. This is achieved by means of three different proteins: endonuclease DICERLIKE 1 (DCL1), the RNA binding protein HYPONASTIC LEAVES 1 (HYL1), and the zinc-finger protein SERRATE (SE). A second cleavage of the hairpin pre-miRNA, carried out by the DCL1/HYL1/SE complex, leads to formation of a 20–24 bp miRNA duplex. Methylation of the 3' of both miRNAs in the duplex, by ENHANCER 1 (HEN1), improves the miRNA's stability (Park *et al.*, 2002; Vazquez, Gasciolli and Cre, 2004; L. Yang *et al.*, 2006; Z. Yang *et al.*, 2006) (Fig. 1). Mature miRNA, which is developed from the 5' arm of the hairpin is currently referred to as "5p", while '3p' is the term used for the miRNA from the hairpin's 3' arm (de Folter, 2019). MiRNA is the most abundant molecule found in the duplex, and is normally produced from the hairpin's 5' arm. The term miRNA* was used for the molecule produced by the 3' arm of the hairpin, which is also known as the miRNA star strand (Chen, 2009). Gene silencing takes place when a single strand of the miRNA-5p/ miRNA-3p duplex is loaded onto ARGONAUTE1 (AGO1). This produces the RNA-induced silencing complex (RISC). It is the complementarity of the target mRNAs which facilitates their recognition by RISC. When the RISC complex interacts with the target mRNA, this can result in the degradation of mRNA and/or the repression of its translation (Baumberger and Baulcombe, 2005; Yu, Jia and Chen, 2017) **(Figure 1**). Secondary siRNAs may, under some circumstances, be formed from mRNA transcripts through cleavage by miRNAs. These were initially termed trans-acting siRNAs (tasiRNAs) (Yoshikawa *et al.*, 2005), although current nomenclature uses the term phased secondary siRNAs (phasiRNAs) (Fei, Xia and Meyers, 2013; Yu, Jia and Chen, 2017). All the main plant lineages contain miRNAs (Chávez Montes *et al.*, 2014). The machinery required for the biogenesis and degradation of miRNAs, and for their activity, is found in the common ancestor of land plants (You *et al.*, 2017). Throughout the plant kingdoms, there are two classes of miRNA family which have been identified by comparing miRNA plant families. In the first class, the miRNA is highly expressed and evolutionarily conserved. In the second class, miRNAs are generally expressed at low levels, or certain conditions may lead to their induction (Cuperus, Fahlgren and Carrington, 2011).

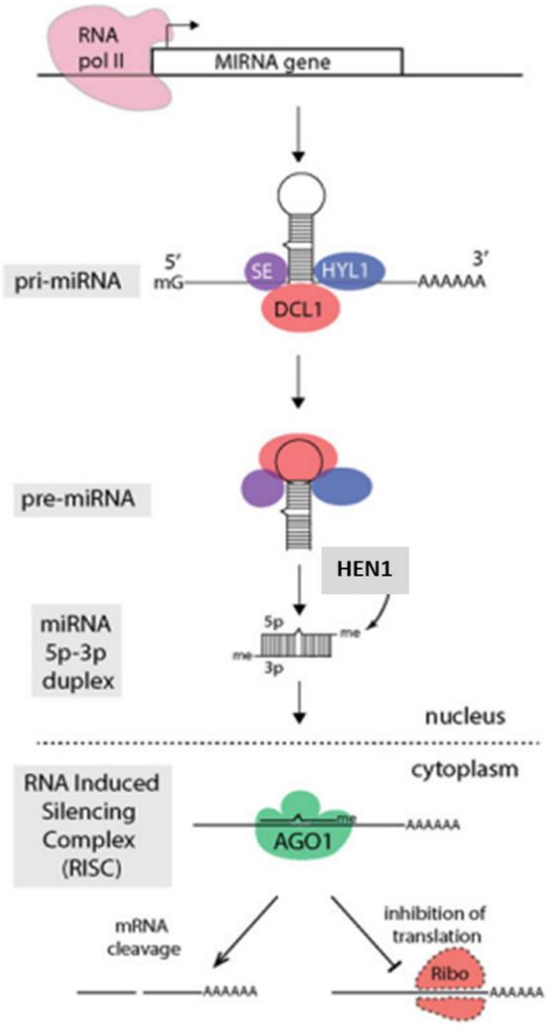


Figure 1. MiRNAs and their functions. Transcription of miRNA genes by mean of RNA polymerase II is shown in pink. This forms a hairpin-shaped structure mRNA consisting of a 5' methylguanylate cap and 3' poly A tail, referred to as pri-miRNA. The 5' and 3' end of this hairpin loop are unpaired and when these are trimmed by DICER-LIKE1 (DCL1) (shown in red), SERPATE (SE) (shown in purple), and HYPONASTIC (HYL1) (shown in blue) a stem-loop pre-miRNA is produced. The next step consists of further trimming by DCL1, SE and HYL1. This result in a 20-40 nucleotide 5p-3p miRNA duplex, which has been termed the miRNA/miRNA* duplex in the past. When methylation of the 3' takes place by means of the ENHANCER 1(HEN1) (shown as cyan), the stability of the structure is improved. Once it has been exported to the cytoplasm, a single strand of this miRNA duplex is loaded onto a protein, AGO1 (shown as green). Thus, the RNA-induce silencing complex (RISC) is formed, into which target mRNAs can be incorporated, depending on their level of complementarity to the miRNA. This may lead to either inhibition of translation, cleavage of the mRNA or both. Figure taken from (de Folter, 2019).

1.4.2 MiRNAs in plant stress response

Stress from drought affects plants in a variety of ways, in terms of their physiology, biochemistry and morphology. Responses to such stresses vary between species, but include toleration, avoidance, escape and recovery strategies which are programmed genetically and subject to complex, synchronised regulation, which is under the control of a variety of non-coding RNA molecules. These develop regulatory networks modulating numbers of different genes to control the aggregate response of the plant (Gelaw and Sanan-Mishra, 2021).

There are two types of non-coding RNA molecules of relevance, small and long. The small non-coding RNA molecules include micro-RNA (miRNA) and small interfering RNA (siRNA) molecules, which are responsible for diverse factors in plant physiology. The long non-coding RNA molecules regulate miRNA by deflecting its actions onto target decoys. Both types operate in intricately complex networks to mediate plant development (Gelaw and Sanan-Mishra, 2021). Indeed, small non-coding RNA can even move between cells, across tissues and along vascular systems, with miRNA regulation of gene expression having a role in plants' long-distance signalling systems so as to maintain nutrient balance (Huen *et al.*, 2017). Although the mechanism of this movement is not understood, evidence from a study of salt stress in soya beans, focusing on a particular miRNA molecule involved in regulating both salt and drought stress, suggests that these miRNA signalling molecules are also likely to be of importance in co-ordinating responses to stress resulting from drought (Gelaw and Sanan-Mishra, 2021). More research is needed into the mechanisms and potential of small non-coding RNA and, in particular, long non-coding RNA for the mitigation of drought stresses (Gelaw and Sanan-Mishra, 2021).

1.4.3 MiRNAs in the regulation of drought stress

As a major impediment to crop productivity, drought as a stress factor has a significant impact on harvests and is expected to become more of a problem as climate change progresses (Kim, Iizumi and Nishimori, 2019). The search for genetic means of increasing crop plant stress resistance has recently begun to focus on the role of micro-RNA (miRNA) as a gene expression regulator. These small, non-coding RNA molecules play an important role in stress conditions, with transcription

factors (TFs) as their key targets, some of which also happen to be key genes for drought response, controlling the expression of many genetic loci which have potential in adaptation to drought (Singroha, Sharma and Sunkur, 2021).

Drought response mediated by miRNA reveals a diversity of physiological, molecular and biochemical processes. In particular, abscisic acid (ABA) is a phytohormone with important functions in the initiation of adaptive responses under conditions of drought stress, and especially the regulation of stomatal conductance, as well as acting in synergy with other phytohormones to use auxin signalling to improve drought tolerance. MiRNA has been shown to regulate these ABA actions, as well as drought resistance strategies which are independent of ABA, so that the manipulation of miRNA has great potential for improving crop tolerance of drought (Singroha, Sharma and Sunkur, 2021).

Where miRNAs are induced by drought stress, they can also be exploited as markers of use in marker-assisted selection (MAS), especially where they are species specific. Plant breeders are likely to continue to find MAS Marker-assisted selection the optimum approach for boosting drought resistance and markers based on miRNA will also be helpful in the mapping of quantitative trait loci (QTL: genome sections coding for particular phenotypic traits) in wheat and other cereals (Singroha, Sharma and Sunkur, 2021).

Whilst the importance of miRNA to gene regulatory networks, and thus to plant growth and stress responses, has been recognised, there is much that still needs to be discovered (Singroha, Sharma and Sunkur, 2021). There has been a concentration on the individual functions of miRNAs in relation to stress tolerance, but the manner in which miRNAs interact has yet to be explored. Further, miRNAs tend to target multiple TFs in the same family, but which may regulate the expression of either separate or overlapping groups of genes. Greater knowledge in these areas will facilitate the efficient concentration of resources on particular miRNA molecules and on their target genes. Other areas, where greater knowledge would be useful for engineering greater resistance to drought stress, include determining which signalling pathways are critical to miRNA regulation in times of stress and understanding how miRNA genes' cis-regulatory elements are characterised, in terms of their corresponding TFs and the regulation of miRNA transcription in situations of drought stress (Singroha, Sharma and Sunkur, 2021).

1.4.4 MiRNA regulation of drought stress in wheat

Significant work has been done in terms of identifying the miRNA molecules involved in resistance to drought stress in key cereal crops: rice, maize, sorghum and barley, as well as wheat. In the case of wheat (*Triticum* spp.), whilst less work has been done to identify the functions of specific miRNA molecules than in maize and rice (Gasparis, Yanushevska and Nadolska-Orczyk, 2017), several studies have identified specific miRNAs conferring drought resistance. Thirteen miRNAs in *T. dicoccoides* show differential expression and are implicated in drought response (Kantar, Lucas and Budak, 2011a). The same number show reversed expression between leaves of drought-tolerant ("Hanxuan10") and drought-susceptible ("Zhengyin1") *T. aestivum* genotypes undergoing dehydration stress (Ma et al., 2015). According to Akdogan *et al.*, (2016), 285 miRNA molecules in wheat leaves and 244 in wheat roots show differential expression in drought stress conditions, with 23 leaf and 26 root examples only expressed at times of drought stress. Chen *et al.*, 2017, note specific miRNA expression changes during drought stress, with "taensmR10" and "tae-miR9654a-3p" beings upregulated and "taecsmR5082-1" and "tae-nsmR5/tae-nsmR6" being downregulated. The miRNA molecule, "TaMIR1119" in *T. aestivum* has been shown to mediate drought tolerance by means of the regulation of the genes which control processes linked to osmotic stress (Shi *et al.*, 2018). In *T. durum*, important miRNAs, related to drought stress responses, have been mapped on each chromosome (Fileccia *et al.*, 2017). This is significant for targeting miRNA for genetic improvement using molecular markers, but the impact of those miRNA molecules which are modulated by drought stresses on their specific target genes is a comparatively unexplored area, requiring more work to investigate miRNA targets in stress situations (Singroha, Sharma and Sunkur, 2021).

The expression and activity of miRNA can be manipulated by such techniques as creating targeted artificial miRNA (amiRNA) molecules, miRNA gene editing mediated by CRISPR/Cas and the use of artificial target mimics (Singroha, Sharma and Sunkur, 2021). AmiRNA strategies, where miRNA sequences are modified by engineered miRNA so as to target particular mRNA (Basso *et al.*, 2019), have shown promise for improving drought tolerance in a number of crops, including wheat, where grain hardiness is improved, although with a loss of protein content (Gasparis, Yanushevska and Nadolska-Orczyk, 2017).

1.5 Rationale and objectives

Wheat is an important staple crop for global food security, providing 20% of all calories consumed by people worldwide. Currently, it is the main food for more than two billion (40%) of the world's population. However, demand for wheat is predicted to increase in the future as the global population increases. With the world's population estimated to reach 9.6 billion by 2050, wheat production will need to be increased by 60% in the coming decades. Wheat is cultivated in over 220 million hectares, covering a wide range of geographic areas and a considerable variety of climatic conditions.

Crop yield is affected by agricultural and environmental variables such as water availability and temperature. There is extensive crop yield variability in many semi-arid regions, which are owed to water limitation and year-to-year fluctuations in climatic conditions.

Drought has been the most important limiting factor for crop productivity and significantly reduces cereal production on a global scale via negative effects on plant growth, physiology, and grain development. The effect of drought on crops is driven by a complex pattern of interactions between genetic, physiological, morphological and ecological factors.

MiRNAs are emerging as key modulators for controlling drought responsive genes expression and, subsequently, the functional proteins involving in positive or negative drought response. Many drought stress-induced miRNAs were identified and proved to play key roles in stress adaption in some model plants such as *Arabidopsis*, *Medicago truncatula* and *Populus trichocarpa*. In wheat, few studies were conducted to identify deferentially expressed miRNAs during drought stress. However, for such an important, diverse and globally widespread crop more studies are required to understand the role of miRNAs in drought response in the different wheat genotypes and under the different agronomic and environmental conditions.

Based on the work of earlier researchers, this thesis examines the following three hypotheses:

Hypothesis1. The expression level of some miRNAs is different in a drought resistant variety of wheat compared to a drought sensitive variety in normal conditions.

Hypothesis2. The expression level of some miRNAs is different in a drought resistant variety of wheat compared to a drought sensitive variety when both types of plant are subjected to drought conditions.
Hypothesis3. The expression level of certain miRNAs changes in response to drought stress.

Therefore, the overall objective of this work is to gain insights on the role of miRNAs in drought stress using two wheat varieties (Yecora Rojo and Pavon76) with different responses to drought.
This objective was achieved through the following steps:
1- Prepare and sequence small RNA libraries from my test conditions.
2- MiRNAs sequence analyses (quality control, mapping to a reference genome, counts and expression level calculation).
3- The read numbers of miRNAs were compared between the two varieties and two treatments to identify differentially expressed miRNAs.
4- Northern Blot technique was applied to validate the results.
5- Target identification for miRNAs were predicted.
6- 5’ RACE technique was applied to validate whether the targets were real.
Therefore, the current study presents an in-depth analysis of the differential expression of miRNAs in response to drought stress in two wheat varieties, which provides vital information to improve our understanding of the effect of drought on wheat and help in minimize the effect of drought on its production.

Chapter 2. Material and methods

2.1 Plant material

2.1.1 Triticum aestivum

Drought-tolerant (Pavon76) and drought susceptible (Yecora Rojo) spring bread wheat (*Triticum aestivum* L.) cultivars were kindly provided by the John Innes Centre (Norwich, UK).

2.1.2 Growth conditions

Wheat seeds were kept in a fridge overnight at a temperature of 4℃. The following day, they were sterilized in 70% ethanol for one minute and subsequently washed with distilled water twice. Seeds were germinated in a petri dish on two layers of filter paper for four days at room temperature. Germinated seeds were transferred to a growing tray with 15 individual cells (26.8cm x 30.7cm), containing Levington Advance (F2) compost (Scotts, Ipswich UK), one seed to each cell. Plants were grown in a controlled environment room (CER) with a 16 hour day and 8 hour night photoperiod. The temperature was 20 ℃ during the light and 12℃ during the period of darkness. The plants were watered twice a week throughout the period of the experiment except in the drought stress treatments where watering was stopped when the plants were 20 days old.

2.2 Drought stress experiment

To determine the effect of drought conditions on sRNA expression in Pavon 76 and the Yecora Rojo 30 plants were used for each variety. The plants were treated as detailed above (in ‘Growth conditions’) until they became 20 days old. At this point, for both varieties, we stopped watering the plants for 10 and 12 successive days in the “water deficit treatments” 10 days and 12 days treatments, respectively. In the control treatments (well-watered treatments) we continued watering the plants normally (as previously described) during the period of 10 days and 12 days. Leaf

and root samples were collected from the plants immediately after the end of the drought application period (10 and 12 days) for both water deficit and well-watered treatments. For all treatments, 1.6 g of tissue was taken separately from leaves and roots, immediately snap frozen in liquid nitrogen in 50 ml Falcon tubes (Corning, Corning, USA) and stored at -80ºC until RNA was extracted. A total of 48 samples were collected from the experiment, which are the results of the combination of 2 wheat varieties (Pavon 76 and the Yecora Rojo) x 2 tissues (leaves and roots) each x 2 conditions (well-watered, water deficit) x 2 periods of application (10 days and 12 days) x 3 replicates.

2.3 RNA Extraction

To extract total RNA from each sample a pestle and mortar and liquid nitrogen were used to grind the sample to powder. The powder was placed into a 50 ml Falcon tube (Corning) containing 16 ml TRIzol® Reagent (AM9738, Thermo Fisher Scientific, Altrincham, UK) and vortexed for 20 seconds. To facilitate the subsequent steps, the solution of tissue/TRIzol mix was split up into ten 2-ml Eppendorf tubes, with each containing 1600 µl. Then 320 µl chloroform was added to each tube, thoroughly shaken, incubated for eight minutes at room temperature, followed by centrifugation at 12,000 g (rcf) and 4ºC for 15 minutes. Approximately 800 µl of the upper aqueous phase from each tube was transferred into a new tube. An equal volume of isopropanol was added to each tube and samples were thoroughly shaken, then stored at -80ºC overnight. The following day, the tubes were removed from the freezer, thawed, and centrifuged at 12,000 g and 4ºC for eight minutes. After that, the supernatant was discarded and the pellet contained the RNA was washed with 80% ethanol and centrifuged again at 7,500 g for three minutes. The ethanol was then discarded carefully without disturbing the pellet. To remove any residual ethanol the tube was then centrifuged again at 7,500 g for two minutes. The pellet was air dried for three minutes to make sure that ethanol residuals were completely removed. Finally, the RNA pellet was dissolved in 10 µl of RNase-free distilled water, then each of the ten tubes with RNA originated from the same sample were amalgamated in one Eppendorf tube with final volume of 100 µl of RNA. A Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific) was used to ascertain the concentration

of RNA in each sample. 1.5 µg of each sample was run on a 1.5% agarose gel to ascertain the quality of the RNA by checking for degradation.

2.4 Small RNA Library Construction and Sequencing Using High Definition Adapters

High Definition (HD) adapters are standard Illumina adapters which have 4 degenerate nucleotides (NNNN) at the ends facing the sRNA. This reduces ligation bias between sRNA and adapter and therefore increases representation of sequences (Xu *et al.*, 2015).

2.4.1 Total RNA clean up

*Mir*Vana™ miRNA isolation kit (AM1560, Ambion, Austin, Texas, USA) was used to clean 20 µg from total RNA (from leaf and root samples). Following the manufacture's protocol, a master mix tube contained 20 µg of the total RNA, 5 volumes of lysis/binding buffer and 1/10th volume of miRNA Homogenate Additive was made for each sample. The master mix was thoroughly mixed and placed on ice for 10 minutes. After 10 minutes, the tube was taken and 1.25 volumes of 100% ethanol were added to the tube, mixed thoroughly, and passed through a *mir*Vana™ filter cartridge by centrifuging for 30 seconds at 10,000 rcf. The flow-through was then discarded. 700 µl of miRNA wash solution 1 was then added and the cartridge centrifuged for 30 seconds at 10,000 rcf. The flow-through was discarded. 500 µl of miRNA Wash Solution 2/3 was added and the cartridge was centrifuged for 30 seconds at 10,000 rcf. The flow-through was discarded and this wash step was repeated a second time. An additional centrifugation for 1 minute at 12,000 rcf was used to ensure that all residual wash buffer was removed. The filter was then transferred to a new collection tube and 50 µl of Elution Solution was added (Elution Solution was pre-heated to 95°C before added to the filter). This was left for 1 minute at room temperature and then the column was centrifuged for 1 minute at 20,000 rcf. The elution was repeated with a second volume of 50 µl of Elution Solution, therefore the final eluted volume was 100 µl. 10 µl of 3M NaOAc (S2889, Sigma-Aldrich,) and 300 µl of 100% ethanol were added to the 100 µl of purified RNA. This was precipitated at -80°C overnight.

The following day, the RNA was pelleted by centrifugation for 20 minutes, at 25,000 rcf. The supernatant was removed from the tube very carefully, as the pellet contained the RNA. The RNA was washed using 1000 µl of 80% ethanol and centrifuged at 10,000 rcf for three minutes. The ethanol was discarded without disturbing the pellet. The centrifuge was repeated for 1 minute at 12,000 rcf to ensure that all ethanol was removed. The pellet was air dried for 3 minutes until it turned clear. 10 µl of distilled water was added to the tube to resuspend the total RNA. A Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific) was used to quantify the total RNA.

2.4.2 3' Adapter adenylation

A master mix tube was prepared for each sample with 2 µl of the phosphorylated DNA oligonucleotide (the 3' HD Adapter; Sigma Aldrich; **Table 1**), 4 µl of 10x 5' DNA adenylation reaction buffer, 4 µl of 1mM ATP, 4 µl of Mth RNA ligase (New England Biolabs), 26 µl of nuclease-free water, making a total of 40 µl. The master mix was then transferred to a PCR tube and incubated at 65°C for one hour. The enzyme was then inactivated by incubation at 85°C for five minutes. The reaction was purified using Oligo clean and Concentrator^{TM} kit (D4061, Zymo, Orange, California, USA). The sample was quantified using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific).

2.4.3 3' Adapter ligation

To prepare an RNA sample for 3' adapter ligation 1.8 µg RNA was mixed with 1.5 µl adenylated 3'-HD adapter (from paragraph 2.4.2) and 5.5 µl of distilled water, incubated at 70°C for 2 minutes, then placed on ice. A 3'adapter ligation master mix was prepared consisting of 2 µl of T4 RNA ligase 10x buffer, 0.75 µl of Recombinant Ribonulease Inhibitor (RNaseOUT) (Thermo Fisher), 1 µl of T4 RNA ligase 2 (New England Biolabs) truncated and 3.5 µl of distilled water, making a total volume of 7.25 µl. 9 µl prepared RNA sample, 4 µl of 50% Polyethylene Glycol (PEG) solution (NEB) and 7 µl of the 3' adapter ligation master mix were mixed and incubated at 26°C for 2 hours. The RNA was then purified using the RNA Clean and Concentrator-5 kit (R1015, Zymo) according to the manufacturer's protocol.

To deadnylate the adapter, 12.1 µl of the cleaned RNA was added to 1.6 µl of 10x deadenylase buffer, 0.8 µl of 100mM DithiothreitoI (DTT) (New England Biolabs), 0.5 µl of RNaseOUT (Thermo Fisher), 1 µl of 5' deadenylase and 1 µl of distilled water. The mixture was incubated at 30°C for 30 minutes, after which 4 µl of 25 mM Ethylenediamine tetraacetic acid (EDTA) was added to stop the reaction. The EDTA was mixed in thoroughly and centrifuged briefly. To remove the excess 3' adapter the following components were added to the sample: 2 µl of 500 mM Tris-HCL pH 9, 7 µl of 50 mM $MgCl_2$, 1 µl of RecJ exonuclease (New England Biolabs, M0264S). The sample was incubated at 37°C for 30 minutes.

2.4.4 5' Adapter ligation

To denature the 5' HD adapter (**Table 1**), 2 µl was heated at 70°C for 2 minutes, after which it was placed on ice. A master mix was prepared using 1 µl of 10x T4 RNA ligase, 1 µl of 10mM Adenosine Triphosphate (ATP), 2 µl of 5' HD adapter and 1 µl of T4 RNA ligase (Thermo Fisher) and then added to the sample, followed by 7 µl of 50% PEG. This produced a total ligation reaction volume of 45 µl. The reaction was incubated for 2 hours at 26°C. 5 µl of distilled water was added to bring the reaction volume to 50 µl. The RNA was then purified using the RNA Clean and Concentrator-5 kit (R1015, Zymo) with the final volume of the eluted RNA being 30 µl.

2.4.5 cDNA synthesis

To synthesise complementary DNA (cDNA) a reverse transcription master mix was added to 30 µl of purified RNA for each sample. The mix contained 4 µl of 10x MMLV Reverse transcription buffer (Lucigen, MM070150), 2 µl 10mM of Deoxyribonucleotides (dNTP), 2 µl of 100mM DithiothreitoI (DTT), 1 µl of RT primer (standard Illumina RNA PCR primer; **Table 1**) and 1 µl of high performance MMLV reverse transcriptase (Sigma). The reaction was incubated at 37°C for 20 minutes, after which the reaction was terminated by heating for 15 minutes at 85°C. The sample was then kept on ice for the purpose of PCR amplification. The total volume at this stage was 40 µl.

Table 1. A list of the oligonucleotides used in the small RNA library construction

Oligonucleotide name	Oligonucleotide sequence (5' to 3')	Intended use
3' HD Adapter	NNNNTGGAATTCTCGGGTGCCAAG	To ligate to the 3' end of the RNA
5' HD Adapter	GUUCAGAGUUCUACAGUCCGACGAUCNNNN	To ligate to the 5' end of the RNA
RT primer	GCCTTGGCACCCGAGAATTCCA	A standard illumina RNA PCR primer for cDNA synthesis

2.4.6 PCR amplification

For each sample, it is usual to run three different PCR programmes. In this case, 12, 14 and 16 cycles were run to ensure that there is adequate amplification of sRNA populations - this is empirical and differs from sample to sample, using 4 µl for each reaction from the 40 µl of cDNA. The following components were added to each PCR : 9.3 µl of nuclease-free water, 0.2 µl Phusion DNA polymerase (Thermo Scientific™, F530L), 0.5 µl of 10 mM dNTP, 4 µl 5x high fidelity Phusion buffer, 1 µl of Illumina forward RP-1 primer (10 µM; AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCG), 1 µl of Illumina reverse primer: (CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCCTTGGC ACCCGAGAATTCCA) as this is the size that is the 5'adapter + sRNAs + 3' adapter containing different index primer 10 µM. The bit I have highlighted is the index. This particular index belongs to index primer 19. It's essentially a barcode which is unique to that primer, so that multiple libraries made with different indexes can be

sequenced at the same time in the same lane and later demultiplexed based on these known but unique sequences (**Table 2**).

The PCR conditions were: 30 seconds at 98°C, then the specified number of cycles of 10 seconds at 98°C, 30 seconds at 55°C, and 15 seconds at 72°C**,** followed by 10 minutes at 72°C. After the PCR, 5 µl was taken from each of the three reactions (12, 14 and 16 cycles) and added to a fourth tube. For each tube 5 µl of 5X loading buffer Novex™ (Invitrogen™, LC6678) was added.

Table 2. A list of the Illumina index primers used for miRNA sequencing from the 48 samples

Index No.	Base sequence	Index No.	Base sequence	Index No.	Base sequence
1	ATCACG	17	GTAGAG	33	CAGGCG
2	CGATGT	18	GTCCGC	34	CATGGC
3	TTAGGC	19	GTGAAA	35	CATTTT
4	TGACCA	20	GTGGCC	36	CCAACA
5	ACAGTG	21	GTTTCG	37	CGGAAT
6	GCCAAT	22	CGTACG	38	CTAGCT
7	CAGATC	23	GAGTGG	39	CTATAC
8	ACTTGA	24	GGTAGC	40	CTCAGA
9	GATCAG	25	ACTGAT	41	GACGAC
10	TAGCTT	26	ATGAGC	42	TAATCG
11	GGCTAC	27	ATTCCT	43	TACAGC
12	CTTGTA	28	CAAAAG	44	TATAAT
13	AGTCAA	29	CAACTA	45	TCATTC
14	AGTTCC	30	CACCGG	46	TCCCGA
15	ATGTCA	31	CACGAT	47	TCGAAG
16	CCGTCC	32	CACTCA	48	TCGGCA

2.4.7 Library size selection

PCR products were separated by polyacrylamide gel electrophoresis (PAGE). 8% polyacrylamide gels were prepared using 10 ml water, 3 ml of 19:1 acrylamide/bis solution, 1.5 ml 5X TBE (Tris-Borate EDTA buffer), 150 µl of 10% ammonium persulphate and 7.5 µl of Tetramethlethlethylenediamine (TEMED). This was poured between 1 mm glass plates (Bio-Rad, 1651824) and a 1 mm comb (Bio-Rad, 1653359) with 10 wells inserted. The glass plates containing the gel were then transferred to Mini-ProteanR Tetra Cell tank (BioRad, 185-8000) and the comb was removed. The first well contained 20 µl of the 12 cycling sample, the second well contained the 20 µl of the 14 cycling sample, and the third well contained the 20 µl of the 16 cycling. The fourth well contained a mix of each of the three previous samples. The fifth well was left free to avoid the ladder mixing with the samples and the sixth well contained the 10 µl of 20 bp DNA ladder. The gel was run at a constant 120V for 2 hours with 0.5 X TBE running buffer. The gel was then stained with 5 ml of 0.5 X TBE buffer and 5 µl of SYBR^{TM} Gold Nucleic Acid Gel Stain (InvitrogenTM, S11494) to facilitate visualization during scanning. The gel was scanned using the Typhoon FLA 9500 (GE Healthcare Life Sciences). Scanning revealed the presence of the required band of 145 bp. This was carefully cut out with a scalpel and placed in a tube. We cut this size as this is the size that is the 5'adapter + sRNAs + 3' adapter. 400 µl of 1X NEB-2 buffer was used for buffering of cDNA after elution from acrylamide slice. The tube was centrifuged at 20,000 rcf for 5 minutes. The cDNA was eluted by shaking the tubes overnight at 4°C to insure the release of total amount of cDNA from the gel. The gel debris was removed from the cDNA by applying it to a Spin-X column (Corning CoStar, Fisher Scientific) and centrifuging for 5 minutes at 2600 rcf, after which the elute contained the cDNA. The cDNA was then further purified by ethanol precipitation. 300 µl of elute from the Spin-X column was transferred into a tube containing 30 µl of sodium acetate (NaOAc), 2 µl of Glycoblue and 975 µl of 10% ethanol and incubated at -80°C for 5 hours. To pellet the cDNA the tube was centrifuged at 20,000 rcf for 20 minutes at 4°C. The pellet was washed with 500 µl of 70% ethanol and centrifuged for 5 minutes at 12,000 rcf, the supernatant was discarded and the pellet dried in the tube with the lid open at 37°C for 5 minutes. The pellet was then resuspended in 15 µl of distilled water.

To ensure that the cDNA samples had been cut accurately a second PAGE gel was run. An 8% PAGE gel was run as described above. The first, second, third and fourth wells contained 3.75 µl each of the cDNA. The fifth well was left free to avoid the ladder mixing with the samples and the sixth well contained the 10 µl of 20 bp DNA ladder. The 145 bp band was cut and the cDNA purified as previously described.

2.4.8 Library normalisation

cDNA concentration was quantified using 8% PAGE gel, run as described in 2.4.7, to enable cDNA library quantities to be normalised. For each sample 1 µl of cDNA was mixed with 2 µl of loading dye and 7 µl of distilled water. ImageQuant (GE Healthcare), pixel counting was used to quantify the amount of library present on gel. These pixel counts were then normalised relative to a sample and concentrations were re-adjusted so that all of the libraries were the same concentration. This is important as the more similar the libraries are in concentration the more even the read distribution will be between them, and therefore the more reliable the results.

2.4.9 sRNA library sequencing

Normalised sRNA libraries were sequenced by Novogene (Cambridge, UK) resulting in Illumina 100 bp single end reads.

2.5 sRNA Northern Blots

2.5.1 RNA size separation by urea polyacrylamide gel electrophoresis

To prepare two 15% polyacrylamide gels, 6.3 g urea (Fisher Scientific, 10142740) powder was added to 3.75 ml sterile water and 1.5 ml of 5X TBE. The mixture was heated in the microwave for 20 seconds until it dissolved. Once cool, 5.5 ml of 19:1 acrylamide/bis solution (Bio-Rad, 1610144) was added, followed by 7.5 µl of TEMED (Sigma-Aldrich, 110-18-9) and 150 µl of 10% of ammonium persulfate solution (APS) (Thermo-Fisher, 17874). The gel was poured between 1 mm glass plates (Bio-Rad, 1651824) and a 1 mm comb (Bio-Rad, 1653359) was then added

and the gel was allowed to set, which took approximately twenty minutes. Once the gel had solidified the comb was removed.

For Northern blot analysis we used RNA from leaf samples of plants subjected to 12 days of water deficit as the effect of drought stress is expected to be clearer at 12 days than 10 days. 10 μg of RNA from leaf samples under well _watered and water deficit at 12 days (from Pavon 76 and Yecora Rojo), 7.5 μl of Ambion gel loading buffer II (Thermo Fisher Scientific, AM8546G) and water in a volume of 3 μl were denatured for 2 minutes at 70°C and kept on ice until loaded into the gel. The urea polyacrylamide gel was run in a Mini-ProteanR Tetra Cell tank (BioRad, 185-8000). To check that the gel was functioning properly, 15 μl of loading buffer was only added to the first well and the gel was pre-run on its own for 15 minutes at 100V. The samples were then loaded and run for 2 hours at 100 V. The gel was then placed in a square plastic dish which was half filled with 0.5X TBE and 5 μl of ethidium bromide (Fisher Scientific, 1239-45-8) for 5 minutes at room temperature with shaking. The gel was then scanned using a Typhoon FLA 9500 (GE Healthcare Life Sciences).

2.5.2 Transfer of RNA to nylon membrane

To transfer the RNA from the 15% urea polyacrylamide gel to the nylon membrane six pieces of Whatman filter paper (ThermoFisher Scientific, 3030-335) and one piece of Hybond-NX membrane (GE Healthcare Life Sciences) per gel were cut to the same size as the gel (9 cm by 7 cm). The Whatman papers and nylon membrane were soaked in 0.5X TBE and layered one at a time as follows: three Whatman papers; the nylon membrane; the urea polyacrylamide gel; a further three Whatman papers were placed on the base plate of the Semidry apparatus (Fisher). This was to allow the RNA to be transferred from the gel to the membrane. After one hour and 15 minutes, the membrane was removed. The membrane was cut in the top left corner to indicate the orientation. A Typhoon FLA 9500 was used to re-image the gel to ensure that the RNA had been successfully transferred to the membrane.

2.5.3 Chemical crosslinking

To improve the detection of sRNA by northern blotting the RNA was crosslinked to the nylon membrane. 10 ml of water, 122.5 µl of 12.5 M 1-methylimidazole (Sigma-Aldrich, M50834) and 10 µl of concentrated HCL (Sigma-Aldrich, H1758) were mixed in a 50 ml falcon tube. A 15 ml falcon tube was filled with 0.373 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermo-Fisher, 22980). The EDC was dissolved in 10 ml of the solution from the 50 ml falcon tube, the solution return to the original 50 ml falcon tube and made up to a total volume of 12 ml with water. A piece of Whattman paper was cut to be just slightly larger than the membrane, placed onto saran wrap and soaked with 5 ml of the crosslinking solution. A tissue was used to dry the excess crosslinking solution which had not been absorbed. The nylon membrane, which contains RNA from the transfer stage, was then placed on the soaked membrane, with the RNA side facing upwards. The saran wrap was carefully sealed and placed in an oven (Thermo) at 60°C for an hour. The membrane was then washed with water on a shaker then wrapped in saran wrap and stored at 4°C. U6 probe (TCATCCTTGCGCAGGGGCCA) was used to ensure that similar amount of U6 RNA (10 µg) was used in all tested samples, thus allowing more precise quantification of the miRNA from the hybridization signal. U6 probe was added to the membrane in the hybridization solution and incubated overnight at 37°C.

2.5.4 Probe preparation

Probe preparation was conducted in a "hot lab" due to the use of a radioactive isotope of phosphous (^{32}P). A master mix was made containing 2 µl of 10 µM DNA oligo probe specific to selected miRNA (Sigma) without 5' phosphate, 2 µl of 10X polynucleotide kinase (PNK) FORWARD buffer, 13 µl of water, 2 µl γ-^{32}P (Perkin Elmer 10 mCi/mL) and 1 µl of T4 polynucleotide kinase (PNK) (New England Biolabs, M0201S), giving a total volume of 20 µl. The mix was incubated for an hour at 37°C in a heating block in the "hot lab" behind a plastic radiation cover.

2.5.5 Membrane hybridisation, washing and imaging

The nylon membrane from the chemical crosslinking step was placed into a 200 ml tube, with the RNA side facing into the tube, and 5 ml of ULTRAhyb^{TM}-Oligo buffer (Thermo-Fisher, AM8663) added. The tube was then placed into a rotating oven (Thermo) at 37°C for 10 minutes. 30 µl of water was added to the 20 µl probe solution (from 2.5.4) to give a total volume of 50 µl. The probe solution was then placed into the tube with the membrane, with care being taken not to touch the membrane during this process. The tube was then sealed and placed into a rotating oven where it was left overnight at 37°C. The following day, the hyb-buffer/probe solution was poured away into the sink in the hot lab, using extreme caution as the solution was radioactive. The membrane was washed by 50 ml with 0.2% saline-sodium citrate (SSC): 0.1% sodium dodecyl sulphate (SDS) at room temperature. The solution was then poured away. The tube was then half refilled with fresh SSC/SDS and the tube was then sealed and placed into a rotating oven for 20 minutes at 37°C. The washing process was repeated and a final fourth wash carried out in the same way as the first wash, using brief manual shaking with fresh wash buffer. The SSC/SDS was then poured away, the membrane removed from the tube using forceps and then placed on saran wrap with the RNA side facing upwards. The wrap was carefully sealed. The wrapped membrane was then placed on a phosphor-image plate and exposed for 10 hours at 4ºC. After exposure, the phospho-image plate was scanned using the Typhoon FLA 9500.

2.5.6 Membrane stripping

The probe was stripped away from the membrane and stored at -20°C for future use. The membrane was placed in a 200 ml tube and filled with 50 ml of 0.1 % SDS then placed in a rotating oven at 85°C for an hour. The tube was then put on a shelf until the SDS had cooled down to room temperature.

2.5.7 Data collection

The number of pixels to be found in each band was quantified using ImageQuant (GE Healthcare) software, once each hybridisation had been imaged. One sample

was chosen as the control against which the other samples would be normalized. The control band intensity was allotted as 1. The intensity for all the other bands was given as a ratio of the normalising sample. The loading control was U6 and its intensity was quantified by the same method using ImageQuant. For each of the membranes the normalizing sample, whose value had been expressed as 1 in the earlier hybridisation, was allocated 1 in the U6 hybridisation. For each band used in the initial hybridization, the normalised intensity value was divided by the normalised U6 intensity value for the same sample. The values identified were shown on each northern blot figure.

2.6 5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

2.6.1 Isolation of mRNA

RACE is a common technique for validation of miRNA targets. This is because miRNA guided endonucleolytic cleavage results in two fragments of an mRNA and the 5'P of the 3' cleavage fragment can be exploited to ligate an adapter. A nested PCR can then be employed to amplify regions with ligated adapters and primers complementary to a sequence of a suspected target. This is a standard technique for target mRNA in plants.

The volume of the 100 μg total RNA (from leaf samples under well-watered at 10 days from Pavon 76 and Yecora Rojo) were adjusted to 100 μl using distilled water. It was heated for 2 minutes at 65 ° C to disrupt any secondary structures, before being placed on ice.

200 μl of oligo dT (Dynabeads, 61002) were calibrated in a tube using binding buffer. The chilled total RNA was added to the Dynabeads and binding buffer and thoroughly mixed by rotating on a roller for 5 minutes at room temperature, enabling the mRNA to anneal to the oligo (dT)25 on the beads. The tube was then placed on a magnet until the solution cleared, after which the supernatant was removed. The tube was then taken off the magnet and the complex of mRNA and beads was washed using 200 μl Washing Buffer B (from Dynabeads kit). The tube was returned to the magnet to ensure that all the supernatant had been removed. The complex was

rewashed using 200 µl of Washing Buffer B. Elution was carried out by adding 10 µl of 10 mM Tris HCl pH 7.5, then heating for 2 minutes at 65°C, after which the tube was immediately placed on the magnet. The eluted mRNA was then transferred to an RNAse-free tube.

2.6.2 5' Adapter Ligation

To ligate the 5' adapter to cleaved mRNAs a reaction containing 200 ng mRNA, 1.25 µl of 200 ng/ µl RNA oligo adapter (GeneRacer™ RNA Oligo; **Table 3**) and water in a total volume of 7.75 µl was set up. This was then incubated for 5 minutes at 65°C before being chilled for 2 minutes on ice. 1 µl 10X ligase buffer, 0.5 µl RNaseOUT and 1 µl T4 RNA ligase were added, mixed thoroughly and the tube contents spun down. The reaction was then incubated for an hour at 37°C. The RNA was then purified using the RNA Clean and Concentrator-5 kit (R1015, Zymo) with the final volume of the RNA eluted in water being 12 µl.

2.6.3 Reverse Transcription

10 µl of the RNA produced in 2.6.2 was mixed with 1 µl of 50 µM GeneRacer Oligo dT primer (**Table 3**), 1 µl of 10 mM dNTP (Thermo Fisher) and 1 µl H_2O and incubated for 5 minutes at 65°C. It was then chilled for 2 minutes on ice. A master mix was then made using 4 µl 5X first strand buffer (Superscript™, 18091050), 1 µl 0.1M DTT (NEB), 1 µl RNAseOUT (Thermo Fisher) and 1 µl Superscript II (Thermo Fisher). This master mix was then added to the mixture made in the first step, mixed well and spun down and then incubated for 10 minutes at 55°C, deactivated at 80°C for 10 minutes and chilled on ice for 2 minutes.

2.6.4 Touch down PCR

Two tubes were prepared. The first tube contained 1 µl of RT Template and 1.5 µl of 10 µM Reverse gene-specific DNA primer (GSP). While the second tube contained the following components: 4.5 µl of 10 µM GeneRacer™ 5' Primer (**Table 3**), 10 µl of 5X Phusion HF buffer (ThermoFisher Scientific, F-530,XL), 1.5 µl of 10 mM dNTP Solution (Thermo Fisher) and 0.5 µl of Phusion DNA polymerase (Thermo

Fisher) and 31 µl of sterile H_20. This brought the total volume of the second tube to 47.5 µl. The contents of the two tubes were then mixed together in a fresh PCR tube. The PCR conditions were: 2 minutes at 98°C, then 5 cycles of 30 seconds at 98°C, 40 seconds at 73°C, and 5 cycles of 30 seconds at 98°C, 40 seconds at 71°C, then 25 cycles of 30 seconds at 98°C, 30 seconds at 69°C and 40 seconds at 69°C, followed by 10 minutes at 68°C.

2.6.5 Nested PCR

The nested PCR was also made in two separate tubes. The first contained 1 µl of 10µM GeneRacer™5' Nested (**Table 3**), 1 µl dNTP Solution (Thermo Fisher), 10 µl 5x Phusion HF buffer (ThermoFisher Scientific, F-530,XL), 0.5 µl Phusion DNA polymerase (Thermo Fisher) and 34.5 µl H_20. The second tube contained 1 µl Reverse Nested GSP and 2 µl Initial PCR (from step 2.6.4). The two tubes were then mixed together in a fresh PCR tube and placed in the PCR machine. The PCR conditions were: 2 minutes at 98°C, then 15 cycles of 30 seconds at 98°C, 30 seconds at 70°C, and 2 minutes at 70°C, followed by 10 minutes at 68°C.

Table 3. List of the primers used in 5'RACE PCR

Primer name	**Primer sequence** (5' to 3')
GeneRacer™RNA Oligo	CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA
GeneRacer Oligo dT	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$
GeneRacer 5' Primer	CGACTGGAGCACGAGGACACTGA
GeneRacer 5' Nested	GGACACTGACATGGACTGAAGGAGTA

2.6.6 Gel purification of PCR product

25 µl of the nested PCR product (from step 2.6.5) was run on a 1.5% agarose gel with a 100 bp ladder (6x bp ladder, SM0241). The required band were then cut from

the agarose gel (between 350 bp and 450 bp), weighed and the DNA purified using a Zymoclean gel DNA recovery kit. The DNA was eluted using 6 µl H_20.

2.6.7 Cloning with pJET vector

The Blunt End cloning reaction was made using the following components: 5 µl of 2X Reaction Buffer, 1 µl of purified PCR product, 0.5 µl of pJET 1.2/blunt Cloning Vector, 3 µl of nuclease-free H_20 and 0.5 µl of T4 DNA Ligase; and incubated at room temperature for 5 minutes. The reaction was then placed on ice for 5 minutes. 5 µl of the reaction was added to the component DH5 alpha *E.coli* cells and incubated for 30 minutes on ice. *E.coli* cells were then heated at 42 °C to heat shock the bacteria, before returning to ice for 2 minutes. 300 µl of Lysogeny broth (LB media) was then added and the bacteria incubated while being shaken for 1.5 hours at 37°C and 180 rpm. Subsequently, the 100 µl bacteria were plated out on ampicillin (100 µg/ml) LB agar plates, ensuring aseptic conditions, and incubated at 37°C overnight. Subsequently, ten colonies were selected for each miRNA-candidate target pair and overnight cultures were started in disposable tubes with 5 ml LB and ampicillin (100 µg/ml). These were incubated overnight at 37°C and 180 rpm.

The following day plasmid was extracted from each culture using QIAprep Spin Miniprep Kit, (Qiagen, USA) as per manufacturer's instructions, it was determined that 10 colonies contained inserts. 2 µl of the corresponding nested PCR primer was then mixed to 15 µl of extracted plasmid DNA in a fresh tube and sent to Eurofins Scientific (Luxembourg) for sequencing.

2.6.8 Clone sequence analysis

Sanger sequencing results were aligned against the expected cleavage products for each miRNA target using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Sequences which aligned perfectly were taken as confirmation of cleavage.

Chapter 3. Generating small RNA libraries for next generation sequencing

3.1 Experimental design and plant material collection

Our experiment involves two hexaploid spring wheat varieties: Pavon 76 and Yecora Rojo. Pavon 76 is a spring wheat variety developed by the National Institute of Forestry, Agriculture and Livestock Research (INIFAP) and the Mexican International Wheat and Maize Improvement Centre (CIMMYT) in Mexico and was first released in 1976. It is characterized to be tolerant to drought and rust diseases and has a crop duration of 112-117 days (https://wheat.pw.usda.gov).

Yecora Rojo is a spring wheat variety that was developed by CIMMYT in Cooperation with the Mexican Ministry of Agriculture in Mexico. It derives from a single red-grained plant selected from the cross (Ci and X Sonora 64 - Klein Rendidor) x 8156. This variety was first introduced to Saudi Arabia in 1981 being a high-yielding, short-statured variety with a short crop duration (90-100 days). However, it is vulnerable to environmental changes such as temperature, which is particularly significant in the phase of the swelling grain. The temperature rises at this time give more rapid maturation, making grain filling quicker and decreasing kernel weight, so cutting grain yield (Mason *et al.*, 2010). This is one of the reasons why we chose this variety (being drought susceptible) together with Pavon 76 (drought tolerant) to be the material of this study.

Our experiment consisted of 16 treatments as a result of the different combinations of four factors (variety, watering regime, time period and collected tissue). Each treatment had three biological replicates as shown in (**Table 4**).

Table 4. The experimental design showing number of treatments, replicates and sample IDs. WW is well-watered; WD is water deficit

Variety 1	**Pavon76**	**Pavon76**	**Pavon76**	**Pavon76**	**Pavon76**	**Pavon76**	**Pavon76**	**Pavon76**
Watering regime	WW	WW	WD	WD	WW	WW	WD	WD
Time period	10 days	12 days	10 days	12 days	10 days	12 days	10 days	12 days
Tissue	Leaf	Leaf	Leaf	Leaf	Root	Root	Root	Root
Sample ID (R)	1, 2 ,3	4, 5, 6	7, 8, 9	10, 11, 12	31, 32, 33	34, 35, 36	37, 38, 39	28, 29, 30
Variety 2	**Yecora Rojo**	**Yecora Rojo**	**Yecora Rojo**	**Yecora Rojo**	**Yecora Rojo**	**Yecora Rojo**	**Yecora Rojo**	**Yecora Rojo**
Watering regime	WW	WW	WD	WD	WW	WW	WD	WD
Time period	10 days	12 days	10 days	12 days	10 days	12 days	10 days	12 days
Tissue	Leaf	Leaf	Leaf	Leaf	Root	Root	Root	Root
Sample ID (R)	13, 14, 15	16, 17, 18	19, 20, 21	22, 23, 24	40, 41, 42	43, 44, 45	46, 47, 48	25, 26, 27

A total of 48 samples were collected, where half of these (24 samples) were from leaf tissue and the other half consisted of root tissue. All samples were stored at -80°C until RNA extraction.

3.2 Assessment of quality and integrity of the extracted RNA

3.*2.1* RNA Quality

Total RNA was isolated from leaf and root samples from the two wheat varieties using TRIzol® reagent, chloroform and liquid nitrogen. Then, MirVana miRNA kit was uesd to clean 20 µg from the total RNA per sample and RNA was quantified using a Nanodrop 8000 spectrophotometer. A good amount of total RNA was obtained from 1.6 g of the tissue (leaves and roots), as the mean yield of RNA per sample ranged between 400 µg and 410 µg. The A260/A280 ratio varied among samples and was 2.09 on average for leaf samples (**Figure 2A)**, whiles the root sample, the A260/A280 ratio varied among samples and was 2.34 on average (**Figure 2B**).

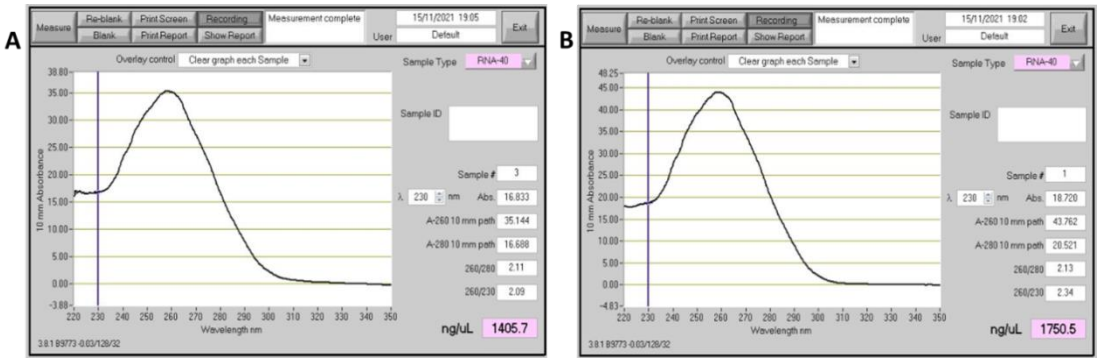


Figure 2. An example of the results obtained by Nanodrop 8000 spectrophotometer for 1 µl of total RNA. **(A)** shows an example for wheat leaves RNA sample. **(B)** shows an example for wheat roots RNA sample.

3. 2.2 RNA integrity

Total RNA integrity for all samples was tested using agarose gel electrophoresis to ensure that the RNA had not been degraded during the extraction and purification procedures. After separating 1.5 µg of each sample, the fragments of 28S, 18S and 5S rRNA are visible as clear, defined bands for the wheat leaves (**Figure 3A**) and roots (**Figure 3B**) on a 1.5% agarose gel. This demonstrates that the RNA had not been degraded and was therefore suitable for generating small RNA libraries.

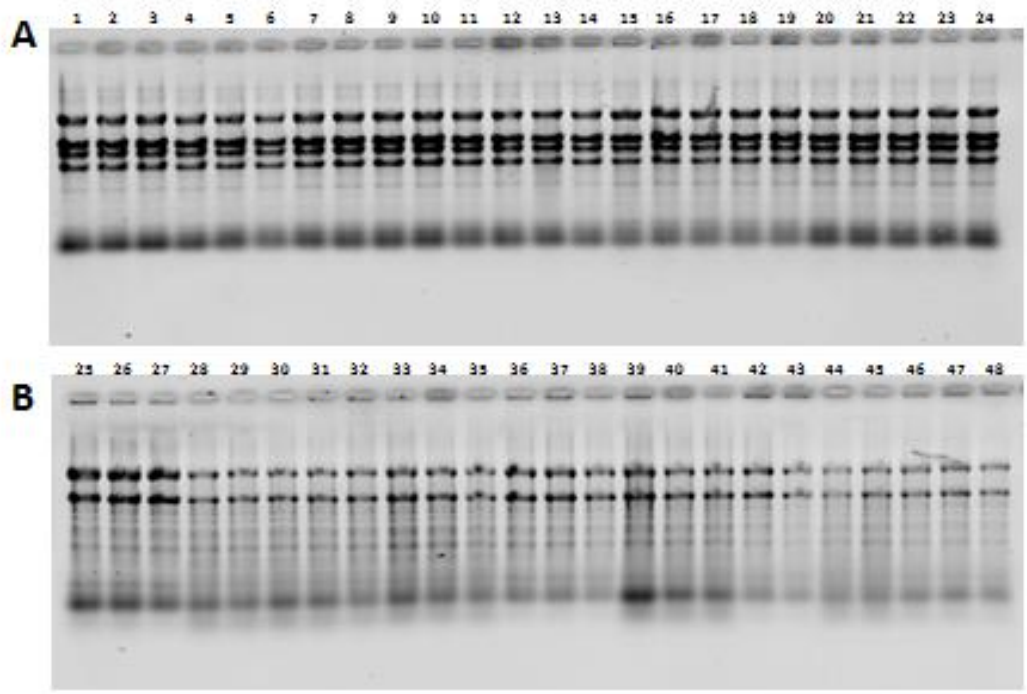


Figure 3. RNA integrity of the 48 samples from wheat leaves and roots is shown on a 1.5% agarose gel. (**A**) The total RNA extracted from 24 wheat leaf samples. (**B**) The total RNA extracted from 24 wheat root samples. The gel was allowed to run at 110V for forty-five minutes and then stained by ethidium-bromide.

3.3 Generating cDNA libraries for sRNAs using high-definition adapters

3'adaptor adenylation was carried out and 16% Urea-PAGE gel was used to check the efficiency of the adenylation as shown in **(Figure 4)**. Non-adenylated 3' adapters were used as markers. The double bands indicate that the 3'adaptors are adenylated compared to the single bands of the non-adenylated 3'adaptors.

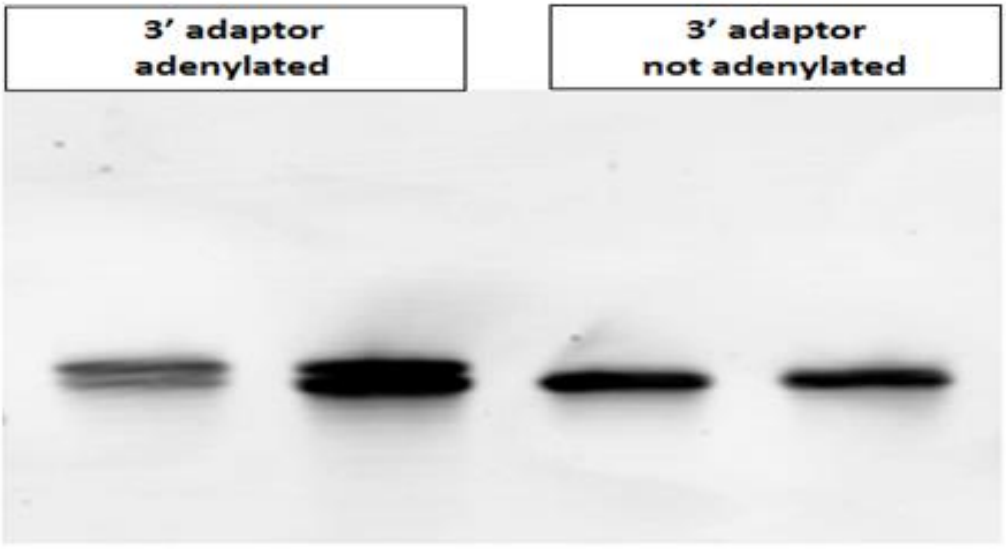


Figure 4. Checking the efficiency of the adenylation of 3' adapters using urea-PAGE gel. The first and second lanes are the adenylated 3' adapter samples, and the third and fourth lanes are the non-adenylated 3' adapter samples. The gel was allowed to run at 110V for 1 hour.

Once the efficiency of the adenylation had been confirmed, the 3' and 5' adapter were prepared. Cleaning of the reaction was achieved using an RNA clean and concentrator kit™ (Zymo Research). First the 3' adenylated adapter was ligated to the extracted total RNA samples and then the 5' adapter was ligated to the product of the first ligation reaction. The next step was to prepare the cDNA synthesis followed by three different cycles of PCR amplification. The cDNA was primed by an oligonucleotide reverse complementary to the 3' adapter. The PCR reaction used the same reverse primer and a forward primer identical to the 5' adapter. Three different PCR programmes were normally run for each sample. This was to ascertain the optimum cycle for each sample. In the current study, 12, 14 and 16 cycles were run and 8% PAGE gel was prepared, after which each lane was loaded with 20 µl PCR product. Staining the gels using Sybr Gold to reveal the required bands of 145 bp (**Figure 5A**).

The required band of 145bp was cut out and placed in a tube. The gel was then re-imaged to ascertain that the samples had been accurately cut out (**Figure 5B**). The cDNA was eluted from the cut-out gel pieces and precipitated at -80°C for 5 hours. A

second PAGE gel was run to check that the cutting of the cDNA samples was accurate and that no other bands were present (**Figure 5C**).

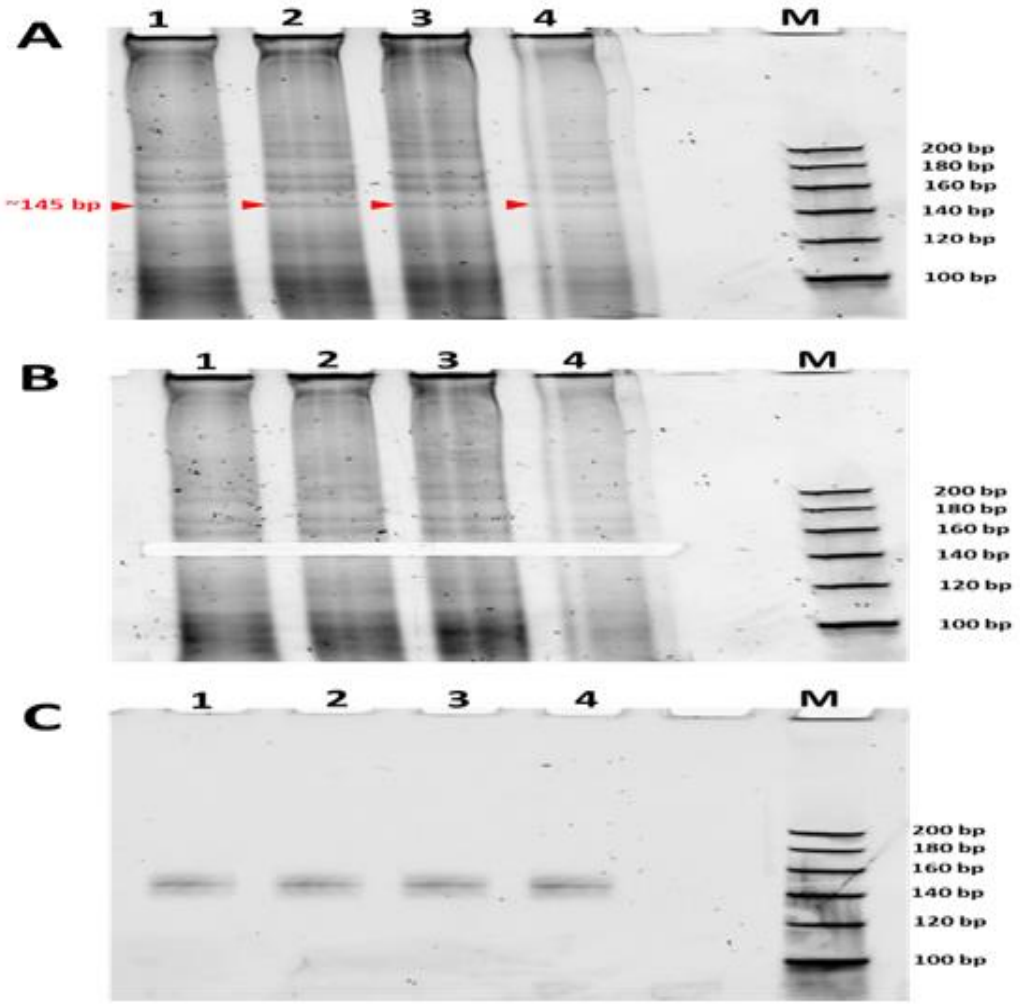


Figure 5. PAGE gels prepared during the generation of cDNA libraries for sRNAs using high-definition adapters. (**A**) PAGE gel shows the efficiency of cDNA synthesis after different thermo-cycles. Red arrows indicate the cDNA bands with *ca.* 145 bp in size, which is required for this experiment. (**B**) PAGE gel shows the gel after cutting out the cDNA bands with the size *ca.* 145 bp. (**C**) PAGE gel shows the cDNA bands with the size *ca.* 145 bp after it had been extracted from the cut slices of polyacrylamide gel. In **A**, **B** and **C**, lanes 1, 2 and 3 represent cDNA obtained from 12, 14 and 16 thermo cycles. Lane 4 is the mix of 5 µl from each of the three cDNA. Four microliters from each cDNA sample were loaded on 8% PAGE gel. Lane M contains a 20 bp ladder. The gel was allowed to run at 120V for two hours.

The entire procedure described here was for a single sample, therefore all steps were repeated 48 times, to generate cDNA libraries for all samples. It was important that each sample would be easily identifiable by the sequencing company, therefore a

different Illumina reverse index primer was used during the final PCR step to distinguish them from each other. Having prepared all the libraries, cDNA quantification was conducted, once again using 8% PAGE gel. Altogether, 48 tubes were prepared (i.e. one for each sample). The concentration of each sample was checked using ImageQuant Analysis software, and adjustments were made as required (**Figure 6**). The 48 samples were divided into four tubes each tube contained 12 samples with different index primes and was sent to Novogene (Cambridge, UK), where they were sequenced using NovaSeq 6000 system - Illumina, next-generation sequencing (NGS).

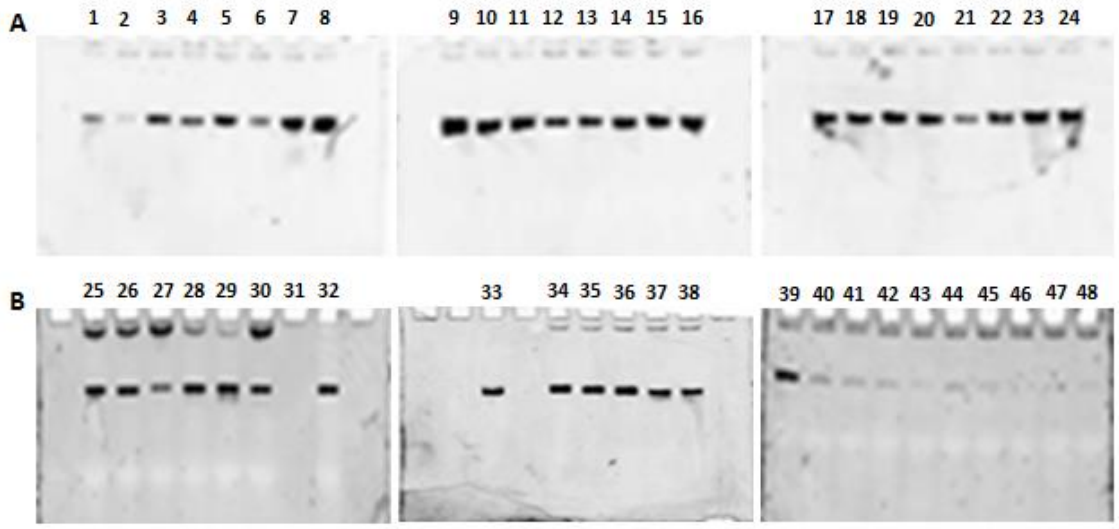


Figure 6. cDNA quantification of the 48 samples from wheat leaves and roots as shown in 8% PAGE gel. (A) shows the quantity of cDNA for 24 samples from wheat leaves. Lane 1, 2 and 3 are Pavon 76 leaves under well-watered conditions at 10 days. Lane 4, 5 and 6 are Pavon 76 leaves under water deficit at 10 days. Lane 7, 8 and 9 are Yecora Rojo leaves under well-watered conditions at 10 days. Lane 10, 11 and 12 are Yecora Rojo leaves under water deficit at 10 days. Lane 13, 14 and 15 are Pavon 76 leaves under well-watered conditions at 12 days. Lane 16, 17 and 18 are Pavon 76 leaves under water deficit at 12 days. Lane 19, 20 and 21 are Yecora Rojo leaves under well-watered conditions at 12 days. Lane 22, 23 and 24 are Yecora Rojo leaves under water deficit at 12 days. **(B)** shows the quantity of cDNA for 24 samples from wheat roots. Lane 25, 26 and 27 are Pavon 76 roots under well-watered at 10 days. Lane 28, 29 and 30 are Pavon 76 roots under water deficit at 10 days. Lane 31, 32 and 33 are Yecora Rojo roots under well-watered conditions at 10 days. Lane 34, 35 and 36 are Yecora Rojo roots under water deficit at 10 days. Lane 37, 38 and 39 are Pavon 76 roots under well-watered conditions at 12 days. Lane 40, 41 and 42 are Pavon 76 roots under water deficit at 12 days. Lane 43, 44 and 45 are Yecora Rojo roots under well-watered conditions at 12 days. Lane 46, 47 and 48 are Yecora Rojo roots under water deficit at 12 days. When all samples were amalgamated to be sent for sequencing, the concentration of each was adjusted to match that of lane 32. The low-density bands were due to the low RNA concentration in some cDNA samples. For these samples, I added the total volume (15 µl) of the synthesised cDNA to maximise the quantity in each tubes. The gels were allowed to run at 110V for one hour.

3.4 Discussion

Small RNAs (sRNAs) consist of approximately 21 to 24 nucleotides and are part of the machinery that helps to silence genes in eukaryotes. Among the two major groups of small RNAs, short interfering (siRNAs) and microRNAs (miRNAs), miRNAs have been well elucidated as being involved in post-transcriptional repression which allows regulation of expression of certain genes. Both these groups of RNAs are very important because they play a major role in controlling developmental timing in plants and animals and even regulate cell proliferation and death along with hematopoietic stem cell differentiation in humans (Lewis *et al.*, 2003). Small RNA libraries are made to investigate the intricate complexities of these molecules and to sequence them for quantification in both plant and animal systems (Lu *et al.*, 2005). After the library has been prepared they can be sequenced by a variety of methods, although these days the Illumina platform is used exclusively. Even though it has been observed that methods used for digital gene expression (DGE) profiling of small RNAs are biased towards certain small RNAs, thereby giving a wrong quantification, this is independent of the sequencing method adopted (Lee, Hormozdiari and Alkan, 2009). Next generation sequencing is the most common method of sRNA sequencing at present because it broadens scope for quantification and allows discovery of new sRNAs. Before sRNA libraries are sequenced, library construction of sRNAs includes numerous steps (Xu *et al.*, 2015). The main point of concern is adapter ligation to the 3'end of sRNAs. The ligation efficiency of sRNA to an adapter is higher if they can anneal to each other compared to sRNAs that cannot anneal to the adapter because in the latter case the chance of an sRNA getting close to the adapter is lower than if they can anneal to each other (Hafner *et al.*, 2011)**.** The steps for sRNA library construction start after total RNA extraction and consequent purification of the sRNA fraction. At present high definition (HD) adapters are synthesised with four degenerate nucleotides at the ligating ends of common commercial adapters such as Illumina (Sorefan *et al.*, 2012). After preparation of 5' and 3' HD adapters, sRNAs are ligated at the 3'end using truncated T4 RNA ligase 2 followed by cleaning and elution in water. Then the remaining 3' adapters are deadenylated and degraded to avoid formation of adapter-adapter products later. The 5' adapter is then ligated to sRNA, and after the sRNAs were ligated to both HD adapters, reverse transcription is carried out to generate

cDNAs corresponding to the sRNA. These cDNA molecules are then PCR amplified and the products run on an 8% polyacrylamide gel (PAGE). After separation by PAGE, bands are excised out from the gel, the DNA is purified, measured and finally sent off for sequencing (Xu *et al.*, 2015). The analysis of the sequencing result is carried out using genome information of the species (from which RNA was isolated) and other related annotations. After trimming of the 3' adapter sequence (by a software that is part of the UEA sRNA workbench), the sequence reads obtained are mapped to the reference genome (Stocks *et al.*, 2012). There are many types of software that are able to identify conserved miRNAs (e.g. miRProf), locations of their hairpin and secondary structures (e.g. ViennaRNA package) thus giving us a comprehensive picture of the entire sRNA profile (Package *et al.*, 2011).

Chapter 4. Identifying differentially expressed miRNAs during drought stress in wheat

4.1 Analyses of sequencing results

All bioinformatics analysis were performed by Rachel Rusholme Pilcher (Earlham Institute, Norwich). The first step was to check the quality of the sequences by using the FastQC (Fast Quality Control) software (Andrews, 2015). The second step used Cutadapt software (Martin, 2011) to remove the 3' adapter to leave only the small RNA sequences. The third step used Burrows Wheeler Aligner (BWA.aln) software (Li and Durbin, 2010) to align the short RNA sequences to the whole genome of wheat (Appels *et al.*, 2018). Subsequently, Shortstack software (Johnson *et al.*, 2016) was used to identify groups of mapped reads and filter the reads to miRNA, het-siRNA and phasiRNA as shown in (**Figure 7**). Then, the number of counts was calculated for each mapped read (considering the positions of the reads on the wheat chromosomes). These counts were used to identify differentially expressed miRNA in the subsequent analysis.

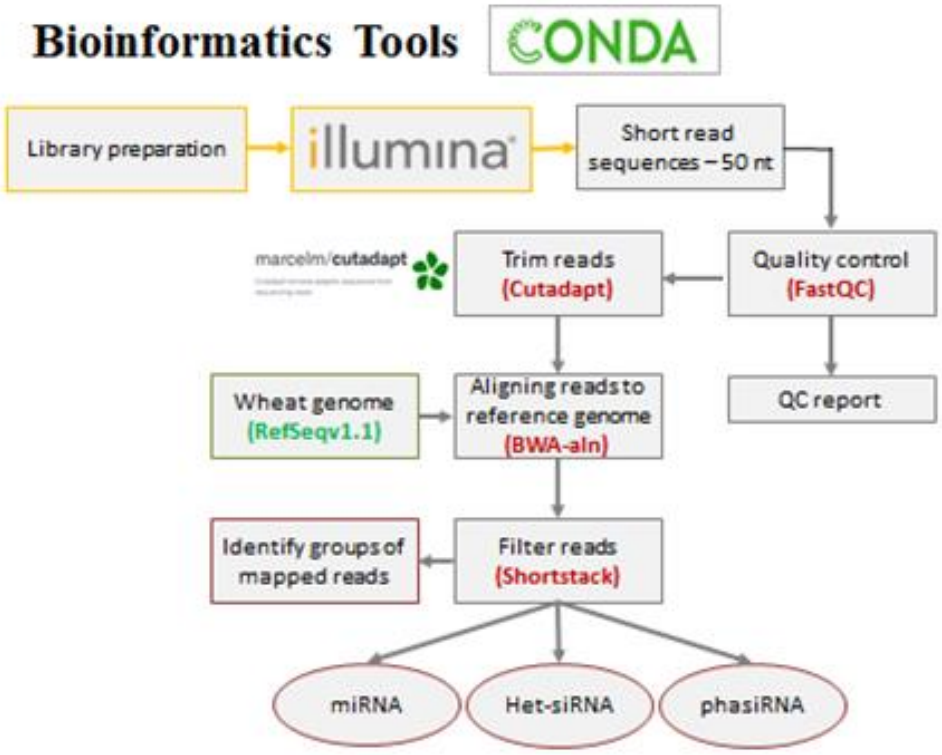


Figure 7. The bioinformatics tools used to analysis the sequencing data.

4.2 Quality control of the Illumina sequences

Four different libraries were constructed, each of which contained 12 samples with different index primers. The number of reads varied considerably among the different samples ranging from 257,498 to 73,364,201 (**Figure 8**). In leaf samples the mean number of reads per biological replicate of the same treatments ranged between 5.88 ± 9.07 million (in Yecora Rojo samples under water deficit at 10 days) and 11.29 ± 1.20 million (Pavon 76 samples under water deficit at 10 days), while, in root samples the minimum mean number of reads per biological replicate of the same treatments was 1.54 ± 1.12 million (Pavon 76 samples under water deficit at 12 days) and the maximum mean number of reads was 37.01 ± 34.24 million in Yecora Rojo samples under water deficit at 10 days (**Table 5**). Further information about each sample's number of reads and quality control outcomes can be found in Appendix 1.

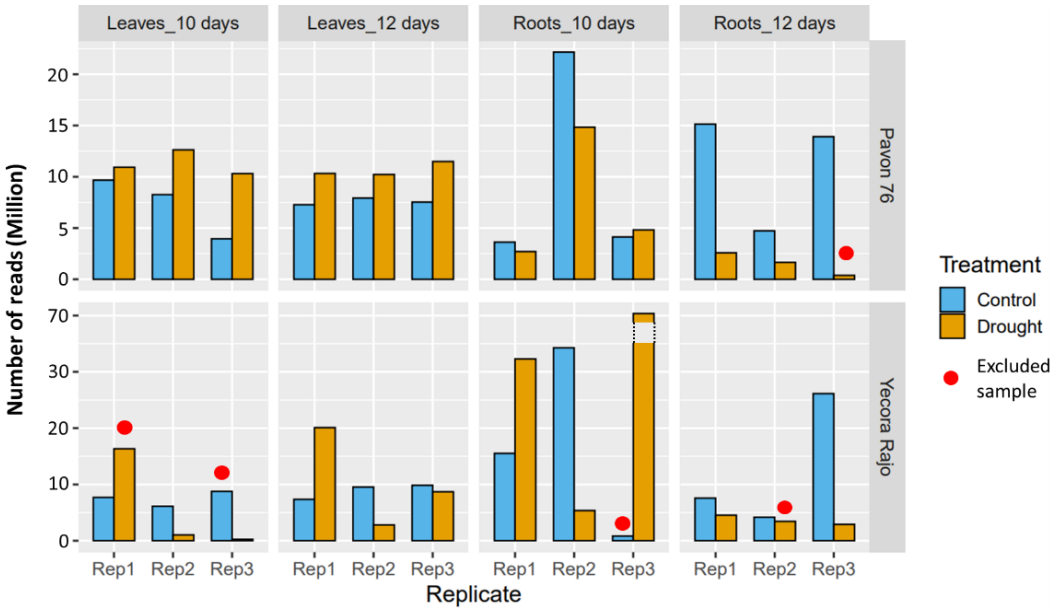


Figure 8. Number of sequencing reads for the 48 samples. The red dots refer to the samples excluded from the differential expression analysis. Five samples were excluded because they have a low sequence quality and showed outlier according to the MA plot analysis.

Table 5. Mean number of sequencing reads for the different treatments in the experiment.

Variety	Treatment	N	Number of reads (Million)	
			Leaves	Roots
Pavon76	Control _ 10 days	3	7.30 ± 2.98	9.98 ± 10.56
	Control _ 12 days	3	7.58 ± 0.32	11.27 ± 5.69
	Drought _ 10 days	3	11.29 ± 1.20	7.45 ± 6.48
	Drought _ 12 days	3	10.68 ± 0.71	1.54 ± 1.12
Yecora Rojo	Control _ 10 days	3	7.53 ± 1.33	16.87 ± 16.75
	Control _ 12 days	3	8.91 ± 1.36	12.63 ± 11.82
	Drought _ 10 days	3	5.88 ± 9.07	37.01 ± 34.24
	Drought _ 12 days	3	10.54 ± 8.79	3.68 ± 0.83

Numbers are mean ± Standard deviation (SD). N refers to number of biological replicates.

4.3 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) tool was used to compare the replicates to each other within one treatment and also to other treatments. The largest difference was between all leaf samples compared to all root samples, indicating that the small RNA content of the two tissues are very different, regardless of the watering conditions and time points (**Figure 9**).

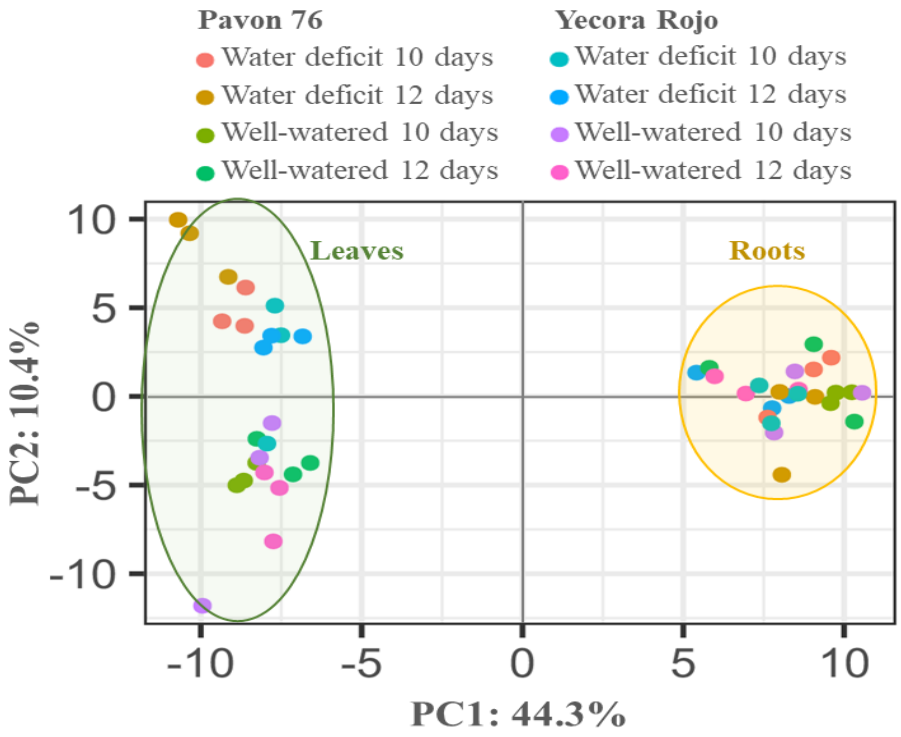


Figure 9. PCA plotting of all samples showing their clustering based on the tissue type for each variety. Leaf samples were separated clearly from roots regardless of the variety, watering regime and time point. For leaf samples, all water deficit samples from Pavon 76 and Yecora Rojo clustered together and clearly separated from the well-watered samples of the two varieties.

PCA was also conducted on samples from leaf tissue. The results showed a clear clustering among the samples from both varieties based on drought conditions, as the samples under drought stress were separated from those under well-watered conditions**.** Interestingly, Pavon 76 and Yecora Rojo samples under drought stress were clustered in two distinguished groups, while in the well-watered treatments; there was no clear separation between samples based on the variety. Samples under drought stress were separated from each other according to the timing of drought application (**Figure 10**).

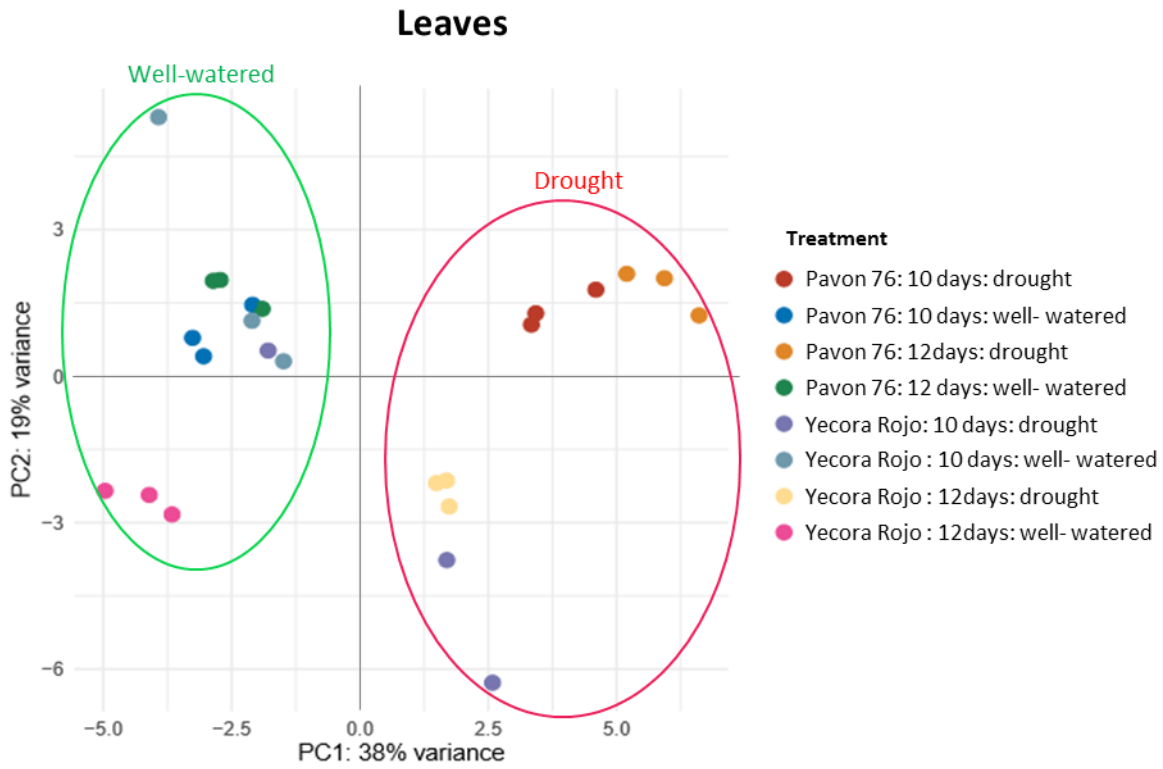


Figure 10. PCA plotting of leaf sample showing their clustering based on treatment. Samples under well-watered conditions were clearly separated from those under drought conditions. Pavon 76 and Yecora Rojo samples under drought stress were clustered in two distinguished groups, while in the well-watered treatments; there were no clear separation between samples based on the variety. Furthermore, samples taken at different time points during drought stress are also separated from each other for both varieties.

PCA was also applied on root samples; however, no clear separation of the samples was obtained in any of the comparisons as shown in (**Figure 11**), where the plotted samples were coloured based on the conditions applied variety, timing of treatment and the treatments themselves.

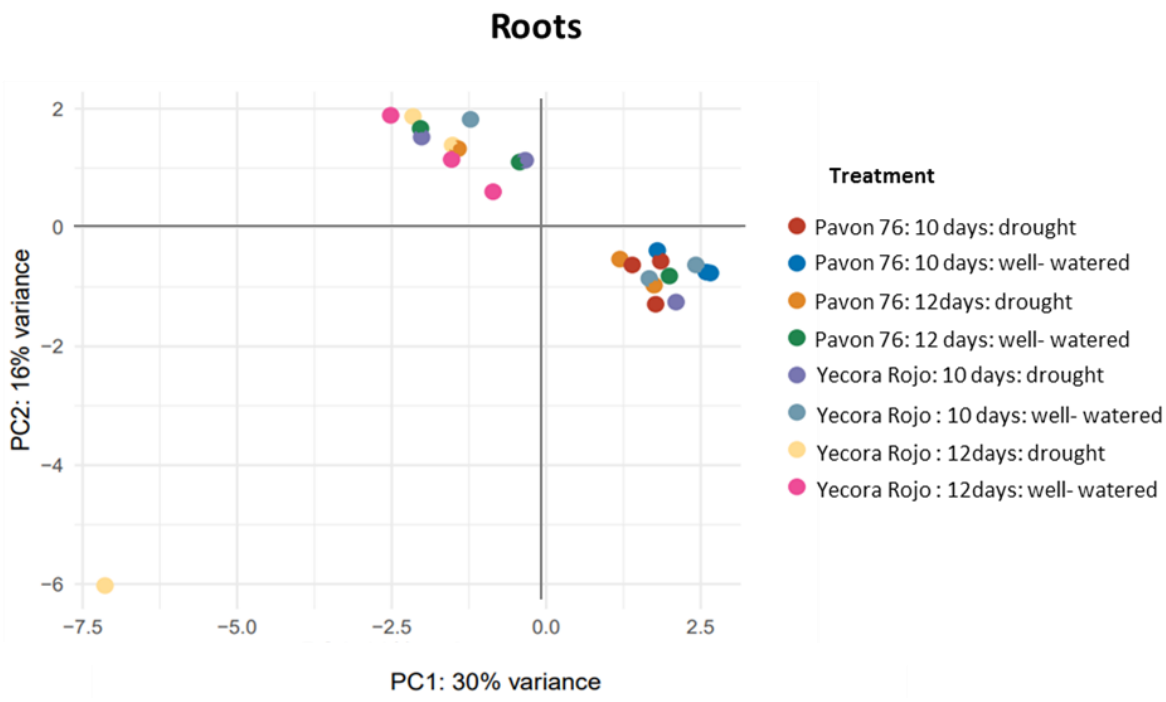


Figure 11. PCA plotting of root samples based on the stress conditions, variety, timing of stress application and the combinations of the different treatments. No clear separation of the samples was obtained in any of the comparisons.

In our study, the application of PCA on the samples demonstrated that leaf samples are very different in sRNA profile from the root samples. Furthermore, this analysis showed that the well-watered leaf samples were clearly clustered in a separate group from the leaf samples under drought conditions. Most importantly, PCA gave us a clear evidence of the differential effect of drought stress on the two varieties under study, considering that one of those varieties (Pavon76) is known to be tolerant to drought stress and the other one (Yecora Rojo) is more sensitive to drought. Thus, the outcomes of PCA analysis support our initial hypotheses, which suggested that the expression level of some miRNAs is different in a drought resistant variety of wheat compared to a drought sensitive variety in normal and drought conditions.

PCA analysis was performed on root samples as well, but there was no clear separation observed among the samples in any of the comparisons. This suggests that the effect of drought stress on root tissues is less pronounced compared to leaf tissues, as the number of differentially expressed miRNAs in root samples was considerably lower. The quality of the extracted RNAs from the roots was found to

be poor, which can provide a possible explanation of these findings. However, this difference in the number of differentially expressed genes between roots and leaves could also be attributed to the distinct histological (e.g., chlorophyll and stomata) and physiological aspects, as well as the different surrounding environment of these two organs, which lead to varying levels of exposure and interaction with drought stress.

4.4 Samples excluded from the miRNA analyses

In theory, the three biological replicates of the same treatment should be very similar to each other. Accordingly, MA plot was used to visualize the pairwise replicate-replicate variation by transforming the data onto M (log ratio) and A (mean average) scales, then plotting these values. MA plotting was done for the replicates of each of the 16 treatments in this study. The samples that were very different from the other replicates of the same treatment were excluded because these samples showed a significant deviation in their expression levels compared to the corresponding replicates. The deviation could be caused by technical errors, such as sample contamination or biological variations. These outlier samples were excluded from the analysis to avoid misleading or inaccurate results in the MA plot analysis.

As a result, five samples (2 leaves and 3 roots samples) that also have low sequence quality were excluded from the analysis (**Table 6)**. **Figure 12** shows MA plots that visualize the pairwise replicate-replicate variation in gene expression levels, which used to represent log fold-change versus mean expression between two samples. This is visually displayed as a scatter plot with base-2 log fold-change along the y-axis (M-value) and normalized mean expression along the x-axis (A-value). The M-value on the y-axis represents the log fold-change of the gene expression levels between the two replicates, with positive values indicating higher expression in the first replicate and negative values indicating higher expression in the second replicate. In **Figure 12A**, the two replicates are very similar to each other, as shown by the tight clustering of points around the horizontal axis (A-value). The majority of genes have M-values close to zero, indicating no or very little difference in expression levels between the two replicates. In **Figure 12B**, the two replicates are quite different from each other, as shown by the spread of points across the y-axis (M-value). The genes with high M-values represent those that are differentially expressed between the two replicates. More detailed information about the MA plots for all replicates in this study can be found in can be found in Appendix 2.

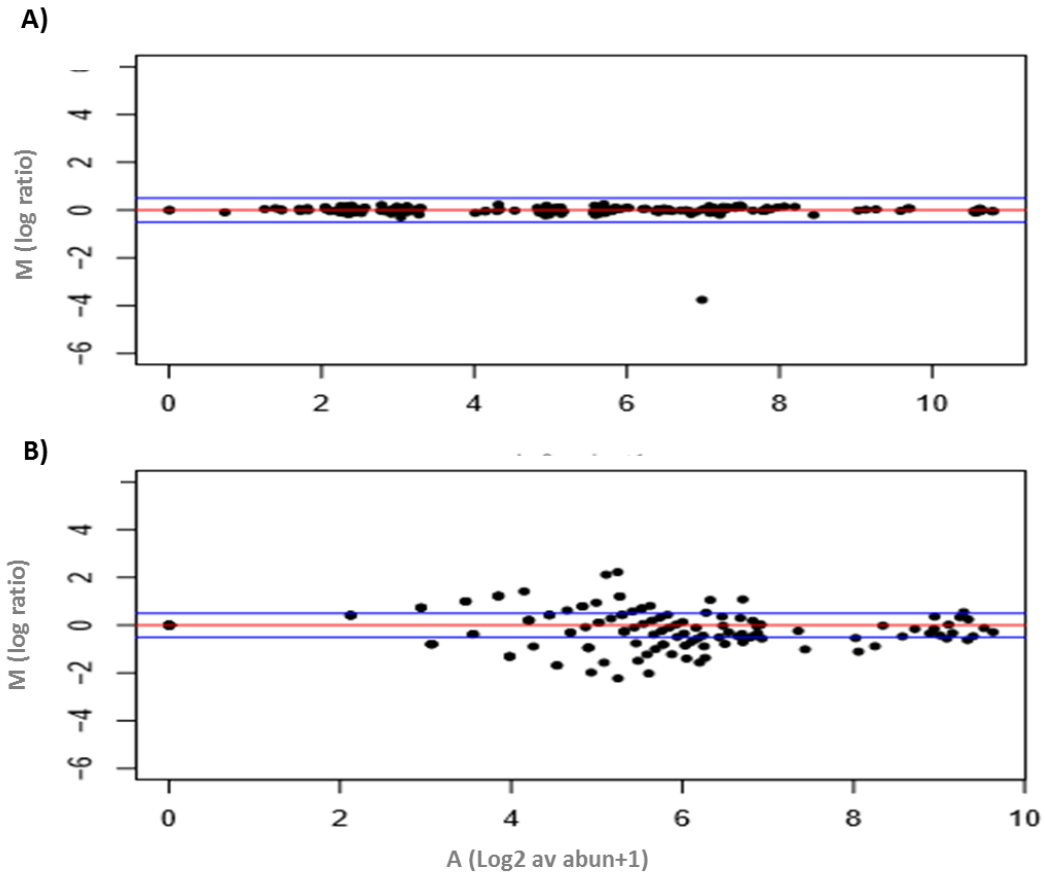


Figure 12. Examples of MA plots used to identify samples to be excluded. A) Pairwise comparison between two similar samples, which are replicates 1 and 2 of the treatment Pavon 76 leaves under well-watered conditions at 10 days. B) Pairwise comparison between two different samples, which are replicates 2 and 3 of the treatment Pavon 76 roots under drought conditions at 12 days, where replicate 3 was excluded, as it was also different from replicate 1 of the same treatment. The blue lines represent standard thresholds for log fold-changes of the gene expression. The points above the blue line are considered upregulated and the points below the line are considered downregulated between the two compared samples.

Table 6. A list of the five excluded samples from the differential expression (DE) miRNA analysis.

Sample	Sample description	Replicate	Index primer	Number of reads	%GC
R15	Yecora Rojo_Leaves_Well-watered (WW)_10 days	Rep3	12	8763339	50
R19	Yecora Rojo_Leaves_Water deficit (WD)_10 days	Rep1	8	16339919	49
R26	Yecora Rojo_Roots_ Water deficit (WD)_12 days	Rep2	12	3437215	57
R30	Pavon76_Roots_ Water deficit (WD)_12 days	Rep3	20	384974	56
R42	Yecora Rojo_Roots_ Well-watered (WW)_10 days	Rep3	20	834614	55

In all cases, not more than one replicate was excluded out of the three biological replicates of each treatment, which means that at least two biological replicates were included in the differential expression analysis.

4.5 Identification of miRNAs differentially expressed during drought-stress

Drought-related miRNAs were identified using a differential expression (DE) analysis in the 43 of 48 samples that passed the quality control analyses. Number of counts was calculated for each mapped read (referred to as miRNA ID, which consists of the sequence of the miRNA and its mapped positions on the wheat chromosomes). The resulting expression matrix of the consensus miRNA raw counts across 43 samples was normalized using DESeq2 package in R (Love, Huber and Anders, 2014). Eight different comparisons were conducted between the treatments of each tissue samples involved in this study. The comparisons were made between the same variety at different conditions and between the two varieties at the same conditions in leaf and root tissues independently (**Table 7**). Then, downregulated or upregulated miRNAs were identified based on log2 fold change, where a miRNA can

be considered differentially expressed with adjusted *P*-value < 0.05. Finally, the overlapping differentially expressed miRNAs among the different comparisons were selected and their importance as candidate miRNAs for drought stress in wheat was defined based on the sets of comparisons from which those candidates originated.

As a result, a total of 44 different miRNA sequences were found differentially expressed that can be derived from 90 loci. The miRNA sequences were compared to all plant miRNAs deposited in miRbase (https://www.mirbase.org). We found that 27 of the differentially expressed miRNAs belong to 21 miRNA families and 8 of these are conserved (*miR160, miR166, miR169, miR319, miR396, miR398, miR528* and *miR9657*) while the other 13 are non-conserved miRNA families. While, the family *miR164* have 4 family members and the families *miR172*, *miR390* and *miR9772* have 3 members, each. The other 9 miRNA families *(miR171, miR530, miR827, miR874, miR1120, miR5067, miR5070, miR9674* and *miR9776*) have less than three family members, each.

In addition to the 27 (21 families) known miRNAs, the 44 differentially expressed miRNAs also included 17 miRNAs that could not be assigned to any of the known miRNA families. This group of miRNA sequences could represent new miRNAs, which to be deposited in the miRNAs database.

4.5.1 Differentially expressed miRNAs in leaf tissue

To identify differentially expressed miRNAs in leaf samples we compared samples from the same variety but under different conditions. This type of comparison can identify differentially expressed miRNAs that are most likely related to drought rather than to the variety itself. In both varieties, the number of differentially expressed miRNAs increased as a response to increasing the timing of drought application from 10 days to 12 days **(Table 7)** (from 15 to 24 miRNAs in Pavon 76 and from 8 to 21 miRNAs in Yecora Rojo). Moreover, we compared the two varieties in the same conditions in order to identify miRNAs that are related to the genetic background, as the two varieties respond differently to drought condition. When comparing Pavon 76 and Yecora Rojo under well-watered conditions at 10 days, we identified only 3 differentially expressed miRNAs. However, this number increased to 10 under drought stress at 10 days. Similarly, the number of differentially expressed miRNAs was 6 when comparing the two varieties when well-watered at 12 days but it became 24 under drought conditions at 12 days (**Table 7**). Again, the results showed that the number of differentially expressed miRNAs increased with the increased timing of treatments in both comparisons. This could be due to genetic differences between the two varieties. It is possible that Pavon 76 has evolved mechanisms to cope with drought stress more efficiently than Yecora Rojo, such as upregulation of stress-responsive miRNAs or the activation of drought tolerance pathways.

Table 7. Number of differentially expressed miRNAs identified from each comparison of both leaves and roots samples.

Comparison type	Comparison for DE miRNAs		Number of DE miRNAs	
	Treatment 1	**Treatment 2**	**Leaves**	**Roots**
Within the same variety at different conditions	Pavon 76 well-watered at 10 days	Pavon 76 under drought at 10 days	14	3
	Pavon 76 well-watered at 12 days	Pavon 76 under drought at 12 days	24	0
	Yecora Rojo well-watered at 10 days	Yecora Rojo under drought at 10 days	7	0
	Yecora Rojo well-watered at 12 days	Yecora Rojo under drought at 12 days	20	0
Between the two varieties at the same conditions	Pavon 76 well-watered at 10 days	Yecora Rojo well-watered at 10 days	3	6
	Pavon 76 well-watered at 12 days	Yecora Rojo well-watered at 12 days	6	2
	Pavon 76 under drought at 10 days	Yecora Rojo under drought at 10 days	10	3
	Pavon 76 under drought at 12 days	Yecora Rojo under drought at 12 days	23	0

4.5.1.1 Comparisons within the same variety at different conditions

To identify miRNAs that are differentially expressed under different conditions, we compared leaf samples from the same variety but under well-watered and water deficit conditions. Accordingly, the differentially expressed miRNAs identified in each comparison were checked for overlap between the four sets of comparisons. We found that there were some unique differentially expressed miRNAs for each group indicating that they were likely to be false positives. Thus, for the comparison of Pavon 76 plants under well-watered and water-deficit conditions at 10 days, 4 unique differentially expressed miRNA sequences were identified, while 13 were found for the same comparison at 12 days of treatment **(Table 8)**. In the case of the comparison of Yecora Rojo leaf samples under well-watered and water-deficit conditions at 10 days, there were 3 unique miRNAs and 9 in the same comparison but after 12 days of the treatment **(Table 8)**. Further information about differentially expressed miRNAs and their overlapping among the different comparison can be found in Appendix 3.

Table 8. The unique differentially expressed miRNAs identified for leaf samples from the same variety under different conditions.

Group	miRNA name	miRNA sequence	miRNA chromosome*
Pavon 76 water deficit and well-watered for 10 days	miR390	AAGCUCAGGAGGGAUAGCGCC	5D
	miR166m	UCGGACCAGGCUUCAUUCCCU	6D
	Unknown	UUCGCCGGAGAAGCUUACUGC	3B,4B,5A,5B and 7B
	miR9661	UGAAGUAGAGCAGAGACCUCA	5B
Pavon 76 water deficit and well-watered for 12 days	miR530	CUGCAUUUGCACCUGCACCUA	2D
	Unknown	UGAGACGAGAUCUCCCCAUAC	4A and 5D
	miR396e	UCCACAGGCUUUCUUGAACUG	2A,2B,2D and 6A
	miR160a	UGCCUGGCUCCCUGUAUGCCA	5B,5D,6A,6D,7A,7B and 7D
	miR164d	UGGAGAAGCAGGGCACGUGCA	2A,6A,6B and 6D
	miR396a	UUCCACAGCUUUCUUGAACUG	6A
	Unknown	AUGUAGAAGCACCAGGGUAAG	3A
	miR1432	AUCAGGAGAGAUGACACCGA	2B
	miR9674b	AUAGCAUCAUCCAUUCUACCA	4B
	Unknown	UUUGAGACGAACACUGACCAA	5B
	Unknown	CAUCCUAACAUAAGUGUCUCA	3B
	miR396d	UUCCACAGCUUUCUUGAACUU	6A and 6D
	Unknown	UUCGCCGGCUGCGCGUUCCCC	3D
Yecora Rojo water deficit and well-watered for 10 days	miR166c	UCGGACCAGGCUUCAUUCCUU	6A
	miR9772	UGAGAUGAGAUUACCCCAUAC	5B
	miR9776	UGGACGAGGAUGUGCAGCUGC	2D
Yecora Rojo water deficit and well-watered for 12 days	miR172a	AGAAUCUUGAUGAUGCUGCAU	6A,6B and 6D
	miR169h	UAGCCAAGGAUGACUUGCCUG	5A,5B,5D,7B and 7D
	miR398	UGUGUUCUCAGGUCACCCCUU	5B
	miR159	UUUGGUUUGAAGGGAGCUCUG	5D
	Unknown	UUCGCCGGAGAAGCAUGCUGC	7A
	miR1632	UUUUUGGAUGUGCUCCUCUAG	6B
	miR169a	CAGCCAAGGAUGACUUGCCGA	3A
	unknown	CUCGCCGGUCGCGCGUUCUCC	2B and 7D
	Unknown	UUCGCCGGCUGCGCGUUCCCC	5D

* MiRNA chromosome refers to the chromosomes where the same miRNA sequence is located. The exact location of each miRNA is listed in Appendix 3.

On the other hand, there were some overlapping differentially expressed miRNAs among the different groups of comparisons. The overlapping miRNAs are more likely to play a role in drought-stress response as they are consistently differentially expressed in two or more different comparisons. We found that there were 7 differentially expressed miRNAs overlapping between the comparisons of Pavon 76 samples under well-watered and water-deficit conditions at 10 days and 12 days (**Table 9**). In addition, there were 5 differentially expressed miRNAs overlapping between the comparisons of the Yecora Rojo samples under well-watered and water-deficit conditions at 10 days and 12 days (**Table 9**). Further analysis showed that four miRNAs (*miR319, miR390, miR398* and *miR7714*) were differentially expressed miRNAs, which were unique to Pavon76. While only one differentially expressed miRNA (*miR169a*) was unique to Yecora Rojo. Those miRNAs may play a role in drought-stress response that is specific to the variety. (**Figure 13**). On the other hand, there was one overlapping differentially expressed miRNA (*miR528*) between the comparison of the same variety regardless of treatment type (well-watered and water deficit) and treatment time (after 10 days and 12 days). This indicates that *miR528* is related to drought-stress response in both wheat varieties. Interestingly, *miR528* has been previously reported to be responsive to drought in *T. dicoccoides* (Kantar, Lucas and Budak, 2011b), *Brachypodium distachyon* (Budak and Akpinar, 2011) and sugarcane (Ferreira *et al.*, 2012).

Table 9. The overlapping differentially expressed miRNAs identified for leaf samples from the same variety under different conditions.

Group	miRNA name	miRNA sequence	miRNA chromosome
Pavon 76 water deficit and well-watered for 10 and 12 days	miR390a	AAGCUCAGGAGGGAUAGCGCC	5B and 5A
	miR398-tae	UGUGUUCUCAGGUCGCCCCCG	3A and 3D
	miR319b	UUGGACUGAAGGGUGCUCCCU	3D and 3A
	miR7714-like	AUGUCGAAGGUAGUAGCUCGA	7B
	miR5168	UCGGACCAGGCUUCAAUCCCU	5A and 5B
	miR159	UUUGGUUUGAAGGGAGCUCUG	4A
	miR528	UGGAAGGGGCAUGCAGAGGAG	4B, 4D and 5A
Yecora Rojo water deficit and well-watered for 10 and 12 days	miR169a	CAGCCAAGGAUGACUUGCCGA	2B and 3B
	miR398a-ath	UGUGUUCUCAGGUCACCCCUU	5A and 5D
	miR528	UGGAAGGGGCAUGCAGAGGAG	4B, 4D and 5A
	miR7714-like	AUGUCGAAGGUAGUAGCUCGA	2A

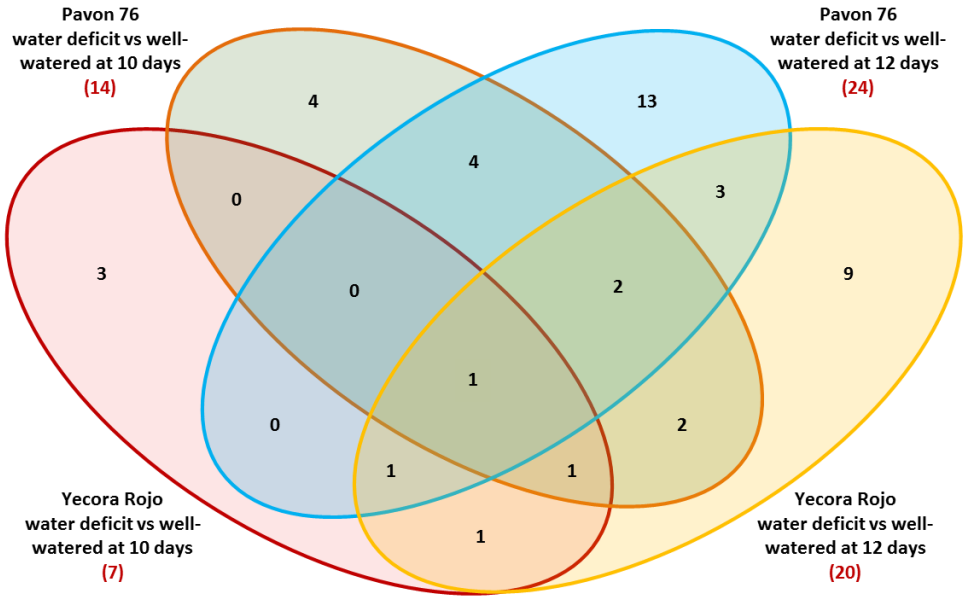


Figure 13. Overlapping differentially expressed miRNAs between the treatments of leaf samples under well-watered and water deficit conditions at 10 and 12 days.

4.5.1.2 Comparisons between the two varieties at the same conditions

To gain more information about the differentially expressed miRNAs that are related to the resistance and sensitivity of the two different wheat varieties to drought stress we compared the miRNAs in the two varieties under the same conditions. Our results showed that there were unique and overlapping differentially expressed miRNAs (**Figure 14)**. In this study, the unique miRNAs that are differentially expressed only at one treatment time (10 days or 12 days) are considered less important than the overlapping differentially expressed miRNA that are consistently differentially expressed at both treatment times. We identified 6 differentially expressed miRNAs that are overlapping between the comparison of Pavon 76 and Yecora Rojo under water deficit treatment after 10 days and the comparison of the same varieties and treatment after 12 days (**Figure 14**). Interestingly, one of those 6 miRNAs was *miR528* mentioned in the previous paragraph as overlapping between all comparisons of the same variety at different conditions (**Table 10**). In addition to that, there were another miRNA (AUGUCGAAGGUAGUAGCUCGA) that showed sequence similarity to *miR7714* that has been experimentally validated to be responsible for drought response in rice (Sunkar *et al.*, 2008) and *Brachypodium*

distachyon (Bertolini *et al.*, 2013). Those miRNAs were taken forward for additional validation. Nevertheless, there were no overlapping differentially expressed miRNAs identified between both the Pavon 76 and Yecora Rojo groups which had received the water deficit treatment for 10 days and the plants of the same varieties which had been well-watered (**Figure 14**). Furthermore, we identified a total of 14 unique differentially expressed miRNAs. Notably, 10 of them were found in the comparison Pavon 76 and Yecora Rojo under water deficit treatment for 12 days (**Table 10**).

Table 10. The unique differentially expressed miRNAs identified for leaf samples from two varieties under the same conditions.

Group	miRNA name	miRNA sequence	miRNA chromosome
Pavon 76 and Yecora Rojo under water deficit treatment for 10 days	miR319b	UUGGACUGAAGGGUGCUCCCU	3D
	miR166c	UCGGACCAGGCUUCAUUCCUU	6A and 6D
Pavon 76 and Yecora Rojo under well-watered for 10 days	miR396d	UUCCACAGCUUUCUUGAACUU	6A and 6D
	miR5168	UCGGACCAGGCUUCAAUCCCU	5B
Pavon 76 and Yecora Rojo under water deficit treatment for 12 days	miR390a	AAGCUCAGGAGGGAUAGCGCC	5A
	miR528	UGGAAGGGGCAUGCAGAGGAG	2A,2B 4D and5A
	miR319b	UUGGACUGAAGGGUGCUCCCU	3A
	miR169b	CAGCCAAGGAUGACUUGCCGA	2D
	Unknown	UUCGCCGGUCGCGCGUUCCCC	2B and 3D
	Unknown	UUCGCCGGAGAAGCUUACUGC	3B,4B,5A,5B and 7B
	miR160a	UGCCUGGCUCCCUGUAUGCCA	5B,7B and 7D
	Unknown	UUCGCCGGCUGCGCGUUCCCC	3D
	Unknown	UUUUUGGAUGUGCUCCUCUAG	6B
	miR171b	UUGAGCCGUGCCAAUAUCACG	2A

Our experimental design and the collected miRNA expression data allowed us to identify differentially expressed miRNAs between the samples at several levels such as the variety (Pavon 76 and Yocora Rojo), tissue type (leaves and roots), stress

conditions (well-watered and water deficit), and different periods of stress application (10 days and 12 days). Then we looked for overlapping differentially expressed miRNAs to answer the initial hypotheses set up at the beginning of our research:

Hypothesis 1. The expression level of some miRNAs is different in a drought resistant variety of wheat compared to a drought sensitive variety in normal conditions. We found that *miR319, miR396, miR827, miR398a, miR9661*, and unknown miR UUCGCCGGUCGCGCGUUCCCC were differentially expressed miRNAs between the drought resistant variety (Pavon76) and drought sensitive variety (Yecora Rojo) in normal conditions at 10 days and 12 days. However, none of them overlapped in both lengths of drought treatment (**Figure 14**).

Hypothesis 2. The expression level of some miRNAs is different in a drought resistant variety of wheat compared to a drought sensitive variety when both types of plant are subjected to drought conditions. We found that there are 10 and 23 differentially expressed miRNAs between Pavon 76 and Yecora Rojo under water deficit conditions at 10 days and 12 days, respectively. Interestingly, six differentially expressed miRNAs (*miR166m, miR169a, miR390a, miR528, miR7714*-like and *miR9776*) were overlapped in both length of drought treatment (**Figure 14)** indicating their role in the differential drought response between the drought resistant and drought sensitive varieties.

Hypothesis 3. The expression level of certain miRNAs changes in response to drought stress. In this study, we identified differentially expressed miRNAs in the same variety but at different drought conditions. In Pavon 76 there were 14 and 24 differentially expressed miRNAs when comparing samples from well-watered and water deficit conditions but at 10 days and 12 days, respectively. There were four differentially expressed miRNAs (*miR319b, miR390a, miR398* and *miR7714*) that were overlapping between both lengths of drought treatment. Similarly, in Yecora Rojo there were 7 and 20 differentially expressed miRNAs when comparing samples from well-watered and water deficit conditions but at 10 days and 12 days, respectively, of which four (*miR169a, miR398a, miR528 and miR7714*-like) were overlapped in both lengths of drought treatment. Detailed averages of the normalized

count values for the identified miRNAs in the different leaf samples can be found in Appendix 4.

At the end of this analysis, three miRNAs were selected based on their similarity to previously reported drought-related miRNAs in other plant species including monocots. These miRNAs are as follow:

- ***miR528***: Overlapped between Pavon 76 vs Yecora Rojo under water deficit at 10 days and Pavon 76 vs Yecora Rojo under water deficit at 12 days, it is also differentially expressed in all comparisons of the same variety under different conditions.
- ***miR7714***: Overlapped between Pavon 76 vs Yecora Rojo under water deficit at 10 days and Pavon 76 vs Yecora Rojo under water deficit at 12 days.
- ***miR319***: overlapped between Pavon 76 under well-watered vs water deficit at 10 days and Pavon 76 under well-watered vs water deficit at 12 days. Thus, it might play a role in drought-stress response specific to the resistant variety Pavon76.

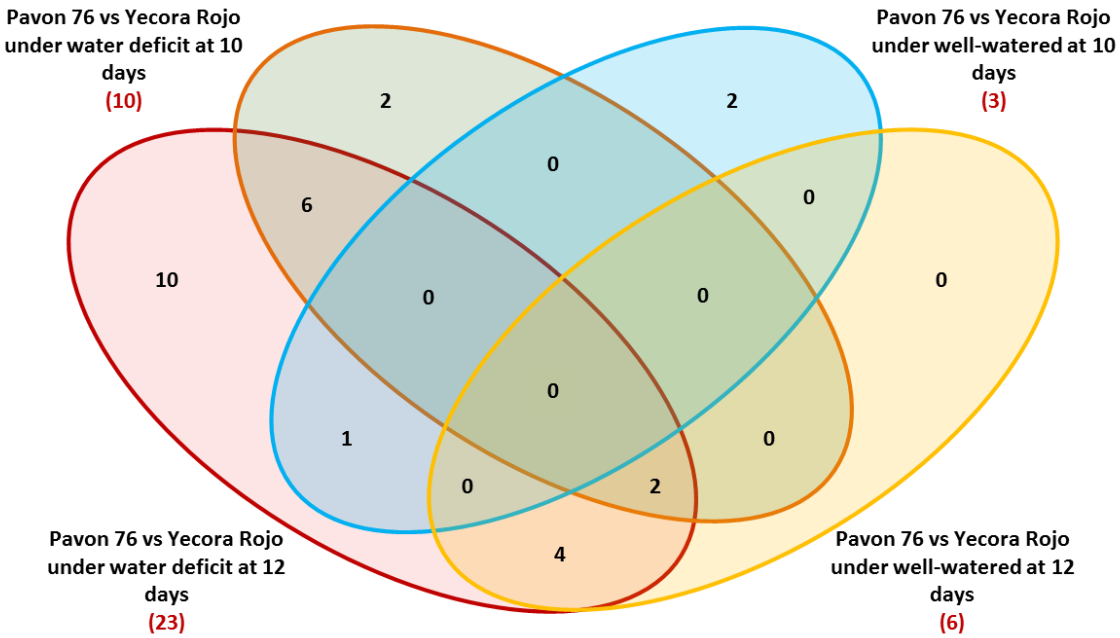


Figure 14. Overlapping differentially expressed miRNAs between the two cultivars Pavon 76 and Yecora Rojo of leaf samples under well-watered and water deficit conditions at 10 and 12 days.

4.5.2 Differentially expressed miRNAs in roots tissue

In the case of the root samples, to identify differentially expressed miRNAs we also compared root samples from the same variety but under different conditions. Three differentially expressed miRNAs (*miR166c*, and other two unknown miRNAs: UUGAUUCCCAUUUCACUAGCU and AUUGAACUAAGGAGGGGUGGA) were identified for the Pavon 76 variety when comparing well-watered samples with those subjected to drought conditions for 10 days. However, no differentially expressed miRNAs were identified for Pavon 76 between well-watered and water deficit for 12 days. Also, no differentially expressed miRNAs were identified for Yecora Rojo between well-watered and water deficit at 10 and 12 days (**Figure 15**).

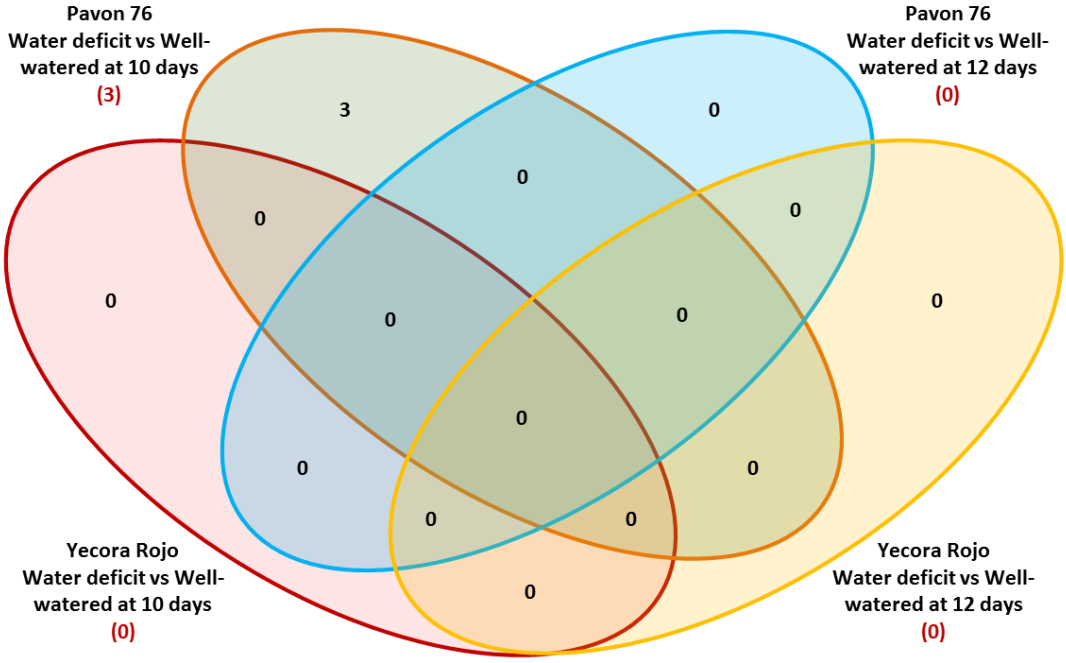


Figure 15. Overlapping differentially expressed miRNAs in root samples between the treatments of the roots from the same variety under well-watered and water deficit conditions at 10 and 12 days.

Comparisons between the two varieties under the same conditions revealed that there were 6 differentially expressed miRNAs for the two varieties when well-watered for 10 days, and 2 differentially expressed miRNAs when well-watered for 12 days. When subjected to drought conditions for 10 days both varieties had 3 differentially expressed miRNAs, while at 12 days there were none (**Figure 16**).

The comparison of Pavon 76 and Yecora Rojo under water deficit for 10 days showed only one unique differentially expressed miRNA, while none was identified at 12 days of water deficit. However, there were 4 unique differentially expressed miRNA under well-watered conditions for 10 days, and 2 unique differentially expressed miRNA when the plants were well watered for 12 days (**Table 11**). On the other hand, two overlapping differentially expressed miRNAs (*miR166c* and unknown miRNA: CUUCUGAUUUACUCGUCGUGG) were identified between both varieties at under well-watered and water deficit.

Table 11. The unique differentially expressed miRNAs identified for root samples from two varieties under the same conditions.

Group	miRNA name	miRNA sequence	miRNA chromosome
Pavon 76 and Yecora Rojo under water deficit treatment for 10 days	Unknown	UCCGUCCCAUACUAUAAGAGC	6A
Pavon 76 and Yecora Rojo under well-watered for 10 days	miR171	UUGAGCCGUGCCAAUAUCACG	2A
	miR9661	UGAAGUAGAGCAGAGACCUCA	5B
	Unknown	AUUGAACUAAGGAGGGGUGGA	2B
	Unknown	UUGAUUCCCAUUUCACUAGCU	3B
Pavon 76 and Yecora Rojo under well-watered treatment for 12 days	miR827	UUAGAUGACCAUCAGCAAACA	2B
	miR1432	AUCAGGAGAGAUGACACCGA	2B

Based on the poor quality of the extracted RNAs from the roots and the lack of miRNAs that are consistently differentially expressed at 10 and 12 days either in one variety during drought-stress or consistently differentially expressed between the two varieties, we concluded that miRNAs are unlikely to play an important role in drought-stress response in roots and focused our work on the miRNAs differentially expressed in leaves. Detailed averages of the normalized count values for the identified miRNAs in the different root samples can be found in Appendix 5.

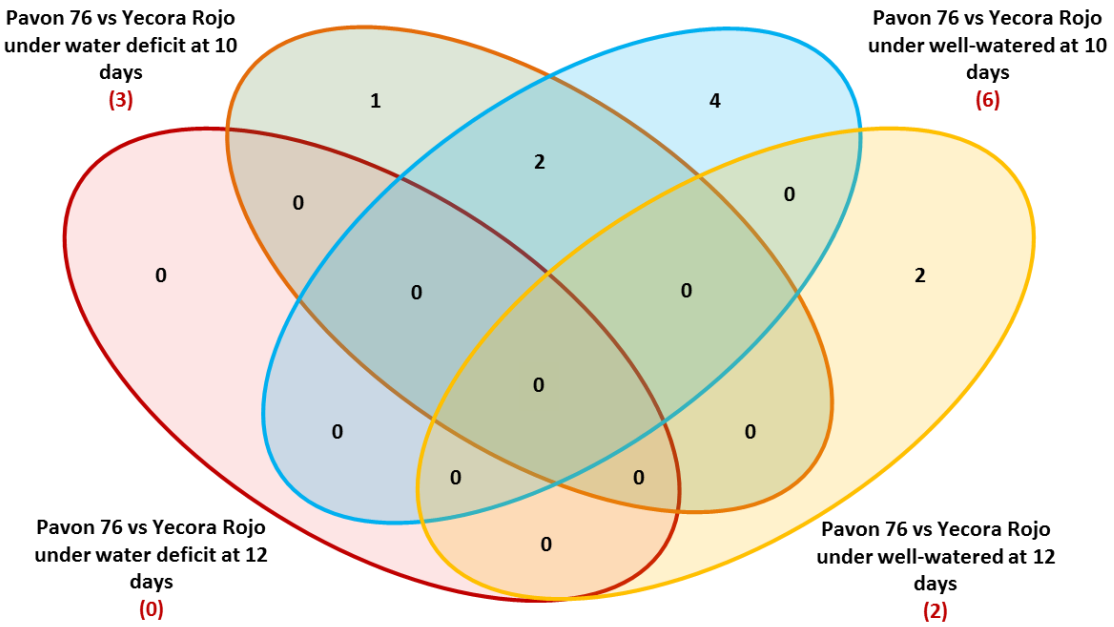


Figure 16. Overlapping differentially expressed miRNAs between the two cultivars Pavon 76 and Yecora Rojo of root samples under well-watered and water deficit conditions at 10 and 12 days.

4.6 Validating differential expression of miRNAs by northern blot

Bioinformatic analysis of the sequencing result is an extremely helpful technique but the results are only prediction. How good the predictions are depend on many factors, such us the quality of the RNA used for generating the libraries, the quality of the library and the analysis itself. The differential expression of miRNAs identified in the bioinformatics analysis requires experimental validation because some of the results may be false positives. Northern blot is a technique which is commonly used in molecular biology to quantify the accumulation level of RNA molecules. Here I used this technique to validate the expression levels of the miRNAs that were predicted to be differentially expressed. The RNA was separated on a urea containing polyacrylamide gel and stained with ethidium bromide (**Figure 17**).

Figure 17. 15% polyacrylamide urea gel with 10 µg of total RNA. The second and third lanes contain the sample from well-watered wheat leaves, as a control from the Pavon 76 variety. The fourth and fifth lanes have the water deficit for 12 days wheat leaves sample from the Pavon 76 variety. The sixth and seventh lanes have samples from well-watered wheat leaves as a control from the Yecora Rojo variety. The eighth and ninth lanes have the sample from the water deficit for 12 days wheat leaves from the Yecora Rojo variety. The gel was allowed to run at 120V for two hours and then stained with ethidium bromide.

The RNA was then transferred from the gel onto a nylon membrane. To confirm that the transfer of RNA to the membrane had worked correctly, the gel was re-scanned (**Figure 18**).

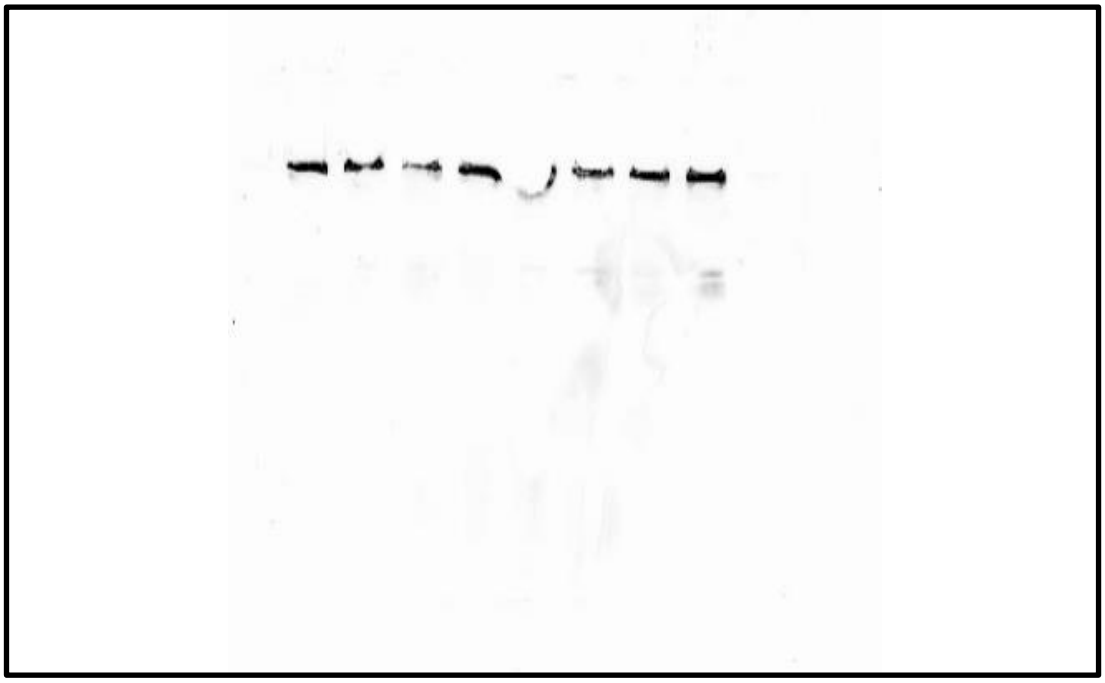


Figure18. 15% polyacrylamide urea gel after the RNA has been successfully transferred to a membrane. The same gel that is shown on (**Figure 17**) was scanned again after the RNA was transferred to a membrane.

After ascertaining the correct transfer of the RNA to the membrane, the RNA was crosslinked to the membrane and hybridised to a radioactive probe specific to a certain miRNA. After a series of washing steps, to remove the probe molecules that are not annealed to the RNA on the membrane, the phospho-image plate with the film was scanned using Typhoon FLA 9500.

Northern blot was performed to validate the results obtained from the miRNA analysis for the three selected miRNAs (*miR319, miR528,* and *miR7714*). This assay was also used to confirm the differential expression patterns of the aforementioned miRNAs of the two different varieties under different conditions. Northern blot was performed using 3 probes with reverse complementary sequences to the miRNAs under study (**Table 12**).

Table 12. Optimizing probes with their Tm and annealing temperature.

Probe name	Probe sequence (5' → 3')	Melting Temperature (T_m)	Applied annealing temperature
miR7714	TCGAGCTACTACCTTCGACAT	61	37°C
miR528	CTCCTCTGCATGCCCCTTCCA	73	37°C
miR319	AGGGAGCACCCTTCAGTCCAA	69.5	37°C

Overall, northern blot analysis showed fairly good signals for the three miRNAs, but the extent of the signal was different for each one.

Northern blot using a *miR7714*-specific probe showed three bands with different sizes. The smallest of these is expected to be *miR7714* with a size of 21 nt, while the middle and the largest ones may be the precursor-miRNAs (pre-miRNA) and primary miRNA (pri-miRNA), respectively. However, as the exact size of the pre-miRNA and pri-miRNA molecules are unknown, different probes, covering the flanking regions of the mature miRNA should be used in the future to further investigate the identity of those two bands. Intensity of the miRNA (the lowest band) signals was normalised for the loading control U6 using ImageQuant (GE Healthcare) software. The highest level of normalised expression (3.01) was found in the Yecora Rojo samples under water deficit conditions, which is considered differentially expressed from the Pavon 76 samples under the same conditions as the error bars did not overlap, where the lowest value (0.73) was reported. Interestingly, the samples from the two varieties under well-watered conditions were not differentially expressed, with normalized values of 1.26 and 1.12 in Pavon 76 and Yecora Rojo, respectively. Furthermore, the number of reads of *miR7714* obtained from miRNAs sequence analysis corresponds with those obtained from northern blot as shown in (**Figure 19**).

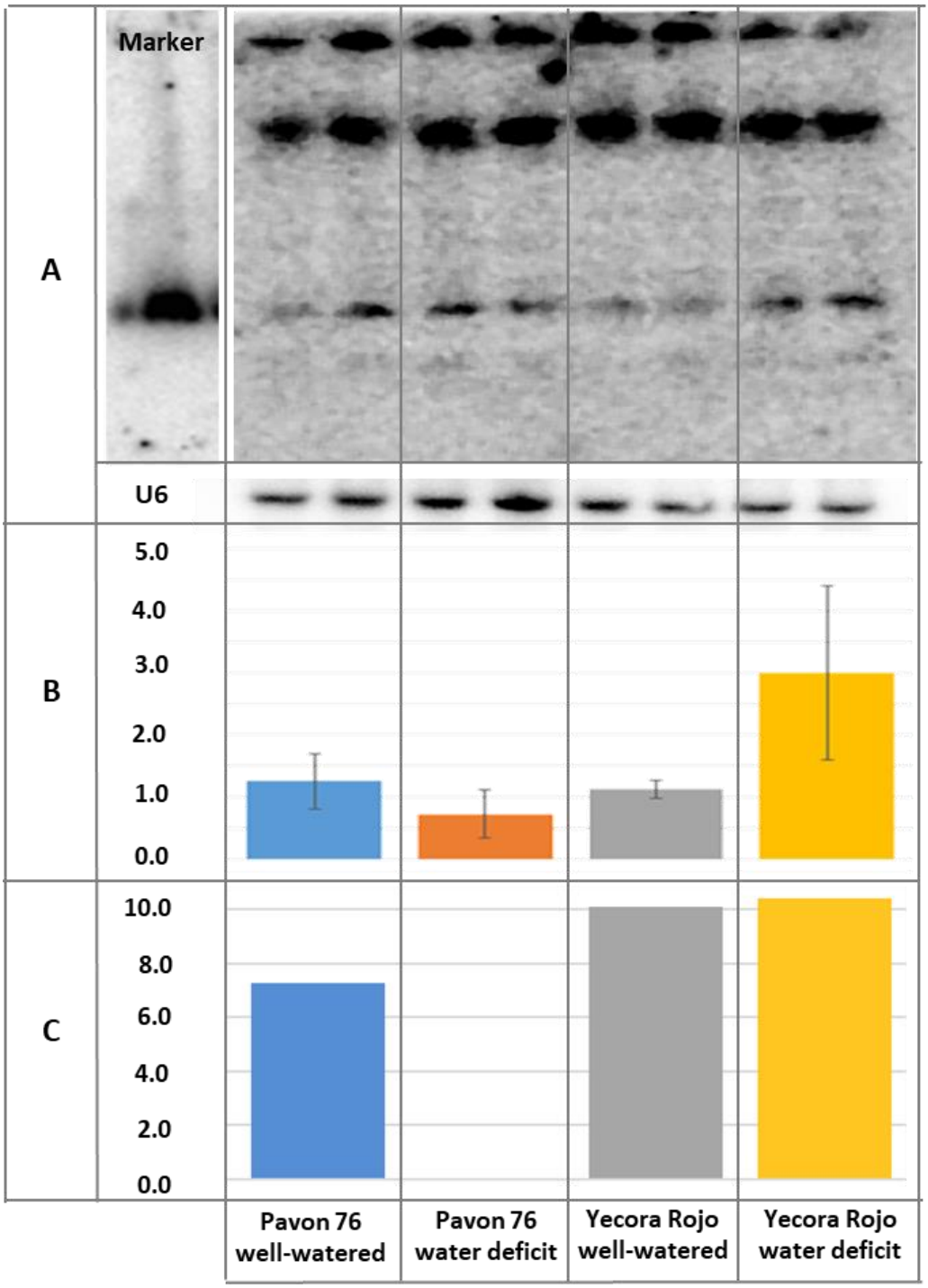


Figure 19. Expression of miR7714. (A) shows the northern blot for miR7714, with the lowest band representing the mature miRNA. The signal for U6 used as a loading control. Marker refers to the miR156 from date palm that was used as miRNA size control. **(B)** shows the mean normalized band intensity. **(C)** shows the mean of the read number of miR7714 following sRNA sequencing.

The northern blot for *miR319* revealed a single signal of approximately 21 nt in size, which was downregulated under water deficit in the Pavon 76 samples but it was

upregulated in the Yecora Rojo sample under same conditions as shown in (**Figure 20**).

The Yecora Rojo samples under water deficit conditions showed the highest level of expression (having a normalized northern blot density of 1.88). This is considered differentially expressed from the Pavon 76 samples treated with water deficit conditions as the error bars did not overlap, which showed the lowest value of 0.71. For leaf samples under well-watered conditions, in the samples from the two varieties the normalized values were 1.74 for Pavon 76 and 0.61 for Yecora Rojo. The number of *miR319* reads obtained from the sequence analysis did not correspond perfectly with those obtained from northern blot as shown in (**Figure 20**) but the sequencing result predicted correctly that *miR319* is expressed at a higher level in Yecora Rojo than in Pavon 76 during drought-stress.

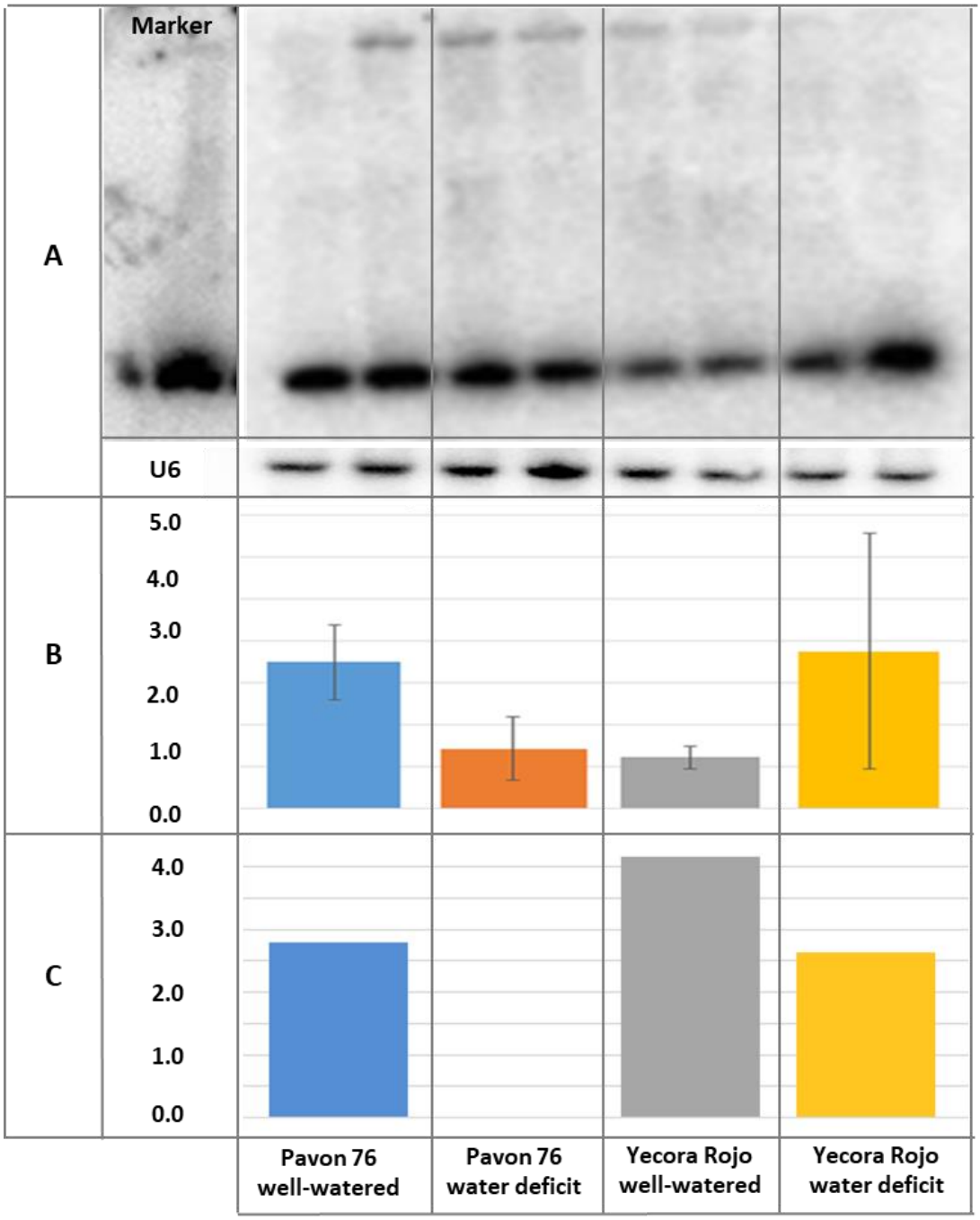


Figure 20. Expression of miR319. (A) shows the northern blot for miR319, with the lowest band representing the mature miRNA. The signal for U6 used as a loading control. Marker refers to the miR156 from date palm that was used as miRNA size control. **(B)** shows the mean normalized band intensity. **(C)** shows the mean of the read number of *miR319* following sRNA sequencing.

The northern blot using the *miR528* specific probe detected three bands with different sizes, as for *miR7714*. *MiR528* was upregulated in the samples from both varieties under water deficit conditions.

The smallest of these is expected to be *miR528* with a size of 21 nt, while the middle and the largest ones may be the precursor-miRNAs (pre-miRNA) and primary miRNA (pri-miRNA), respectively. However, as the exact size of the pre-miRNA and pri-miRNA molecules are unknown, different probes, covering the flanking regions of the mature miRNA should be used in the future to further investigate the identity of those two bands as shown in (**Figure 21**). When the signal density of the miRNA (the lowest band) was compared with the signal from the same samples for the loading control U6 using ImageQuant (GE Healthcare) software, it was shown that the value for the water-deficit samples of Yecora Rojo was 0.9 and for Pavon 76 it was 1.0, indicating that both varieties had been upregulated to a similar level. For the two varieties under well-watered conditions the normalized values were 0.55 and 0.29 in Pavon 76 and Yecora Rojo, respectively. As found with the other miRNAs, the number of reads of *miRNA528* obtained from the sequence analysis corresponded well with those of obtained by northern blot as shown in (**Figure 21**).

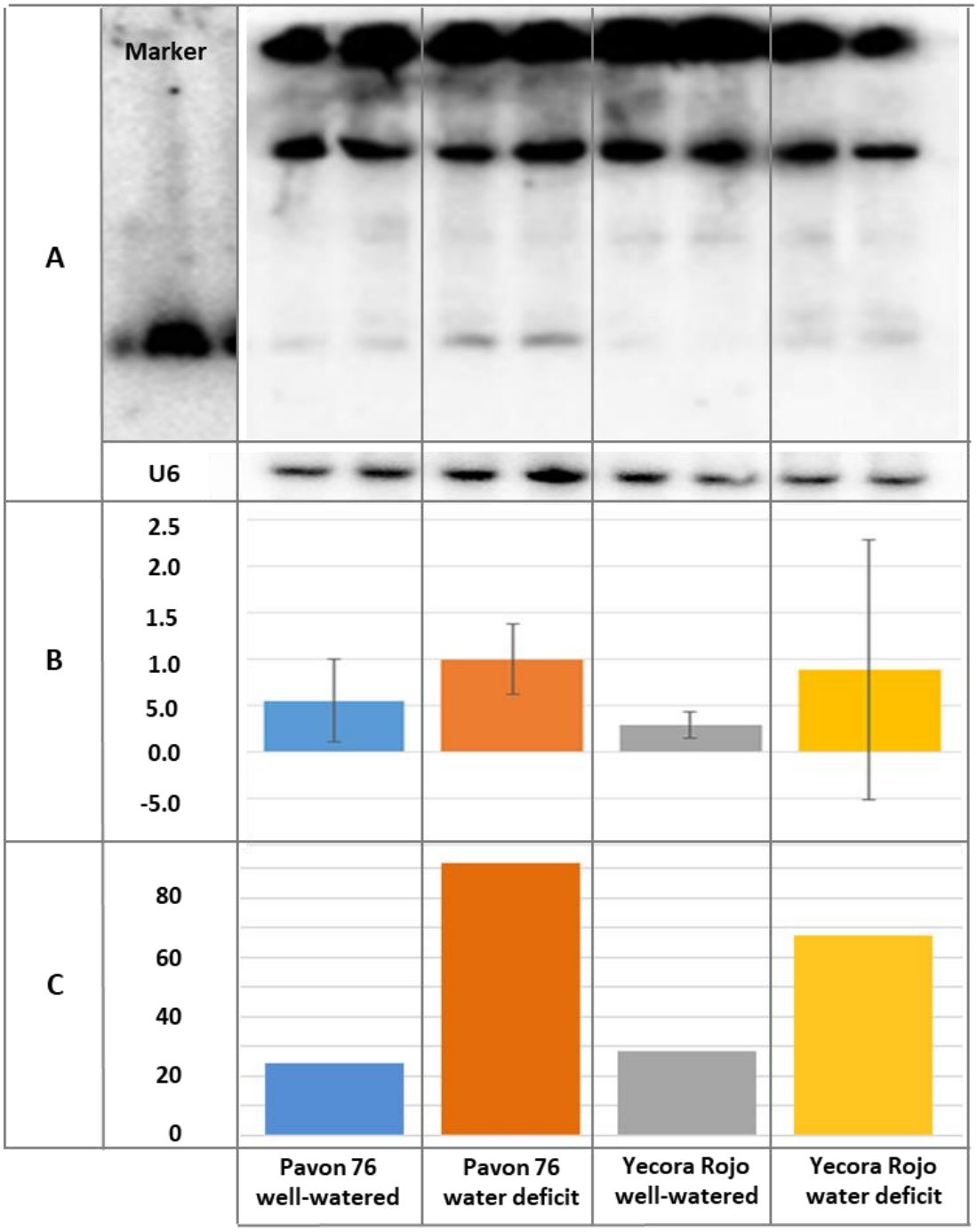


Figure 21. Expression of miR528. (A) shows the northern blot for miR528, with the lowest band representing the mature miRNA. The signal for U6 used as a loading control. Marker refers to the miR156 from date palm that was used as miRNA size control. **(B)** shows the mean normalized band intensity. **(C)** shows the mean of the read number of miR528 following sRNA sequencing.

4.7 Discussion

To summarize, 8 differentially expressed miRNAs were identified and found to be overlapping between the comparison of Pavon 76 and Yecora Rojo under water deficit at 10 days and the same comparison but at 12 days. Those miRNAs represent the group of miRNAs that have different expression level in a drought resistant variety of wheat compared to a drought sensitive variety when both types of plant are subjected to drought conditions (hypothesis 2). Thus, two of these miRNAs (*miR528* and miR*7714*) were selected for further validation using northern blot analysis. The reason why we selected those two miRNAs is that they are both reported to be involved in drought stress response in other cereal plants such as rice, *Brachypodium distachyon* and *T. dicoccoides* (Sunkar *et al.*, 2008; Zhou *et al.*, 2010; Kantar, Lucas and Budak, 2011a; Bertolini *et al.*, 2013). Furthermore, another group of miRNAs are characterized by changing expression level in response to drought stress in the same variety (hypothesis 3). As mentioned before, we identified 13 differentially expressed miRNAs in Pavon 76 at different drought conditions after 10 days and 12 days of treatment. *MiR319* was taken forward for further validation as a representative of this group of miRNAs. *MiR319* has previously been found to be responsive to multiple abiotic stresses such as salinity, drought, cold and its function was validated experimentally using transgenic plants (Zhou and Luo, 2014a).

Northern blot signals obtained from Pavon 76 and Yecora Rojo leaf samples showed clear differences in the expression pattern between well-watered and water deficit treatments at 12 days for the three miRNAs under study (*miR319, miR528,* and *miR7714*). Thus, confirming the results obtained from our miRNAs sequencing data.

Much research has been carried out on miRNAs in drought and other stress tolerance reactions in globally important food crops, including key cereals such as rice (*Oryza sativa*). Many miRNAs have been reported to play role in drought and heat stresses in cereals. *MiR528* is a miRNA molecule that has been isolated in rice, which is proven to be responsive to stress and exhibits differential expression under various stress conditions such as drought, mineral and nutrient deficiency, and the presence of toxins (Liu *et al.*, 2015). A recent study has shown that *miR528* is involved in the regulation of diverse biological processes, including responses to stress (e.g. salinity, drought, and mineral deficiency), nutrient uptake, and plant development (Liu *et al.*,

2015). *MiR528* was amongst the deferentially expressed miRNAs in drought-stressed rice flag leaves. It was upregulated in drought-tolerant cultivars (Vandana and Nagina 22), while it was downregulated in drought-sensitive cultivars (IR64 and Pusa Basmati 1). *MiR528* functions by targeting mRNA, which codes for proteins containing copper, helping to regulate this nutrient element's availability in drought and other copper-deficient environmental conditions. Considering that copper is an important element in the homeostasis of reactive oxygen species (ROS), which in turn are vital in internal plant signaling. This is to ensure drought tolerance by regulating the opening and closure of leaf stomata. Therefore, *miR528* is important for mediating the interaction between the homeostasis of ROS and copper (Balyan *et al.*, 2017). Furthermore, (Cheah *et al.*, 2017) found that in drought conditions miR528 and other miRNAs were downregulated in the leaves of two drought-tolerant varieties of rice (Vandana and Aday Sel) and upregulated in a drought-susceptible variety (IR64).

MiR528 targets a gene known in rice as DWARF3, to reduce plant height by affecting the homoeostasis of gibberellin (GA) and abscisic acid (ABA), reducing the former and increasing the latter. Thus *MiR528* has the effect of reducing plant height through shortening the plant stem internodes (Zhao *et al.*, 2022).

Liu et al. (2015) studied arsenic tolerance in rice, which is a significant issue due to bio-accumulation leading to human exposure to this toxic element. The insertion of *miR582* into transgenic varieties raises plant sensitivity to arsenic and increases copper concentrations for three reasons: 1) copper is not assimilated into proteins because of *miR582*'s actions; 2) *miR582*'s precursor opens up copper transport pathways, even though the miRNA reduces the transcription of proteins containing copper; 3) higher arsenic concentrations are correlated with higher copper uptake (Liu et al., 2015). *MiR528* targets in particular the production of two proteins, ascorbic acid oxidase and a protein responsible for binding copper ions, which respond to both nitrogen deficiency and salinity, as they are downregulated to a significant degree in transgenic *creeping bentgrass* with higher *miR528* expression (Yuan et al. 2015).

Interestingly, *MiR528* was found to be involved negatively in resistance to viral infection in rice (Wu *et al.*, 2017). It cleaves the mRNA responsible for the transcription of L-ascorbate oxidase (AO), which thereby inhibits the accumulation

of ROS, which is critical for fighting the infection. Therefore, sites of infection sequester miR528, as it is preferentially associated with the Argonaute 18 protein, allowing higher AO activity and greater accumulation of basal ROS, thus enhancing antiviral defense. The suppression of *miR528* could be exploited to engineer virus-resistance crop plant (Wu et al., 2017). In addition to that, miRNAs research to increase rice inflorescences and production focus on biological processes such as signalling, plant development and stimulus response (Cheah et al. 2017).

Chapter 5. Identification of miRNA targets

5.1 Validation of predicted miRNA targets by Rapid Amplification of cDNA End (5'RACE)

Simon Moxon (UEA, Norwich) predicted the mRNA targets for *miR319, miR528* and *miR7714* using the toolkit developed at UEA for large-scale plant small RNA datasets (Moxon *et al.*, 2008), where the following criteria were used to predict target mRNAs: 1) no more than 4 mismatches in the alignment of the miRNA to the target (G-U bases count as 0.5 mismatches); 2) no more than 1 bulge in the alignment; 3) no more than 2 adjacent mismatches in the alignment; 4) no two adjacent mismatches in the 5' end of the miRNA (bases 2-12) ; 5) no mismatch in positions 10 and 11 of the miRNA; 6) no more than 2.5 mismatches in position 1-12 of the miRNA; and 7) the ratio of the predicted minimum free energy (MFE) of the duplex divided by the MFE of the perfect complement must be >= 0.73. The resulting targets were listed in **Table 13**.

Table 13. List of the predicted mRNA targets of the three selected miRNAs

MiRNA	Predicted target gene ID	Position	Functional Annotation
miR319	TraesCS2A02G226000	2A:233198968:233207873	Protein kinase family protein (Mad3/BUB1 homology region 1)
	TraesCS2A02G251100	2A:379150934:379159267	TCP transcription factor
	TraesCS2B02G249400	2B:257903257:257911251	Protein kinase family protein (Mad3/BUB1 homology region 1)
	TraesCS2B02G265800	2B:357918017:357924451	TCP transcription factor
	TraesCS2D02G252100	2D:302247032:302253739	TCP transcription factor
	TraesCS3B02G322400	3B:521699476:521702489	ATP synthase subunit g, mitochondrial
	TraesCS5A02G031900	5A:29512544:29516131	TCP transcription factor
	TraesCS5A02G423900	5A:609619180:609621983	TCP transcription factor
	TraesCS5B02G032000	5B:35188411:35189313	TCP transcription factor
	TraesCS5B02G426200	5B:601864073:601867389	TCP transcription factor
	TraesCS5D02G040100	5D:41117072:41120665	TCP transcription factor
	TraesCS5D02G432500	5D:488677442:488679253	TCP transcription factor
	TraesCS7B02G470900	7B:727202092:727204794	BTB/POZ domain containing protein
	TraesCS7D02G394400	7D:509612275:509614746	Serine/threonine-protein kinase
miR528	TraesCS1A02G051200	1A:32549428:32552583	Casein kinase I
	TraesCS2A02G198000	2A:169339150:169344295	Blue copper protein (Plastocyanin-like domain)
	TraesCS2B02G225400	2B:215835145:215841899	Blue copper protein (Plastocyanin-like domain)
	TraesCS2B02G308000	2B:440814041:440825740	Initiation factor 4F subunit (DUF1350)
	TraesCS2D02G206000	2D:158672797:158683036	Blue copper protein (Plastocyanin-like domain)
	TraesCS2D02G305400	2D:391260779:391261981	U-box domain-containing protein
	TraesCS3B02G126900	3B:103907873:103909976	Phosphofructokinase family protein
	TraesCS3B02G249600	3B:397670445:397677173	Acyl-protein thioesterase 2 (Phospholipase/Carboxylesterase)
	TraesCS3B02G393500	3B:618869300:618872260	Laccase (Multicopper oxidase)
	TraesCS3D02G074700	3D:35892972:35896896	Steroid 5-alpha reductase (3-oxo-5-alpha-steroid 4-dehydrogenase)
	TraesCS6D02G372400	6D:457592489:457593175	transmembrane protein, putative (DUF679)
	TraesCS7B02G114700	7B:133422041:133422821	Blue copper protein (Plastocyanin-like domain)
	TraesCS7D02G210300	7D:168251864:168252705	Blue copper protein (Plastocyanin-like domain)
	TraesCSU02G049100	Un:39241254:39245463	Steroid 5-alpha reductase (3-oxo-5-alpha-steroid 4-dehydrogenase)
miR771	TraesCS2B02G255900	2B:296012982:296028469	Asparagine-tRNA ligase (OB-fold nucleic acid binding domain)

However, similarly to differential expression, the bioinformatic approach can only predict miRNA targets but some of them may be false positives. Therefore, it is essential to validate the predictions experimentally. The Rapid Amplification of cDNA End (5'RACE) technique was used for experimental validation of the predicted miRNA target mRNAs. Primers were designed to conduct two rounds of PCR (first PCR and a second nested PCR). The expected final PCR (nested PCR) product sizes ranged approximately between 350 bp and 450 bp, as shown in (**Figure 22**). According to the expression (number of reads) of *miR319*, *miR7714*, and

miR528, total RNA was extracted from leaf samples of well-watered plants (cv. Pavon 76). No controls were used from the other variety or the plants under water deficit conditions as the purpose of this experiment was to validate the presence of the cleavage site in the target mRNAs. Dynabeads Oligo (dT) was used to isolate the mRNA from total RNA. The 5' adapter was ligated to the mRNA and preparation of the reverse transcription (RT) was carried out using oligo (dT) primer. The oligo (dT) primer binds to the complementary poly (A) tails of mRNA. For each RT, two PCR reactions were conducted to amplify the potential cleavage products of the candidate target genes, and then run on an agarose gel using electrophoresis as illustrated in (**Figure 23**).

After purifying the PCR products, they were ligated in to the pJET vector and then transformed into *E. coli* bacteria. Ten colonies were selected to be sequenced for each predicted miRNA target.

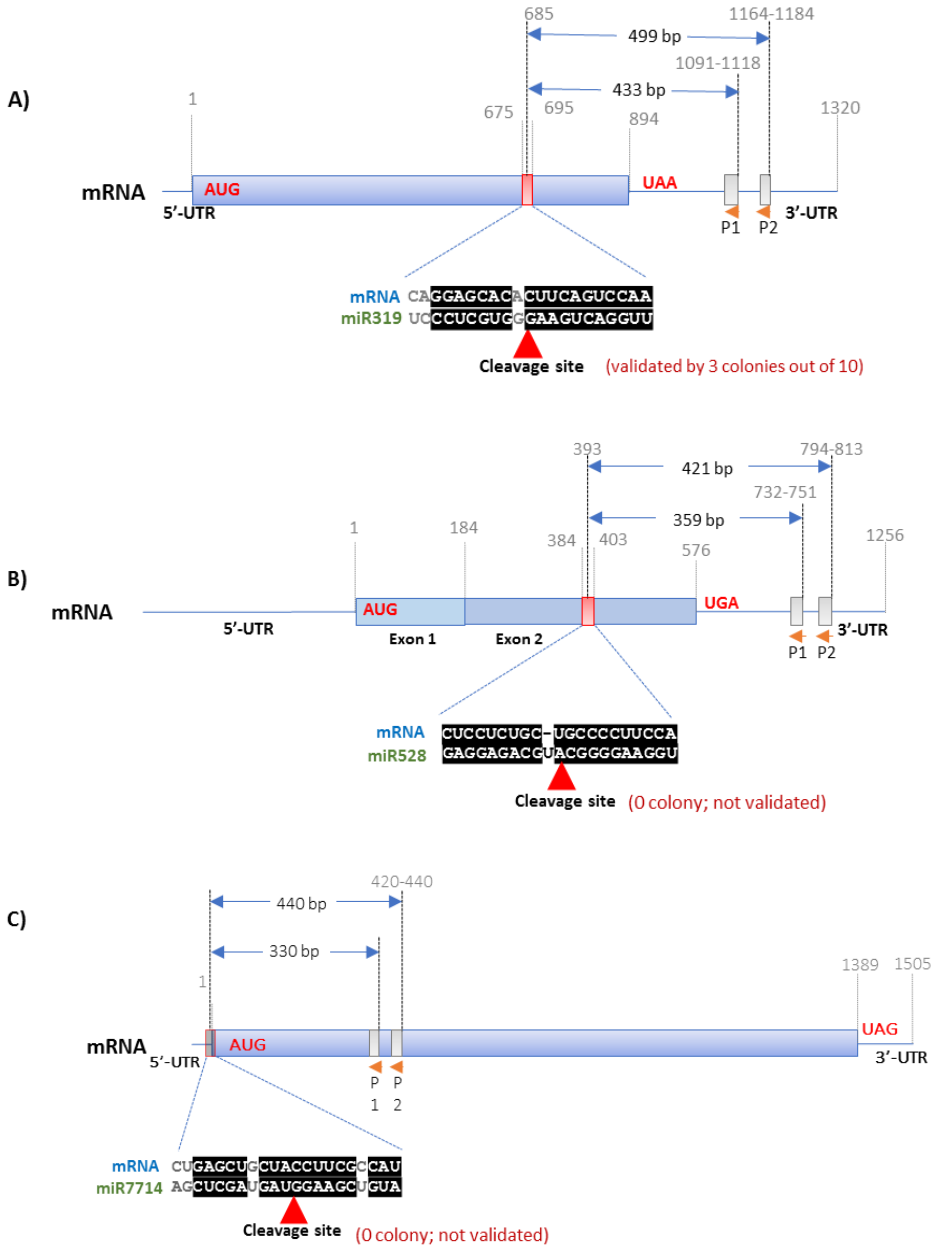


Figure 22. Schematic representation of the predicted cleavage sites in the predicted target mRNA. A) The predicted target mRNA for *miR319* (TraesCS5A02G031900). **B)** The predicted target gene for *miR528* (TraesCS2D02G206000). This gene has two transcripts, each with 12 exons (transcript 1 only is shown to reduce the complexity of the figure). Red rectangles refer to the miRNA binding site. P1 and P2 indicate the two reverse primers used for the first and the second (nested) PCRs. The red thick arrows point to the precise cleavage site in the mRNA and miRNA sequences. Gray numbers refer to the different positions on the target genes showing the start and end of the exons, the position of the miRNA binding site on the mRNA and the locations of the used primers. Black numbers refer to the expected PCR product sizes. The start and stop codons are also shown in red. **C)** The predicted target mRNA for *miR7714* (TraesCS2B02G255900).

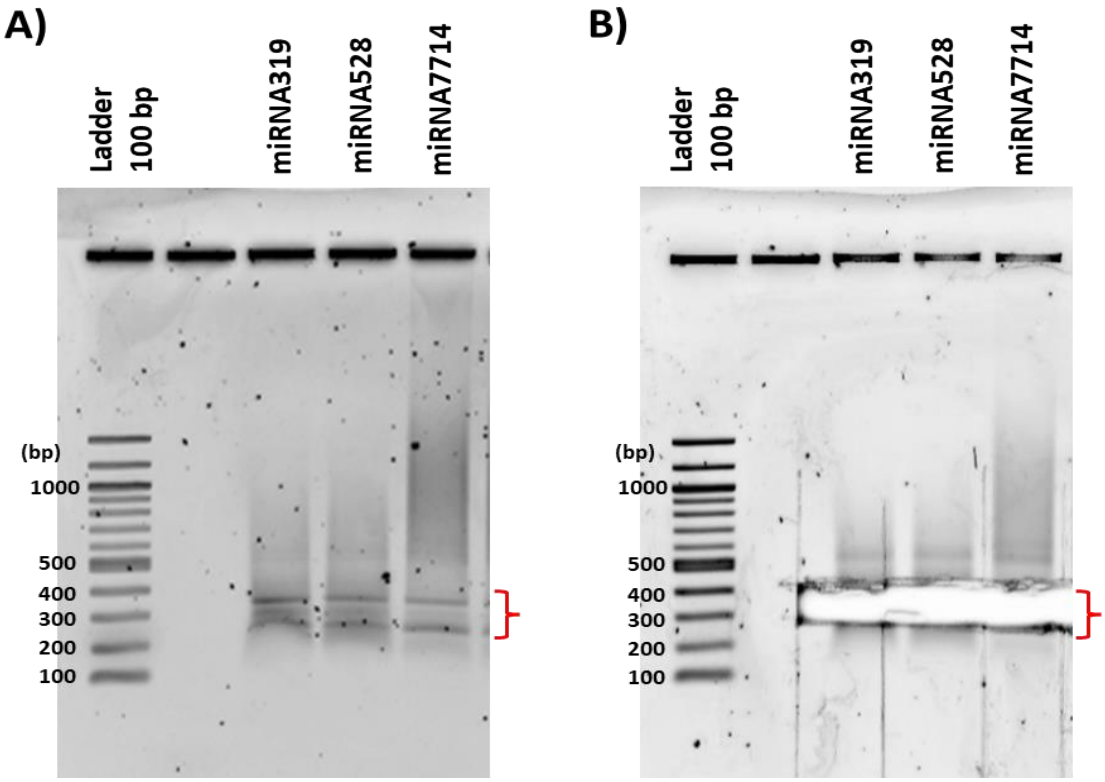


Figure 23. 5' RACE PCR products of the three predicted targets in 1.5% agarose gel before (A) and after (B) cutting the required bands. The gel contained a 1000 base pair ladders in the first lane. The second lane was then left empty, and the third lane contained 25 µl of PCR product for the predicted target mRNA of *miR319*. The fourth lane contained 25 µl of the PCR product for the predicted target mRNA of *miR528* and the fifth lane contained 25 µl of PCR product for the predicted target mRNA of *miR7714*. The PCR products can be seen at approximately 350 bp. The gel was allowed to run at 110V for forty-five minutes and then stained with ethidium bromide.

For *miR319* target, the results showed that three out of ten sequenced colonies contained inserts that start by the complementary sequence of the 5'end of *miR319*. This indicates that the predicted mRNA target was cleaved by *miR319*. Unfortunately, seven out of the ten sequenced colonies resulted with vector and human sequences indicating low efficiency of cloning the 5' RACE PCR products and the occurrence of undesired contamination during the PCR or cloning procedures. Although our validation method could show that the target was cleaved by *miR319* at the predicted site, higher number of colonies with the target sequence is required to be completely sure of this finding. As illustrated in (**Figure 24**), the

sequenced inserts start with TTCAGTCCAA, which is complementary to the first ten 5’ end nucleotides of *miR319* (3’-UCCCUCGUGGGAAGUCAGGUU-5’).

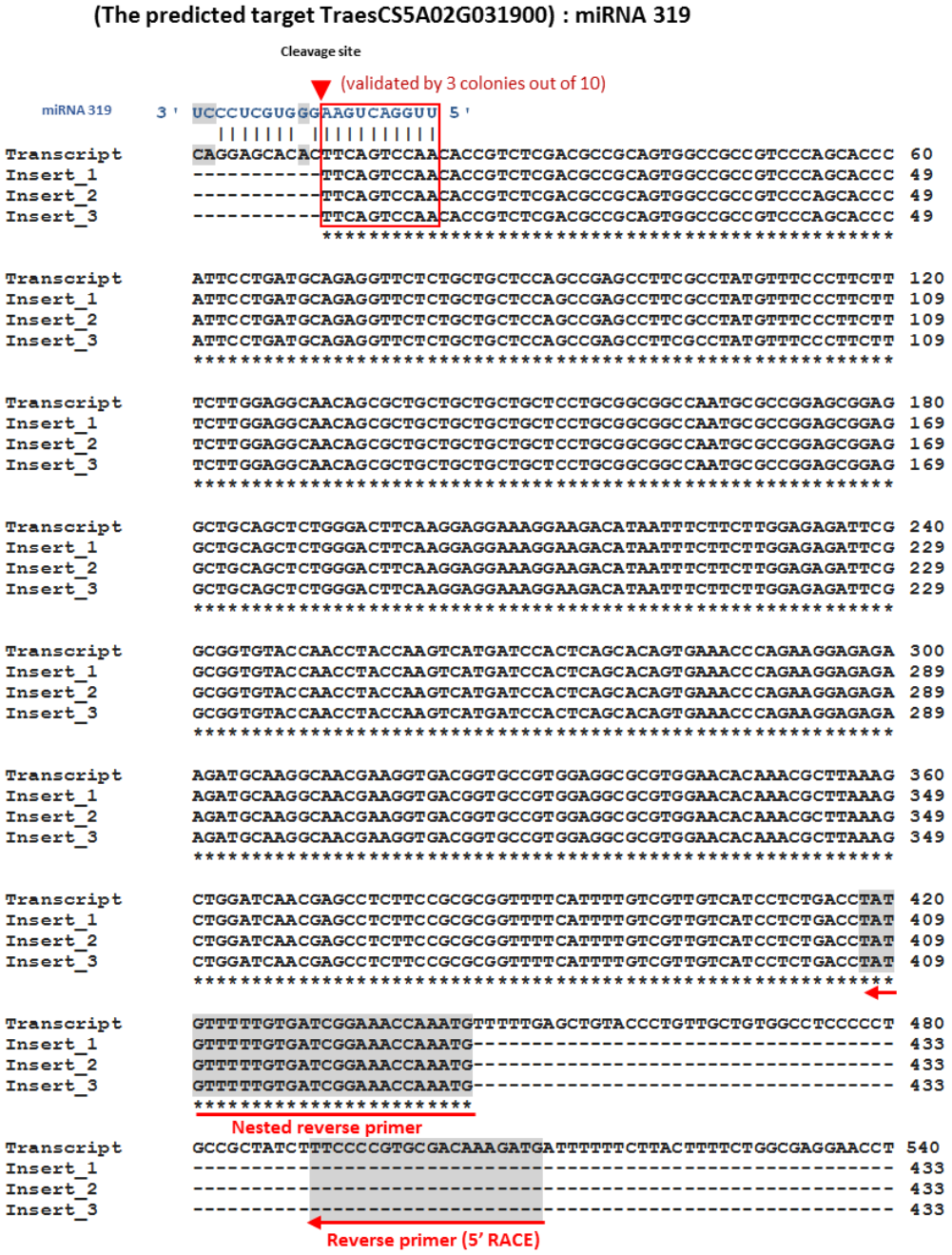


Figure 24. Multiple sequence alignment of the three inserts with the transcript sequence of the predicted target gene TraesCS5A02G031900. The sequence alignment was produced using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Sequences inside the red rectangle shows that they are reverse complementary to the first 10 nucleotides of *miR319*. Red arrows refer to the sequence of the two reverse primers used to amplify the sequenced inserts.

As for *miR7714* and *miR528*, it was not possible to validate the mRNA of the targets as being cleaved at the miRNA binding site. All the obtained sequences for both *miR7714* and *miR528* targets were of human origin. These unwanted outcomes were most probably due to a severe contamination problem that competed the presence of our targets PCR products and hampered their insertion and cloning inside the plasmid. Another explanation of this result could be that the predicted targets of *miR7714* and *miR528* are not the actual targets. Although, for *miR528* there are several targets reported and validated in other studies on monocot plants (Luján-Soto *et al.*, 2021), no targets have been reported so far for *miR7714*, up to the best of our knowledge.

5.2 Discussion

The function of miRNAs is to regulate the expression of protein coding mRNAs. It is possible to predict the targets of miRNAs using bioinformatic tools because the miRNAs are reverse complementary to the target sites on the mRNAs. The predictions are particularly good for plant miRNAs as they are nearly perfectly reverse complementary to their target sites. However, the predictions are still not perfect and there can be some false positives among the predicted targets. Therefore, it is essential to experimentally validate the predicted miRNA targets. I used 5' RACE to validate the predicted targets of the miRNAs that were differentially expressed during drought stress. This is an accepted method to validate miRNA targets because miRNAs cleave their target mRNAs at a specific position, which is opposite to the 10^{th} and 11^{th} nucleotide of the miRNA. MRNAs can be randomly degraded but that leads to different size fragments with different 5' ends. However, if a mRNA is regulated by a miRNA, it is consistently cleaved at that specific position leading to many mRNA fragments that start at exactly the same position. Therefore, if the majority of the PCR products at the end of a 5'RACE experiment starts at the expected cleavage position mediated by a miRNA, that proves that the given mRNA is indeed targeted by the miRNA. In this study, 5' RACE was applied to amplify the cleaved mRNA fragments of the predicted targets of three miRNAs *(MiR319, miR528 and miR7714).* We succeeded to amplify and sequence the cleaved fragments from the mRNAs of the predicted targets for *miR319* and showed that some of the fragments start at the expected cleavage position. The number of

colonies starting at the expected position was only 3 out of 10, therefore we cannot conclude with full confidence that the mRNA is targeted by the miRNA. More work is necessary to fully elucidate whether the mRNA is targeted by *miR319*. The proposed target of *miR319* is gene *TraesCS5A02G031900*, which is a *TCP* transcription factor gene.

MiR319 is known to target *TCP* genes which are involved in the regulation of cell proliferation (Palatnik *et al.*, 2003). It therefore shows significantly greater expression in proliferating cells than it does in expanding cells. The nomenclature of *TCP* transcription factor family derives from the first 4 members that have been identified. These are TEOSINTE BRANCHED1 (TB1) found in maize (*Zea mays*), CYCLOIDEA (CYC) found in the snapdragon (*Antirrhinum majus*), and PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1 (PCF1) and PCF2 found in rice (*Oryza sativa*) (Doebley, Stec and Gustus, 1995). *TCP* transcription factors can combine hormonal, environmental and developmental signals for the purpose of modulating a large number of biological processes. For example two apparently unconnected processes - plant development and plant defence responses - involve TCP proteins, which help maximise plant fitness under conditions of biotic stress (Li, 2015).

In our 5' RACE experiment, we did not include mRNA from plants under drought conditions as we do not expect the occurrence of cleavage due to the very low expression of *miR319* in both Pavon 76 and Yecora Rojo under water deficit condition.

MiR319 is involved with hormone signalling (Schommer *et al.*, 2008), with effects in terms of leaf morphogenesis and stress responses in monocotyledons (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). It has been suggested that *miR319* may be a key master regulator of various stress-response pathways (Zhou and Luo, 2014b). There is also strong evidence that *miR319* operates by suppressing genes which code for transcription factors, particularly those in the TCP family involved in stress responses (Fang *et al.*, 2021).

In maize (*Zea mays*), *miR319* is downregulated in some cultivars as a result of drought and salinity stresses, which is in agreement with our results in wheat. This means that differential levels of miRNA expression may be implicated in increased

tolerance of stress (Zhou et al., 2010). Two forms of *miR319* are found in rice (*Oryza sativa*): *Osa-miR319* and *Osa-miR319b*. Both are important to the plant's response to cold and overexpression results in wider leaf blades (YANG et al., 2013; Wang et al., 2014). Therefore, over-expressing *Osa-miR319b* caused delayed development (Wang et al. 2014). A study of transgenic rice with over-expressed *miR319* found that the transgenic plants were shorter in hight as a result of shorter stem internodes (Liu et al., 2017). The effect on cold tolerance in rice appears to be due to *miR319* being down-regulated in cold stress conditions, which resulted in inducing the expression of the genes targeted by this miRNA (YANG et al., 2013; Wang et al., 2014). This may operate through *miR319* targeting the transcription factors *OsPCF6* and *OsTCP21*, which were down-regulated by cold stress (Wang et al., 2014). Zhou *et al*. (2010) found that *miR319* was strongly responsive to drought stress in rice. However, it was either up or down regulated depending on the plant development stage. Nevertheless, in *A. thaliana*, *MiR319* was found upregulated in drought stress (Sunkar and Zhu, 2004) and saline and cold conditions (Liu et al., 2008). In a transgenic *creeping bentgrass* (*Agrostis stolonifera*) that was generated by inserting the rice gene targeted by *miR319*, the *miR319* was found over-expressed and the plants exhibited changes to their morphology and enhanced tolerance for salinity and drought, correlated with higher wax content in the leaves, higher water retention and lower sodium uptake, due to the down-regulation of the genes targeted by *miR319* (Zhou *et al*., 2013).

MiR528 has been shown to be involved in plant responses to a wide range of stressors. These include water deficit, salt, low-temperatures, nitrate starvation and submergence, as well as stresses from arsenite and arsenate in the cases of maize (*Zea mays*), phalaenopsis orchid (*Phalaenopsis aphrodite*), rice (*Oryza sativa) and* sugarcane (*Saccharum officinarum*) (Ye *et al.*, 2008; An, Hsiao and Chan, 2011; Xu *et al.*, 2011; Ferreira *et al.*, 2012; Nischal *et al.*, 2012; Sharma *et al.*, 2015). *MiR528* has been identified as having exactly the same mature sequence (UGGAAGGGGCAUGCAGAGGAG) in many grass species, including rice, sugarcane, maize, *Brachypodium distachyon* and sorghum (*Sorghum bicolor*) http://www.mirbase.org/; (Kozomara and Griffiths-Jones, 2014), This suggests a similar function for *miR528* in various monocot species. *In silico* analysis has allowed the prediction of eleven possible targets of *miR528* in rice, including

processes of oxidation-reduction (Dai and Zhao, 2011), which suggest that the function of *miR528* may be as an integrator of multiple stresses.

MiR528 has been shown to have several targets in different plant species, demonstrating its functional diversity (Chen, Liu and Xia, 2019). *MiR528* targets two copper-containing laccase transcripts in maize, *ZmLACCASE3* and *ZmLACCASE5*, affecting lignin biosynthesis under specific nitrogen luxury growth conditions (Sun *et al.*, 2018). Although multiple targets have been predicted for *miR528*, only a few of them have been experimentally validated (Shen *et al.*, 2013). A recent study examined the accumulation patterns of zma-miR528 and some targets at the total RNA level, as well as their polyribosomal distribution during somatic embryogenesis in the maize (VS-535) (Luján-Soto *et al.*, 2021). *Zma-miR528* is significantly abundant in immature maize embryos and embryogenic calli (Liu *et al.*, 2005; Wu *et al.*, 2015). The abundance patterns of *miR528* suggest its relevance for target regulation during the proliferative stage of de-differentiated callus (Luján-Soto *et al.*, 2021). The MATE, bHLH, and SOD1a genes have been also validated as *miR528* targets, which show an inverse correspondence with the miRNA profile in total RNA samples during somatic embryogenesis (Luján-Soto *et al.*, 2021).

Two *Creeping Bentgrass miR528* targets have also been identified: *ASCORBIC ACID OXIDASE* and *COPPER ION BINDING PROTEIN1* (*AsCBP1*). These are homologs of rice *AAO* and *CBP1*, which may be involved in oxidation and reduction. *AsAAO* and *AsCBP1* have both been found to have a similar response to high salt stress and starvation of nitrogen. In *miR528*-overexpressing transgenic plants they show significant down-regulation (Yuan *et al.*, 2015).

MiR7714 is known to target asparagine genes which respond to drought conditions by the maintenance of osmotic pressure. Nevertheless, asparagine may also be an indirect response to the restriction of protein synthesis under drought conditions. The concentration of proline may increase in plant tissues that are subjected to stress conditions such as drought and salt stress (Delauney and Verma, 1993; Parry, Flexas and Medrano, 2005; VERDOY *et al.*, 2006). However, there are many studies that have proven that asparagine also accrues at the same time as proline (Miflin *et al.*, 1983) in soybean (Fukutoku and Yamada, 1984; King and Purcell, 2005), in

Alfalfa (Fougère, Le Rudulier and Streeter, 1991), pearl millet (Kusaka, Ohta and Fujimura, 2005) and wheat (Carillo *et al.*, 2005).

However, I could not validate the two targets that were predicted to be regulated by *miR528* and *miR7714*, as the 5' RACE was not successful in amplifying the cleaved fragments in these cases. These results could indicate that the *in-silico* predicted target genes are not targeted by the mentioned miRNA, however, further confirmation is needed to make such a statement. The failure of 5' RACE in amplifying the possible targets of *miR528* and *miR7714* could be due to several reasons. Some species-specific miRNAs lack target genes, while others have targets with either tissue-specific or very low level of expression, which prevents identification by 5'RACE. In addition, those species-specific miRNAs which repress their target gene at the translation step cannot be identified either by 5′ RACE methods, since the target mRNA is not cleaved. Furthermore, some small RNAs identified using high throughput sequencing and predicted to be miRNAs based on the hairpin structure of their precursor, may not be miRNAs but false positive predictions. Finally, human error, such as insufficient RNA quantity and incorrect primer design during PCR amplification, could also contribute to the failure of 5' RACE experiments. Other important reason why we failed in validate the targets of *miR528*, in particular, is that the RNA samples were collected from the plant tissues with very low expression of the miRNA. In our experiment, we collected total RNA from Pavon 76 leaves under no stress conditions where the number of *miR528* reads were almost null. The results obtained from RNA-Seq showed that *miR528* was upregulated in Pavon 76 leaf tissues under water-deficit conditions, which suggests that RNA sampling should have been done from this treatment. For future target validation experiments we recommend considering the miRNA expression data for correct and accurate sampling procedure.

Chapter 6. Conclusion

Food insecurity negatively impacts the health of populations. Among the hundreds of thousands of currently known plant species, humans are dependent on a very small number of them to fulfil their food requirements. It is therefore essential that we understand what affects their productivity and how they adapt to the constraints of the environment if the future good of humanity is to be ensured (Chaves *et al.*, 2013). Wheat is one of the most important plant species for global food security, so understanding what influences its productivity is vital to the future of humanity (Chaves *et al.*, 2013), given that wheat is an essential source of calories in the human diet. Identifying and explaining the genetic mechanisms involved in drought tolerance in wheat is of great importance worldwide considering that the challenges of feeding the ever-increasing human population are to be met by developing agricultural practices that can intensify sustainable production of this essential source of calories. Thus, the overall goal of the current wheat breeding programmes is to achieve the United Nations Millennium Development Goals of ending hunger and extreme poverty (MDG1). These concerns about wheat production have been exacerbated by climate change, with associated water shortages that have severe impact on the farming systems in many parts of the world, especially in North Africa and West Asia, where most of the wheat growing areas are rain-fed due to the lack of water for irrigation (Ortiz *et al.*, 2008).

The development of new drought-tolerant wheat germplasms has been hampered by the lack of suitable techniques, and the focus has been on seeking a single Quantitative Trait Locus (QTL), which is therefore commonly seen as an important way of defining molecular markers for phenotypic expression related to drought tolerance (Gahlaut *et al.*, 2017; Appels *et al.*, 2018). Recent focus has been on gene expression by miRNAs during drought stress (Carrington, 2003; Sunkar, 2010) and miRNAs are now regarded as central elements to the control of drought responsive genes expression (Martin *et al.*, 2010; Shuai *et al.*, 2013), as they assist in post-transcriptional repression to control expression and evolution (Bartel, 2009).

MiRNAs are important in RNA silencing because they act as genetic inhibitors, which bind to effector proteins in the Argonaute family and then to the target nucleic

acids (Carthew and Sontheimer, 2009). Under conditions of environmental stress, including drought, plants over-express or down-regulate certain miRNAs as a response to stress (Liu, Fortin and Mourelatos, 2008). In several studies, miRNA expression following drought exposure has been conducted in different plants such as rice (*Oryza sativa*) (Kantar *et al.*, 2011a), *Medicago truncatula* (Zhou *et al*., 2008), *Brassica napus* (Huang *et al.*, 2010), *Arabidopsis* (Ding and Zhu, 2009) and *Populus trichocarpa* (Lu *et al*., 2008). Less work has been done to identify the functions of specific miRNA molecules in wheat than in maize and rice (Gasparis, Yanushevska and Nadolska-Orczyk, 2017). However, some studies have recognized specific miRNAs that play a role in conferring drought resistance in wheat. For instance, thirteen miRNAs in T. dicoccoides have been found to show differential expression and are implicated in drought response (Kantar, Lucas and Budak, 2011a). Additionally, the same number show reversed expression between leaves of drought-tolerant ("Hanxuan10") and drought-susceptible ("Zhengyin1") T.aestivum genotypes undergoing dehydration stress (Ma *et al*., 2015).

Therefore, the current study has been conducted to provide a comprehensive insight into the role of miRNAs in drought stress in wheat, using two hexaploid wheat (bread wheat) varieties that differ in their responses to drought stress. In our experiments, we used the drought-tolerant cultivar Pavon 76 and the drought-susceptible cultivar Yecora Rojo. We identified a set of drought-related, differentially expressed miRNAs through the preparation and sequencing of a small RNA library and miRNAs sequence analyses, the strongest of which were validated by the Northern blot and 5' RACE techniques. Some of these miRNAs (*miR319*, *miR528* and *miR7714*) are also acknowledged as being part of drought stress response in other cereals, particularly rice, Brachypodium distachyon and T. dicoccoides (Sunkar *et al.*, 2008; Zhou *et al.*, 2010; Kantar, Lucas and Budak, 2011a; Bertolini *et al.*, 2013). The RNA library was constructed using high-definition adaptors that allowed sequencing all the samples successfully using 48 Illumina reverse index primers. The outcomes of our work supported other studies in proving this method to be quite feasible, sensible and cost effective in sequencing large number of independent miRNA samples (Xu *et al.*, 2014, 2015).

The RNA sequence data was analysed using several software and bioinformatics methods to identify drought stress-related miRNAs. PCA was largely used to

identify changes in the miRNA profiles among the studied traits (Arribas-Hernández *et al.*, 2016; Moné *et al.*, 2018; Sell *et al.*, 2020). PCA data summary showed a clearly different clustering with the leaf miRNA dataset compared to that from the root samples, reflecting the fundamental biological and physiological differences between those two tissues. Furthermore, PCA also showed that the leaf samples under drought stress clustered separately from leaf samples from well-watered plants, which could be considered the first evidence of the effect of drought stress on the miRNA in our data, which was confirmed later with more detailed analyses. When leaf samples from drought-stressed plants of the two cultivars, Pavon 76 and Yecora Rojo, were compared, the clustering was also seen to be different, whereas no such variation was seen in well-watered conditions, which is to be expected given the specific characteristics of the two cultivars.

One of the most efficient methods is the differentially expressed miRNA analysis was used to identify trait-related miRNA in many plant species like: wheat (Ma *et al.*, 2015), rice (Peng *et al.*, 2014), and maize (Wei *et al.*, 2009). In our study, the differentially expressed miRNA analysis showed that, under drought stress conditions, there are 25 miRNAs which are expressed differently between the drought resistant (Pavon76) and drought sensitive variety (Yecora Rojo), regardless of the period of drought application, while this number was much lower under well-watered conditions. On the other hand, the number of differentially expressed miRNAs increased from 14 to 24 in the drought resistant variety at ten and twelve days of drought application, respectively, while, relatively smaller numbers (7 and 20) were identified in the drought sensitive variety.

MiRNAs that are consistently expressed differently in the same variety under different drought conditions are more likely to play a role in drought-stress response. In our study we identified a set of seven miRNAs (*miR528*, *miR5168*, *miR390a*, *miR319b*, *miR398*, *miR159* and one similar to *miR7714*) that were consistently expressed differently in Pavon 76 samples under well-watered compared to water-deficit conditions over both stress application times (i.e. after ten and twelve days). In Yecora Rojo, a comparable set of four consistently differentially expressed miRNAs were identified (*miR169a, miR398a*, *miR528* and *miR7714*). Interestingly, we identified four differentially expressed miRNAs that were unique to the drought-resistant variety Pavon 76 and one unique to the drought-sensitive variety, Yecora

Rojo (*miR390a*, *miR319b*, *miR169a*, *miR398* and *miR7714*). These miRNAs could have a special importance as they may play a role in drought-stress response specific to the variety. Sets of differentially expressed miRNAs have been identified in previous studies on the effect of drought stress on the miRNA expression profiles in various plants including the tetraploid wheat cv. Emmer (*miR1867*, *miR474*, *miR398*, *miR1450*, *miR1881*, *miR894*, *miR156*, and *miR1432*) (Kantar *et al.*, 2011c), rice (*miR156*, *miR159*, *miR168*, *miR170*, *miR172*, *miR319*, *miR396*, *miR397*, *miR408*, *miR529*, *miR896*, *miR1030*, *miR1035*, *miR1050*, *miR1088*, *miR1126*, *miR169*, *miR171*, *miR395*, *miR474*, *miR845*, *miR851*, *miR854*, *miR901*, *miR903*, and *miR1125*) (Zhou *et al.*, 2010), Arabidopsis (*miR167*, *miR168*, *miR171*, and *miR396*) (Liu *et al.*, 2008), and Populus (*miR171l-n*, *miR1445*, *miR1446a-e*, and *miR1447*) (Lu *et al.*, 2008b).

Generally speaking, the number of differentially expressed miRNAs in root samples was much lower than those reported in leaves. A possible reason for that is the poor quality and quantity of the extracted RNA from roots comparing with that from leaves. To proof that, we suggest repeating the experiment using the same or different wheat varieties. However, we may find explanations of this finding, if confirmed, by the fact that root tissues are probably less affected by drought stress than leaf tissues. This could be partly explained by the different histology (chlorophyll and the stomata), physiological aspects and environment of the two organs, which make their exposure and hence interaction with the drought stress different.

In total, 44 different miRNA sequences, were found differentially expressed that can be derived from 90 loci in this study. A large proportion of those miRNAs (45.5%) showed significant similarity (P-value < 0.05) to miRNA from other cereals, including wheat, rice, maize and others, although 17 miRNAs did not.

The number of reads obtained from our RNA-seq data for three miRNAs (*miR319*, *miR528* and *miR7714*) was checked using northern blot analysis. In most of the cases the northern blot signals were fairly good; however, it was difficult to interpret the results in a few cases due to the bad quality of the obtained hybridisation signal.

In summary, the number of reads of *miR319* was found to be downregulated by water deficit in both Pavon 76 and Yecora Rojo, which is in agreement with a previous

study on the tetraploid wheat (Kantar *et al.*, 2011b). However, our northern blot results showed that *miR319* was downregulated by water deficit in Pavon 76 but upregulated in Yecora Rojo.

The relevance of these findings can be seen by comparison with findings in other plant species. In maize, *miR319* is downregulated due to salinity and drought stress in some cultivars, which concurs with this study's findings for wheat. Differential levels of miRNA expression may therefore be implicated in increased stress tolerance (Zhou et al., 2010). There are two forms of *miR319* in rice (*Osa-miR319* and *Osa-miR319b*), both of which are important in cold response, with overexpression producing wider leaf blades (Wang et al., 2014; Yang et al., 2013), and overexpression of *Osa-miR319b* resulting in delayed development (Wang, 2014) and reduced height, due to shorter stem inter-nodes (Liu et al., 2017). It would appear that *miR319* is downregulated under cold stress in rice, allowing the genes it targets to be induced (Wang et al., 2014; Yang et al., 2013), perhaps because *miR319* targets two transcription factors which are down-regulated in cold stress (*OsPCF6 and OsTCP21*) (Wang et al., 2014). In terms of drought stress, *miR319* has a strong response in rice, although whether it is upregulated or downregulated depends on the development stage the plant has reached (Zhou *et al*., 2010). In *A. thaliana*, *miR319* is upregulated in drought (Sunkar and Zhu, 2004), saline and cold stress (Liu et al., 2008). In a study of transgenic *creeping bentgrass*, modified by the insertion of the gene targeted by *miR319* in rice, *miR319* was overexpressed, downregulating its targeted genes, resulting in altered morphology, more wax in the leaves, better water retention, reduced sodium uptake and, thus, greater drought and salinity tolerance (Zhou *et al*., 2013).

The number of reads and northern blot results showed that *miR528* was upregulated under water deficit conditions in both Pavon 76 and Yecora Rojo varieties. *MiR528* is implicated in plant response to diverse stressors, including both water deficit and immersion, salinity, cold, lack of nitrates and, in the cases of a number of plants including maize, arsenic stress (An, *et al.*, 2011; Ferreira *et al.*, 2012; Nischal *et al.*, 2012; Sharma *et al.*, 2015; Xu *et al.*, 2011; Ye *et al.*, 2008). It has the same exact mature sequence in several grass species, suggesting a similar function across monocotyledons (Kozomara *et al.*, 2014).

The *miR7714* expression was found to be upregulated by water deficit in Yecora Rojo, but not in Pavon 76 according to the results obtained from both RNA sequencing (number of reads) and northern blot analysis. *MiR7714* has been identified only in a drought tolerant genotype of the wild emmer wheat *(Akpinar, et all.*, 2015) indicating that it is upregulated under water deficit conditions, which agrees with our findings.

The targets for *miR319*, *miR528* and *miR7714* were predicted in this study and 5' RACE was used to amplify the cleaved mRNA fragments at the predicted target sites. The proposed target of *miR319* is TraesCS5A02G031900, which is a TCP transcription factor gene, and it was shown that the mRNA was indeed cleaved by *miR319*. Ten colonies were sequenced, and it was found that three had plasmids with inserts that start by the complementary sequence of the 5' end of each the miRNAs used in the analysis. The sequenced insets begin with TTCAGTCCAA, which is complementary to the first ten 5' end nucleotides of *miR319* (3'-UCCCUCGUGGGAAGUCAGGUU-5'), higher number of colonies with the target sequence is required to be completely sure of this finding.

MiR319 is known to target TCP genes involved in the regulation of cell proliferation (Palatnik *et al.*, 2003). It therefore manifests significantly greater expression in cells which are proliferating than in those which are expanding. TCP transcription factors bring together hormonal, developmental and environmental signals to modulate diverse biological processes, e.g. both development and defence responses in plants involve TCP proteins, assisting in the maximisation of plant fitness under biotic stress conditions (Li, 2015). *MiR319* is also involved in hormone signalling (Schommer *et al.*, 2008), affecting leaf morphogenesis and responses to stress in monocotyledons (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). *MiR319* may indeed be a principal master regulator for various stress-response pathways (Zhou and Luo, 2014b). Evidence also exist showing *miR319* to suppress those genes for transcription factors, especially those in the TCP family which are involved in stress responses (Fang et al., 2021).

For *miR528,* multiple targets have been predicted, which indicate its involvement in diverse functions and several stresses (Dai and Zhao, 2011; Chen et al., 2019). Hence, only a few of these targets have been validated experimentally (Shen *et al.*,

2013). As for *MiR7714*, the predicted targets were found to be asparagine genes, which are directly involved in drought response by maintaining osmotic pressure, and indirectly in response to restricted protein synthesis. The concentration of asparagine and praline seems to increase in concert in plant tissues affected by such stresses as drought and salinity (Miflin *et al.*, 1983), including in wheat (Carillo *et al.*, 2005).

Unfortunately, in this study we were unable to validate the targets of *miR7714* and *miR528*. The cleavage of *miR7714* and *miR528* at the miRNA binding site could not be validated, as 5' RACE was not able to amplify the cleaved fragments for these miRNAs. This could be because certain species-specific miRNAs lack target genes, or because they have targets whose expression is either at a very low level or tissue-specific. This would prevent their identification by 5′ RACE methods. In addition, various species-specific miRNAs repress their target gene translation. This also prevents their identification using either degradome sequencing or 5′ RACE methods. There is also a chance that certain miRNAs which have been identified by means of small RNA sequencing and by predicting the precursor hairpin structure is not real (*i.e.* they are false positives). In addition, it should be noted that human error, such as insufficient RNA quantity and incorrect primer design during PCR amplification, can also contribute to unsuccessful 5' RACE experiments.

In this study, regarding the validation of *miR528* targets, RNA samples were collected from the variety Pavon 76 under no stress conditions, where expression level of this miRNA is extremely low (according to the RNA-Seq data). Thus, the target gene is not expected to be cleaved by *miR528* in the collected tissues. We think that better validation results can be obtained when collecting the RNA samples from Pavon 76 leaf tissue suffering drought stress, where *miR528* is upregulated as it shown by the northern blot and RNA sequencing results.

The current research has important implications for providing a basis for further studies which may help the development of drought resistant wheat which will tolerate the conditions associated with climate change and ensure food security for the future. The identification of the drought stress-related miRNA will facilitate the manipulation of their expression using the currently available gene editing techniques. These techniques are mostly applicable where the miRNA of interest is

expressed at a higher level in the drought sensitive cultivars, which can transform a sensitive wheat cultivar to make it more drought resistant.

Due to the time constraints of the programme, this study was limited to analyse miRNAs from only two wheat varieties (Pavon 76 and Yecora Rojo). However, it is possible that studying the miRNA profiles from other drought resistant or drought sensitive varieties could allow identifying other interesting miRNAs. We, therefore, recommend applying the same methodologies used in the current study to investigate more wheat cultivars and different drought stress-related miRNAs. We also recommend extending the current results obtained from *miR319*, *miR7714*, and *miR528*, which were clearly linked to drought resistance in Pavon 76and Yecora Rojo, to other cereals such as rice, maize and *B. distachyon* to investigate their possible role in drought response in these species. These studies are important to gain a broader insight on the epigenetic mechanisms underlying resistance to drought stress in wheat and other crops, which contribute to our food security in a world with a changing climate.

Appendixes

Appendix 1

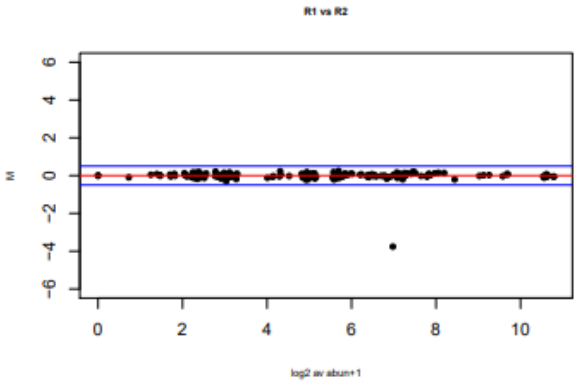
Quality control summary of the 48 sequenced samples.

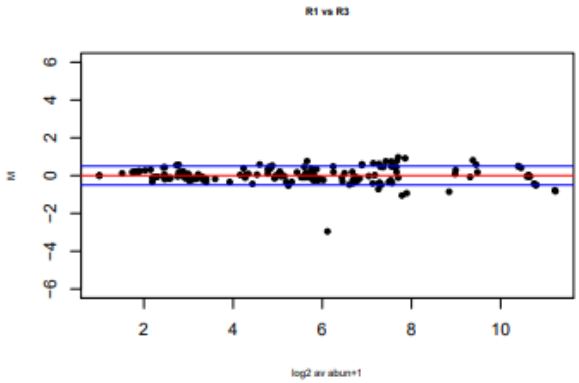
Sample ID	Sample description	Index primer	Total Sequences	Sequence length	%GC	Basic Statistics
R1	Pavon 76_Leaves_Control 10 days	3	9679225	50	50	OK
R2	Pavon 76_Leaves_Control 10 days	9	8260768	50	50	OK
R3	Pavon 76_Leaves_Control 10 days	6	3959020	50	49	OK
R4	Pavon 76_Leaves_Control 12 days	23	7279225	50	51	OK
R5	Pavon 76_Leaves_Control 12 days	20	7922734	50	51	OK
R6	Pavon 76_Leaves_Control 12 days	18	7531898	50	51	OK
R7	Pavon 76_Leaves_Drought 10 days	5	10934314	50	51	OK
R8	Pavon 76_Leaves_Drought 10 days	19	12631297	50	51	OK
R9	Pavon 76_Leaves_Drought 10 days	11	10317804	50	50	OK
R10	Pavon 76_Leaves_Drought 12 days	7	10330728	50	49	OK
R11	Pavon 76_Leaves_Drought 12 days	2	10220576	50	49	OK
R12	Pavon 76_Leaves_Drought 12 days	8	11494142	50	49	OK
R13	Yecora Rojo_Leaves_Control 10 days	10	7693534	50	50	OK
R14	Yecora Rojo_Leaves_Control 10 days	24	6126936	50	50	OK
R15	Yecora Rojo_Leaves_Control 10 days	12	8763339	50	50	OK
R16	Yecora Rojo_Leaves_Control 12 days	22	7343761	50	52	OK
R17	Yecora Rojo_Leaves_Control 12 days	2	9535582	50	50	OK
R18	Yecora Rojo_Leaves_Control 12 days	3	9848425	50	50	OK
R19	Yecora Rojo_Leaves_Drought 10 days	8	16339919	50	49	OK
R20	Yecora Rojo_Leaves_Drought 10 days	7	1033291	50	49	OK
R21	Yecora Rojo_Leaves_Drought 10 days	9	257498	50	50	OK
R22	Yecora Rojo_Leaves_Drought 12 days	10	20103577	50	50	OK
R23	Yecora Rojo_Leaves_Drought 12 days	11	2803832	50	50	OK
R24	Yecora Rojo_Leaves_Drought 12 days	5	8698934	50	50	OK
R25	Yecora Rojo_Roots_Drought 12 days	19	4535392	50	57	OK
R26	Yecora Rojo_Roots_Drought 12 days	12	3437215	50	57	OK
R27	Yecora Rojo_Roots_Drought 12 days	18	2910909	50	56	OK
R28	Pavon 76_Roots_Drought 12 days	19	2587288	50	56	OK
R29	Pavon 76_Roots_Drought 12 days	3	1655277	50	55	OK
R30	Pavon 76_Roots_Drought 12 days	20	384974	50	56	OK
R31	Pavon 76_Roots_Control 10 days	23	3627019	50	55	OK
R32	Pavon 76_Roots_Control 10 days	24	22169456	50	55	OK
R33	Pavon 76_Roots_Control 10 days	6	4135221	50	55	OK
R34	Pavon 76_Roots_Control 12 days	12	15141838	50	55	OK
R35	Pavon 76_Roots_Control 12 days	9	4736899	50	54	OK
R36	Pavon 76_Roots_Control 12 days	18	13918615	50	54	OK

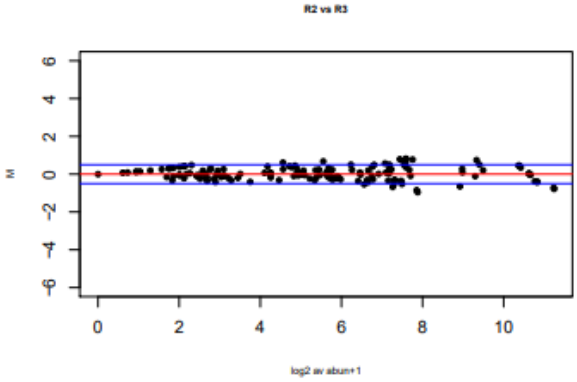
R37	Pavon 76_Roots_Drought 10 days	11	2703890	50	54	OK
R38	Pavon 76_Roots_Drought 10 days	6	14835072	50	54	OK
R39	Pavon 76_Roots_Drought 10 days	23	4819185	50	54	OK
R40	Yecora Rojo_Roots_Control 10 days	8	15522479	50	56	OK
R41	Yecora Rojo_Roots_Control 10 days	22	34262603	50	55	OK
R42	Yecora Rojo_Roots_Control 10 days	20	834614	50	55	OK
R43	Yecora Rojo_Roots_Control 12 days	2	7575215	50	55	OK
R44	Yecora Rojo_Roots_Control 12 days	3	4165280	50	55	OK
R45	Yecora Rojo_Roots_Control 12 days	11	26135020	50	56	OK
R46	Yecora Rojo_Roots_Drought 10 days	7	32294796	50	56	OK
R47	Yecora Rojo_Roots_Drought 10 days	24	5364760	50	56	OK
R48	Yecora Rojo_Roots_Drought 10 days	10	73364201	50	55	OK

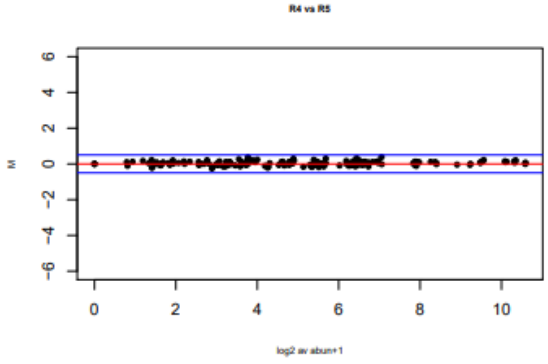
Appendix 2

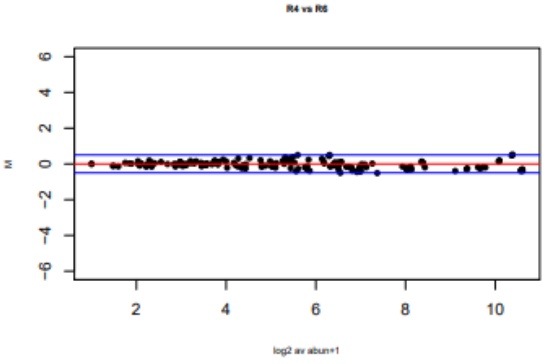
MA dot plots for the pairwise comparison of the replicates of each treatment based on the normalized counts of the identified miRNAs.

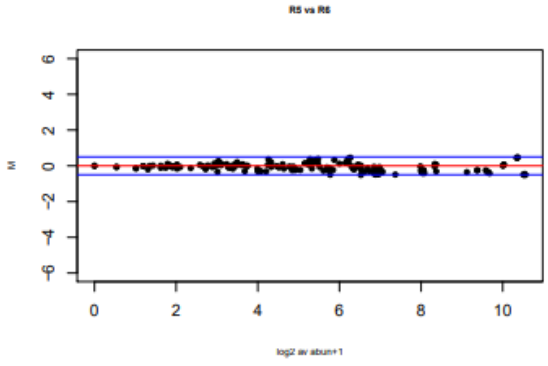


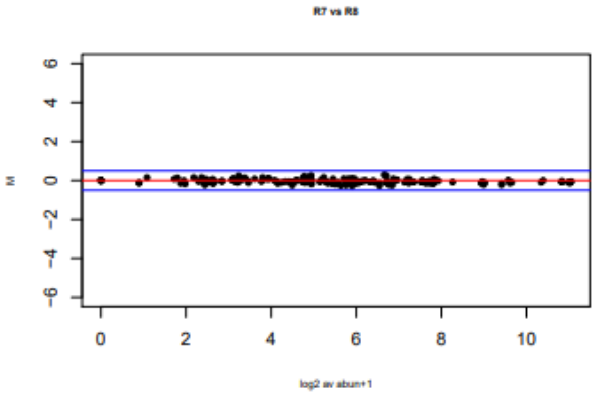


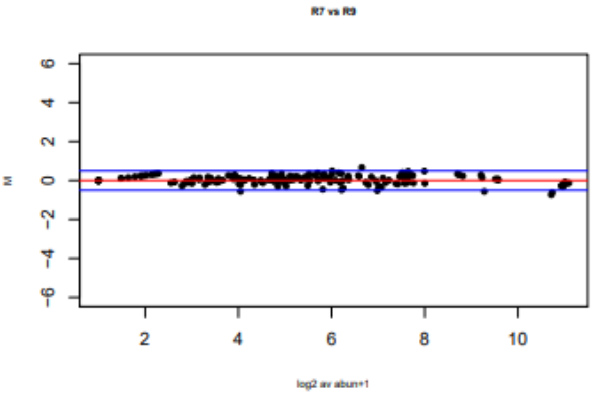


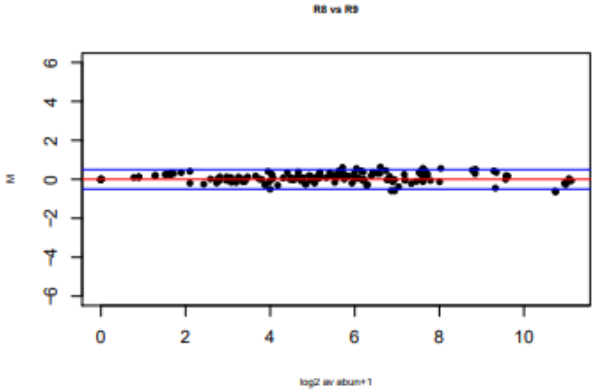


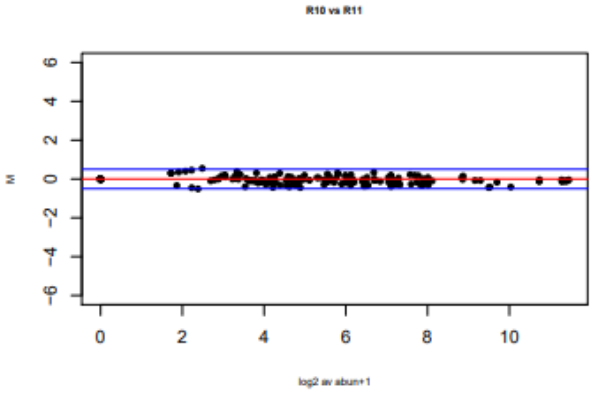


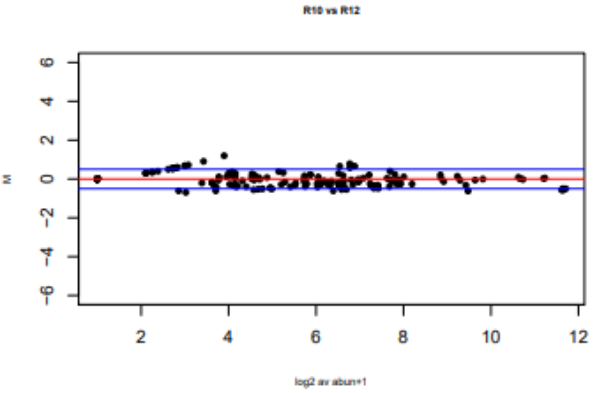


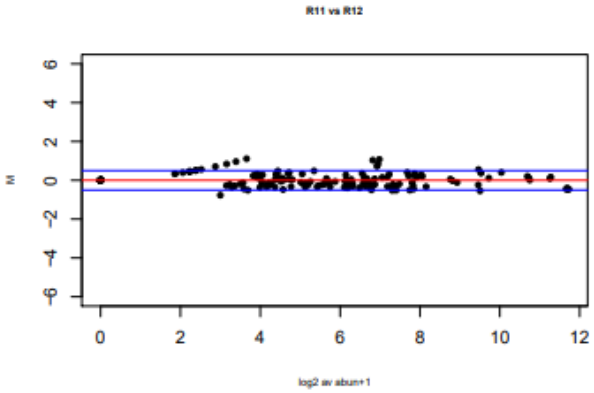

R7 vs R8
M
log2 av abun+1

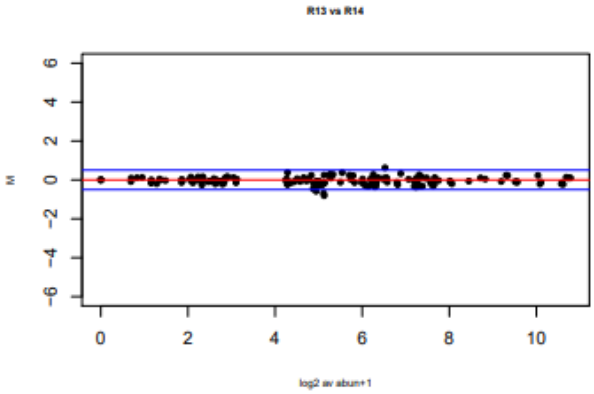

R7 vs R9
M
log2 av abun+1

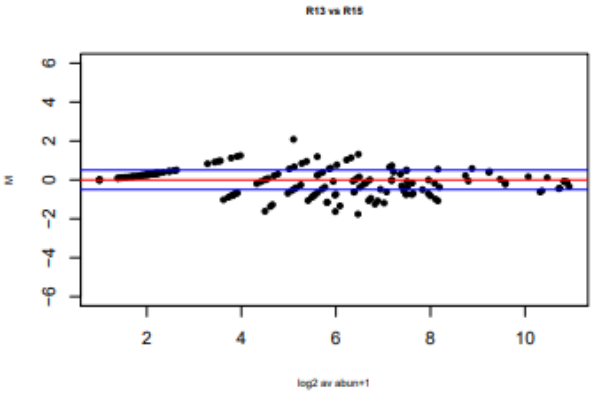

R8 vs R9
M
log2 av abun+1

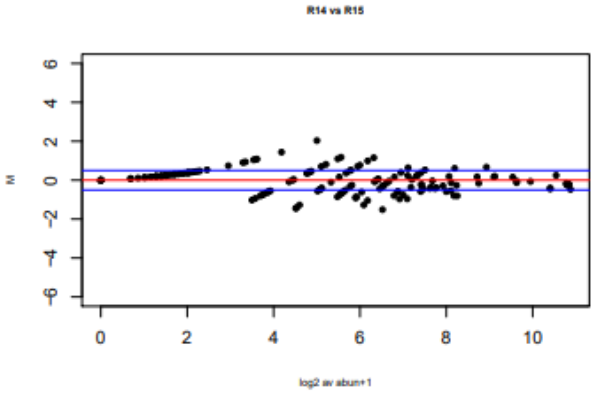

R10 vs R11
M
log2 av abun+1

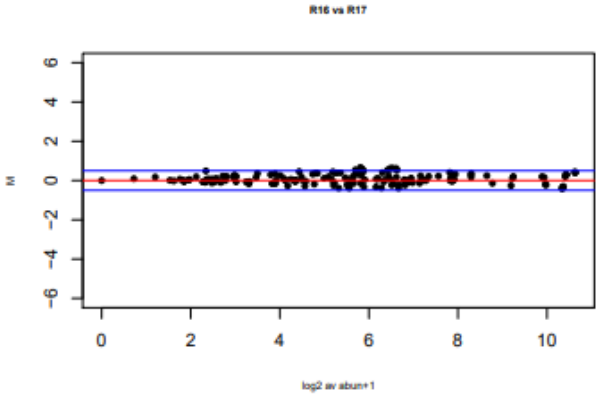

R10 vs R12
M
log2 av abun+1

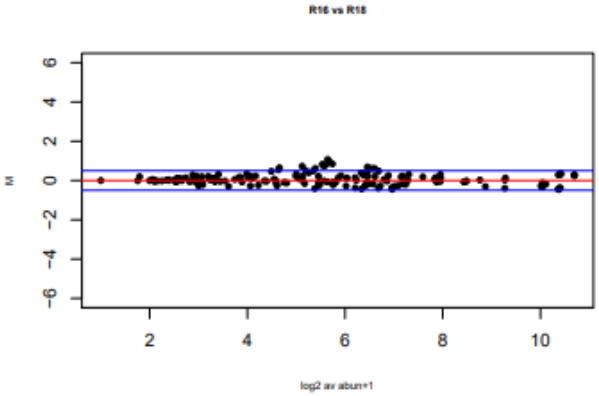

R11 vs R12
M
log2 av abun+1

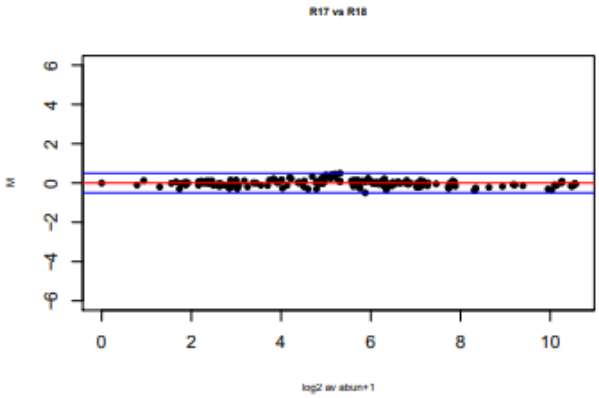

R13 vs R14
M
log2 av abun+1

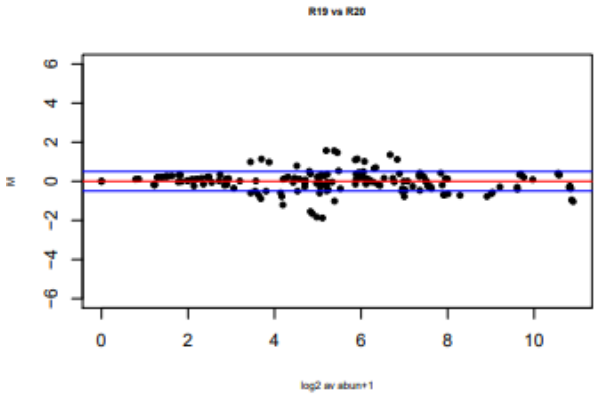

R13 vs R15
M
log2 av abun+1

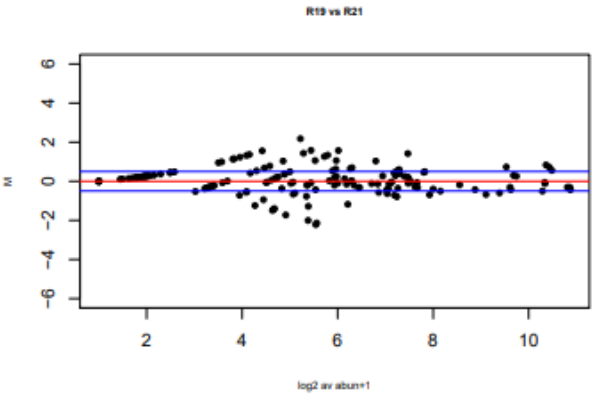

R14 vs R15
M
log2 av abun+1

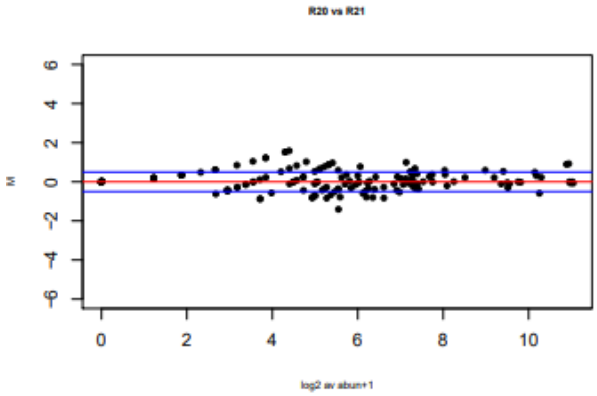

R16 vs R17
M
log2 av abun+1

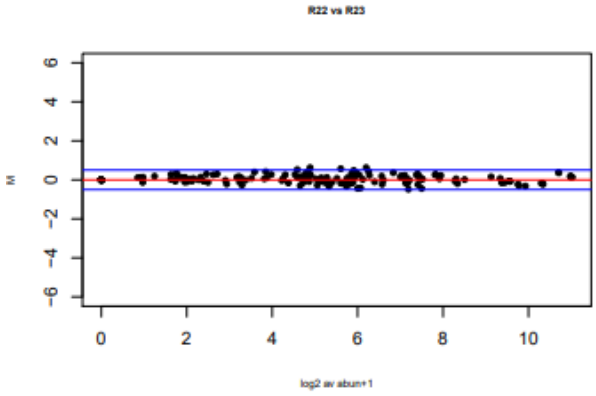

R16 vs R18
M
log2 av abun+1

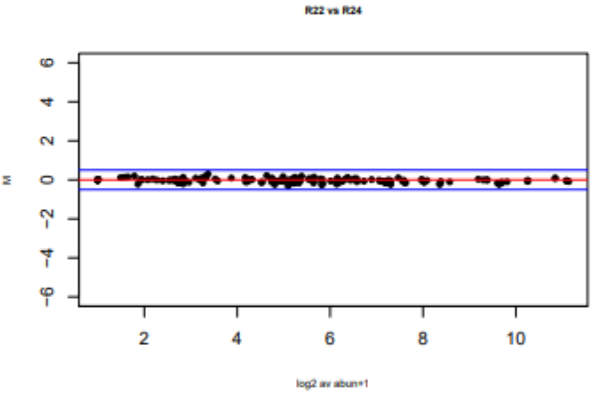

R17 vs R18
M
log2 av abun+1

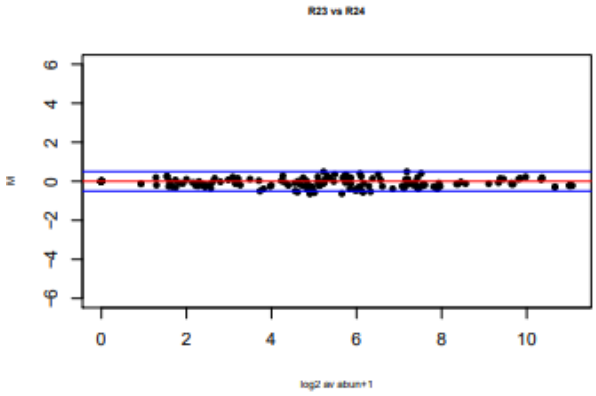

R19 vs R20
M
log2 av abun+1

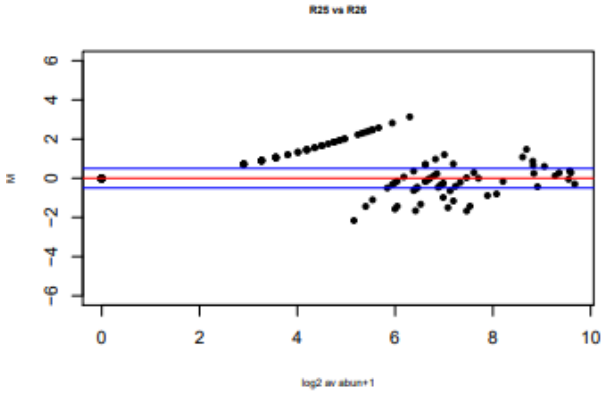

R19 vs R21
M
log2 av abun+1

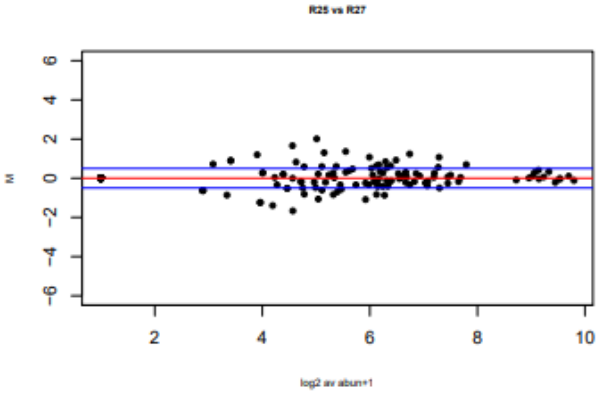

R20 vs R21
M
log2 av abun+1

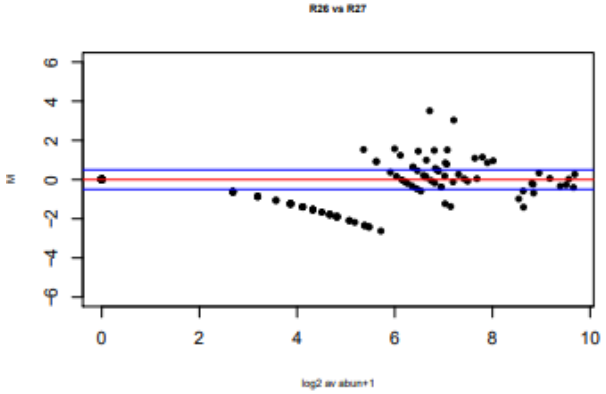

R22 vs R23
M
log2 av abun+1

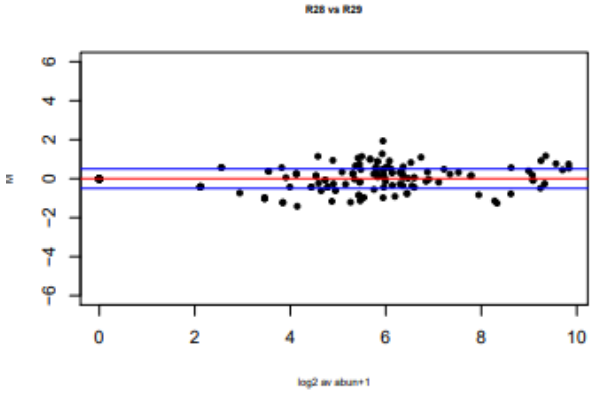

R22 vs R24
M
log2 av abun+1

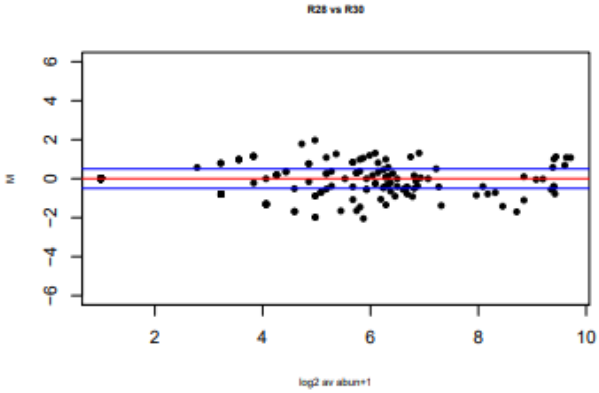

R23 vs R24
M
log2 av abun+1

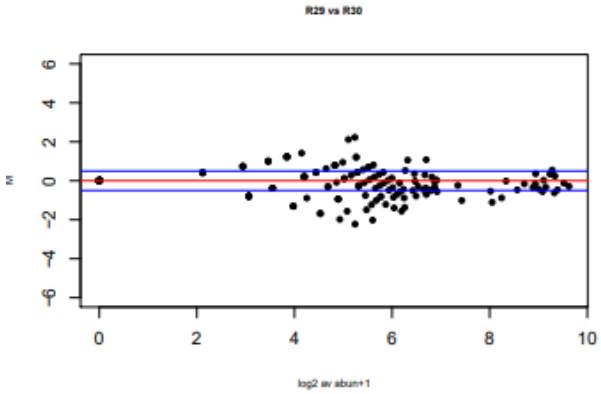

R25 vs R26
M
log2 av abun+1

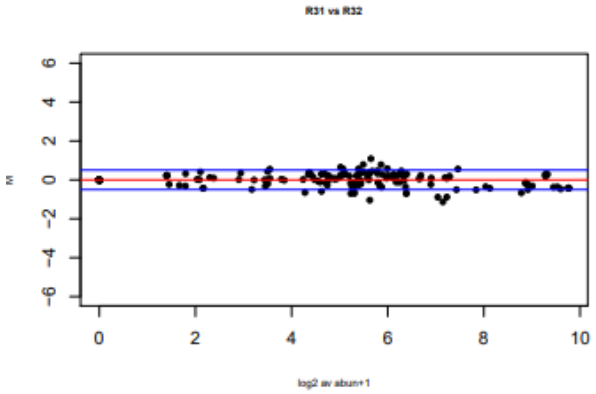

R25 vs R27
M
log2 av abun+1

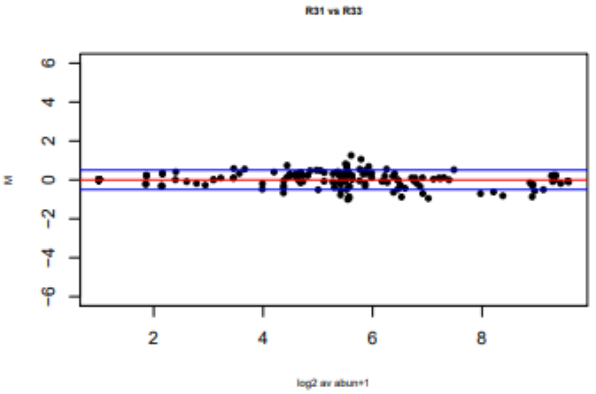

R26 vs R27
M
log2 av abun+1

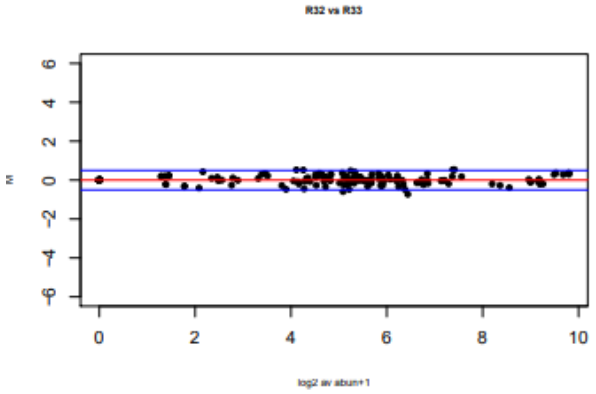

R28 vs R29
M
log2 av abun+1

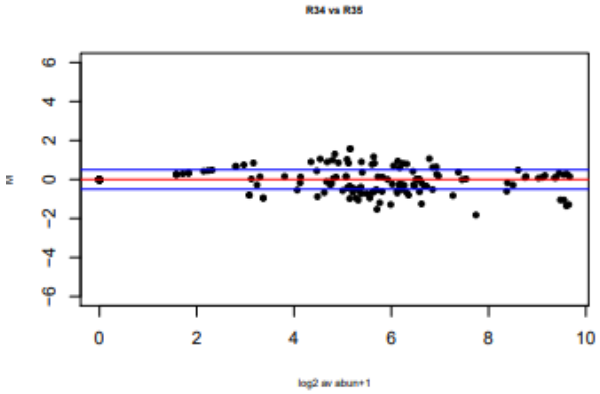

R28 vs R30
M
log2 av abun+1

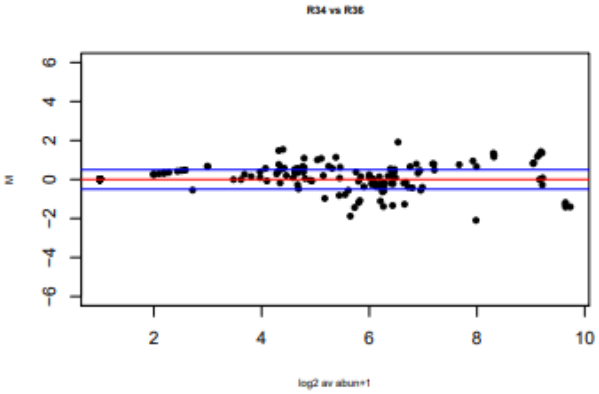

R29 vs R30
M
log2 av abun+1

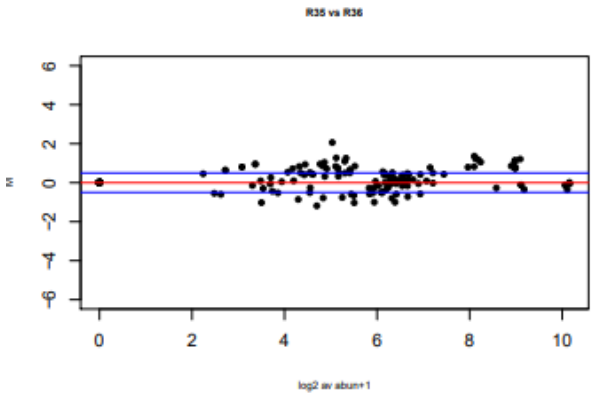

R31 vs R32
M
log2 av abun+1

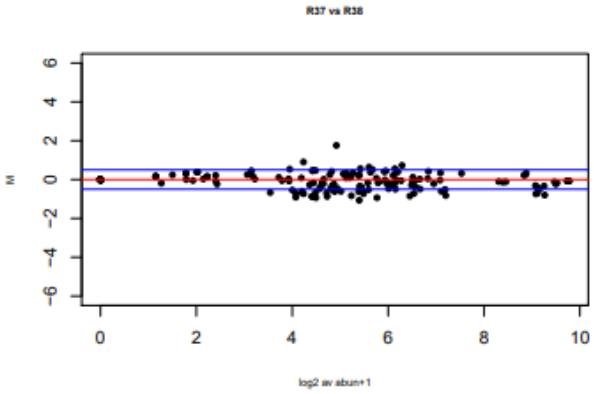

R31 vs R33
M
log2 av abun+1

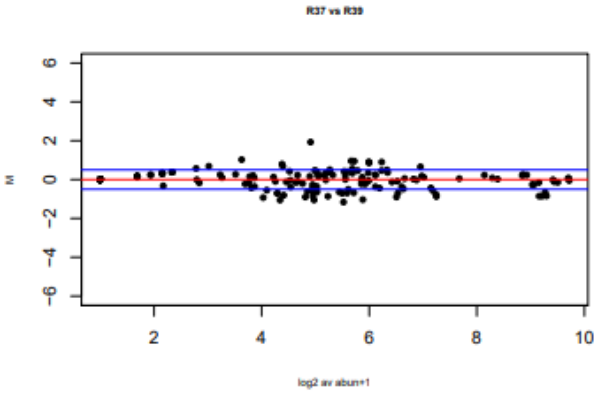

R32 vs R33
M
log2 av abun+1

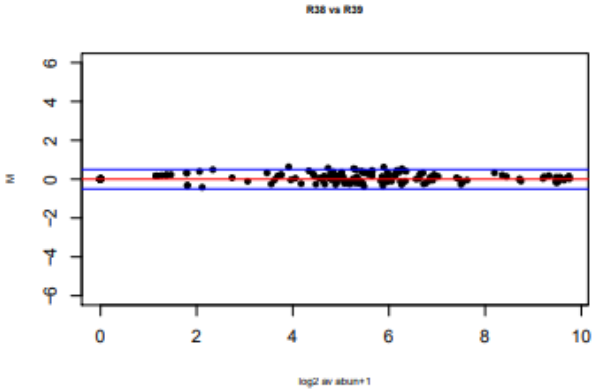

R34 vs R35
M
log2 av abun+1

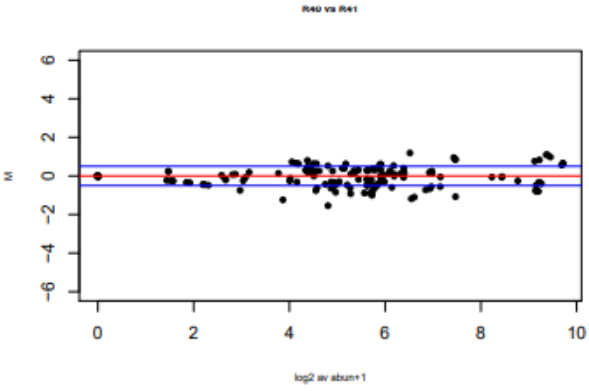

R34 vs R36
M
log2 av abun+1

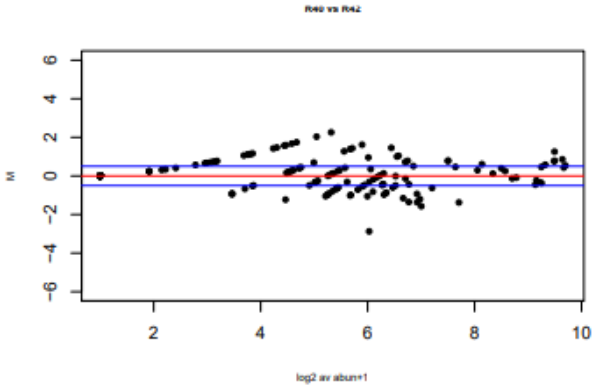

R35 vs R36
M
log2 av abun+1

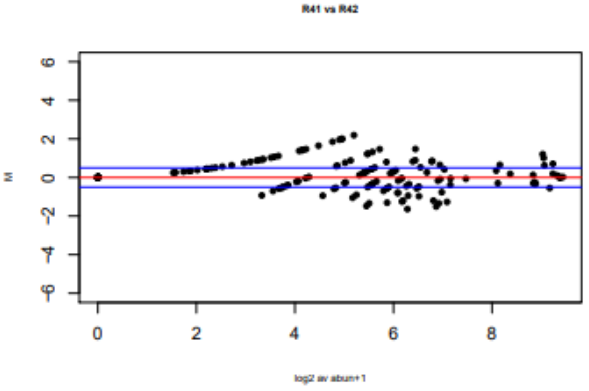

R37 vs R38
M
log2 av abun+1

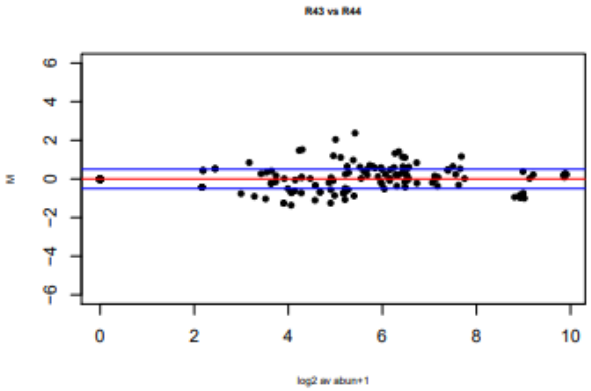

R37 vs R39
M
log2 av abun+1

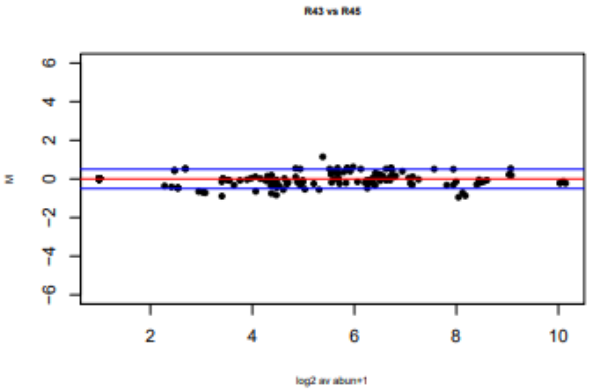

R38 vs R39
M
log2 av abun+1

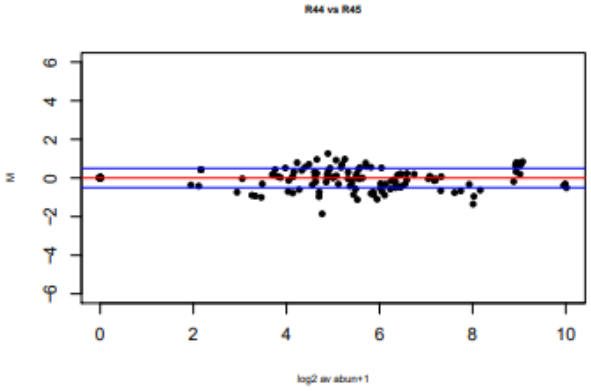

R40 vs R41
M
log2 av abun+1

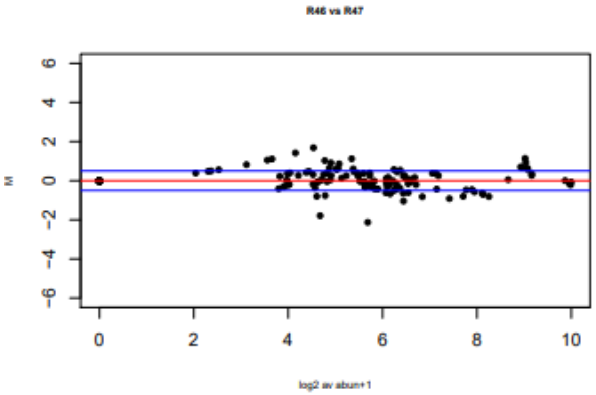

R40 vs R42
M
log2 av abun+1

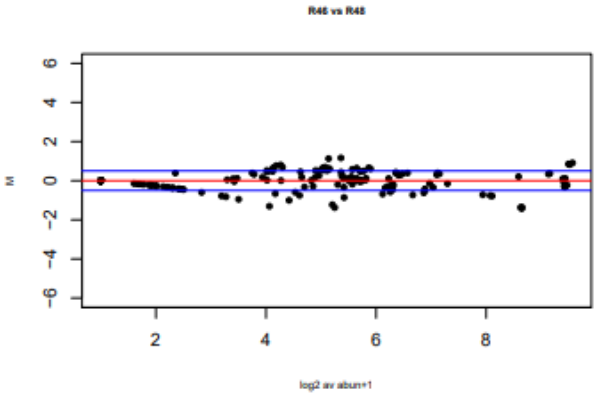

R41 vs R42
M
log2 av abun+1

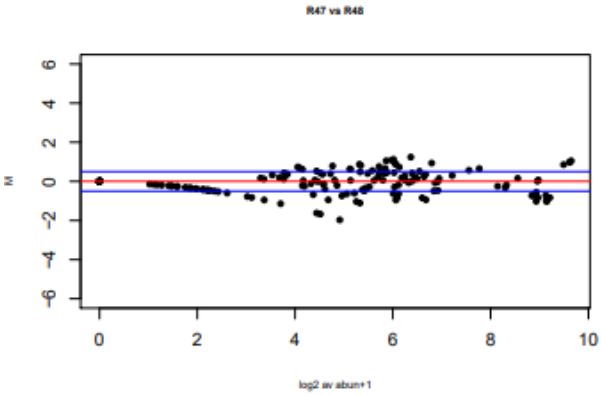

R43 vs R44
M
log2 av abun+1


R43 vs R45
M
log2 av abun+1


R44 vs R45
M
log2 av abun+1


R46 vs R47
M
log2 av abun+1


R46 vs R48
M
log2 av abun+1


R47 vs R48
M
log2 av abun+1

Appendix 3

The identified differentially expressed miRNAs and their overlapping among the different comparison.

miRNA.ID	Overlapping groups	miRNA name (1st hit)	Plant species
AAGCUCAGGAGGGAUAGCGCC_5A:649565390-649565502	L1,L2,L7	miR390a	Arabidopsis thalia
AAGCUCAGGAGGGAUAGCGCC_5B:657146566-657146732	L1,L2,L5,L7	miR390a	Arabidopsis thaliana
AAGCUCAGGAGGGAUAGCGCC_5D:521092217-521092343	L1,L5,L7	miR390a	Arabidopsis thalia
AGAAUCUUGAUGAUGCUGCAU_6A:607452425-607452584	L4	miR172a	Arabidopsis thalia
AGAAUCUUGAUGAUGCUGCAU_6B:702432996-702433283	L4	miR172a	Arabidopsis thalia
AGAAUCUUGAUGAUGCUGCAU_6D:460873256-460873481	L4	miR172a	Arabidopsis thalia
AGUUGAAGAUGAGAUAUUGAA_3B:247570338-247570497	L2,L4	unknown	unknown
AUAGCAUCAUCCAUUCUACCA_4B:654094050-654094305	L2	miR9674b-like	Triticum aestivum
AUCAGGAGAGAUGACACCGA_2B:210258065-210258294	L2,R8	miR1432	Oryza sativa
AUGUAGAAGCACCAGGGUAAG_3A:373156062-373156257	L2	Unknown	Unknown
AUGUCGAAGGUAGUAGCUCGA_7B:737957461-737957552	L1,L2,L5,L7	miR7714-like	Brachypodium
AUUGAACUAAGGAGGGGUGGA_2B:720801630-720801720	R1,R6	Unknown	Unknown
CAGCCAAGGAUGACUUGCCGA_2A:683724053-683724303	L1,L3,L4,L7	miR169a	Arabidopsis thalia
CAGCCAAGGAUGACUUGCCGA_2B:645654934-645655187	L3,L4,L5,L7	miR169a	Arabidopsis thalia
CAGCCAAGGAUGACUUGCCGA_2D:540313472-540313759	L1,L3,L4,L7	miR169a	Arabidopsis thalia
CAGCCAAGGAUGACUUGCCGA_3A:202585447-202585575	L4	miR169a	Arabidopsis thalia
CAGCCAAGGAUGACUUGCCGA_3B:242751068-242751196	L3,L4,L5,L7	miR169a	Arabidopsis thalia
CAUCCUAACAUAAGUGUCUCA_3B:449283776-449283896	L2	Unknown	Unknown
CUCGCCGGUCGCGCGUUCUCC_2B:158881602-158881669	L4	Unknown	Unknown
CUCGCCGGUCGCGCGUUCUCC_7D:421468136-421468314	L4	Unknown	Unknown
CUGCAUUUGCACCUGCACCUA_2D:428671838-428671983	L2	miR530	Oryza sativa
CUUCUGAUUUACUCGUCGUGG_7A:668530071-668530160	R5,R6	Unknown	Unknown
GGUGGGUCUUCUUGGCUAAC_4A:494638262-494638440	L1,L4	unknown	Zea maize
UAGCCAAGGAUGACUUGCCUG_5A:502440252-502440396	L4	miR169h	Arabidopsis thalia
UAGCCAAGGAUGACUUGCCUG_5B:477355303-477355458	L4	miR169h	Arabidopsis thalia
UAGCCAAGGAUGACUUGCCUG_5D:396877526-396877694	L4	miR169h	Arabidopsis thalia
UAGCCAAGGAUGACUUGCCUG_7B:221265370-221265570	L4	miR169h	Arabidopsis thalia
UAGCCAAGGAUGACUUGCCUG_7D:245335473-245335608	L4	miR169h	Arabidopsis thalia
UCCACAGGCUUUCUUGAACUG_2A:758894508-758894652	L2	miR396e	Oryza sativa
UCCACAGGCUUUCUUGAACUG_2B:772062246-772062493	L2	miR396e	Oryza sativa
UCCACAGGCUUUCUUGAACUG_2D:629186110-629186326	L2	miR396e	Oryza sativa
UCCACAGGCUUUCUUGAACUG_6A:614538981-614539249	L2	miR396e	Oryza sativa
UCCGUCCCAUACUAUAAGAGC_6A:212801503-212801721	R5	Unknown	Unknown
UCGGACCAGGCUUCAAUCCCU_5A:450881162-450881313	L1,L2,L4	miR5168	Aegilops tauschii
UCGGACCAGGCUUCAAUCCCU_5B:411091733-411091849	L1,L2,L4,L6	miR5168	Aegilops tauschii
UCGGACCAGGCUUCAUUCCCU_6D:418216628-418216757	L1,L5,L7,R5,R6	miR166m	Oryza sativa
UCGGACCAGGCUUCAUUCCUU_6A:560691652-560691733	L3,L5,R1	miR166c	Aegilops tauschii
UCGGACCAGGCUUCAUUCCUU_6D:418216451-418216534	L5	miR166c	Aegilops tauschii
UGAAGUAGAGCAGAGACCUCA_5B:694314328-694314558	L1,L5,L7,L8,R6	miR9661	Triticum aestivum
UGAGACGAGAUCUCCCCAUAC_4A:609557174-609557261	L2	unknown	unknown

UGAGACGAGAUCUCCCCAUAC_5D:557480963-557481067	L2	unknown	unknown
UGAGAUGAGAUUACCCCAUAC_5B:661123811-661123991	L3	miR9772	Triticum aestivum
UGCCUGGCUCCCUGUAUGCCA_5B:598031772-598031867	L2,L7	miR160a	Oryza sativa
UGCCUGGCUCCCUGUAUGCCA_5D:486487581-486487676	L2	miR160a	Oryza sativa
UGCCUGGCUCCCUGUAUGCCA_6A:113538445-113538531	L2	miR160a	Oryza sativa
UGCCUGGCUCCCUGUAUGCCA_6D:95719039-95719260	L2	miR160a	Oryza sativa
UGCCUGGCUCCCUGUAUGCCA_7A:438858691-438858782	L2	miR160a	Oryza sativa
UGCCUGGCUCCCUGUAUGCCA_7B:383734710-383734802	L2,L7	miR160a	Oryza sativa
UGCCUGGCUCCCUGUAUGCCA_7D:388698517-388698696	L2,L7	miR160a	Oryza sativa
UGGAAGGGGCAUGCAGAGGAG_4B:613386160-613386335	L1,L2,L3,L4,L5,L7	miR528	Triticum aestivum
UGGAAGGGGCAUGCAGAGGAG_4D:483166518-483166697	L1,L2,L3,L4,L7	miR528	Triticum aestivum
UGGAAGGGGCAUGCAGAGGAG_5A:663320628-663320823	L1,L2,L3,L4,L7	miR528	Triticum aestivum
UGGACGAGGAUGUGCAGCUGC_2A:707747459-707747572	L7	miR528	Oryza sativa
UGGACGAGGAUGUGCAGCUGC_2B:680766693-680766982	L7	miR528	Oryza sativa
UGGACGAGGAUGUGCAGCUGC_2D:568229069-568229354	L3,L5,L7	miR9776	Triticum aestivum
UGGAGAAGCAGGGCACGUGCA_2A:62129298-62129500	L2	miR164a	Oryza sativa
UGGAGAAGCAGGGCACGUGCU_6A:574411488-574411628	L2	miR164d	Aegilops tauschii
UGGAGAAGCAGGGCACGUGCU_6B:646613368-646613503	L2	miR164d	Aegilops tauschii
UGGAGAAGCAGGGCACGUGCU_6D:429212150-429212291	L2	miR164d	Aegilops tauschii
UGUGUUCUCAGGUCACCCCUU_5A:505600425-505600630	L2,L3,L4,L5,L7,L8	miR398a-ath	Arabidopsis thalia
UGUGUUCUCAGGUCACCCCUU_5B:480241438-480241607	L4,L5,L7,L8	miR398a-ath	Arabidopsis thalia
UGUGUUCUCAGGUCACCCCUU_5D:400242419-400242534	L2,L3,L4,L5,L7,L8	miR398a-ath	Arabidopsis thalia
UGUGUUCUCAGGUCGCCCCCG_3A:658244028-658244161	L1,L2	miR398- tae	Triticum aestivum
UGUGUUCUCAGGUCGCCCCCG_3D:523327166-523327320	L1,L2	miR398- tae	Triticum aestivum
UUAGAUGACCAUCAGCAAACA_2A:593354662-593354949	L2,L4,L7,L8	miR827	Oryza sativa
UUAGAUGACCAUCAGCAAACA_2B:528817711-528817847	L2,L4,L7,L8,R8	miR827	Oryza sativa
UUCCACAGCUUUCUUGAACUG_6A:596076902-596077022	L2	miR396a	Arabidopsis thalia
UUCCACAGCUUUCUUGAACUU_6A:595988565-595988729	L2,L6	miR396d	Arabidopsis thalia
UUCCACAGCUUUCUUGAACUU_6D:450527450-450527618	L2,L6	miR396d	Arabidopsis thalia
UUCGCCGGAGAAGCAUGCUGC_7A:38967196-38967285	L4	unknown	unknown
UUCGCCGGAGAAGCUUACUGC_3B:755705205-755705387	L1,L7	unknown	unknown
UUCGCCGGAGAAGCUUACUGC_4B:654033954-654034154	L1,L7	unknown	unknown
UUCGCCGGAGAAGCUUACUGC_5A:534566645-534566883	L1,L7	unknown	unknown
UUCGCCGGAGAAGCUUACUGC_5B:272446283-272446353	L1,L7	unknown	unknown
UUCGCCGGAGAAGCUUACUGC_7B:706680829-706680989	L1,L7	unknown	unknown
UUCGCCGGCUGCGCGUUCCCC_3D:453160413-453160571	L2,L7	Unknown	Unknown
UUCGCCGGCUGCGCGUUCCCC_5D:275557431-275557488	L4	Unknown	Unknown
UUCGCCGGUCGCGCGUUCCCCU_2B:408083876-408083976	L7	Unknown	Unknown
UUCGCCGGUCGCGCGUUCCCCU_3D:308784783-308784971	L1,L4,L7	Unknown	Unknown
UUCGCCGGUCGCGCGUUCCCCU_4D:234082939-234082999	L1,L4,L7,L8	Unknown	Unknown
UUGAGACGAAAACAGACCAAC_5D:83923035-83923151	L2,L4	Unknown	unknown
UUGAGCCGUGCCAAUAUCACG_2A:712289716-712289801	L7,R6	miR171b	Arabidopsis thalia
UUGAUUCCCAUUUCACUAGCU_3B:527232708-527232942	L7,L8,R1,R6	Unknown	Unknown
UUGGACUGAAGGGUGCUCCCU_3A:446540933-446541107	L1,L2,L7	miR319b	Brachypodium
UUGGACUGAAGGGUGCUCCCU_3B:429318600-429318775	L6,L7	miR319b	Brachypodium

UUGGACUGAAGGGUGCUCCCU_3D:330699493-330699744	L1,L2,L5	miR319b	Brachypodium
UUUGAGACGAACACUGACCAA_5B:91006912-91007179	L2	unknown	unknown
UUUGGUUUGAAGGGAGCUCUG_4A:614704120-614704296	L1,L2,L4	miR159	Pinus densata
UUUGGUUUGAAGGGAGCUCUG_5D:553856802-553856978	L4	miR159	Pinus densata
UUUUUGGAUGUGCUCCUCUAG_6B:712109341-712109470	L4,L7	unknown	unknown

Legends of Appendix 3

Tissue	Group	Comparison
Leaves	L1	Differentially expressed miRNA in water deficit vs well-watered at 10 days Pavon
	L2	Differentially expressed miRNA water deficit vs well-watered at 12 days Pavon
	L3	Differentially expressed miRNA water deficit vs well-watered at 10 days Yecora Rojo
	L4	Differentially expressed miRNA water deficit vs well-watered at 12 days Yecora Rojo
	L5	Differentially expressed miRNA Pavon vs Yecora Rojo water deficit at 10 days
	L6	Differentially expressed miRNA Pavon vs Yecora Rojo well-watered at 10 days
	L7	Differentially expressed miRNA Pavon vs Yecora Rojo water deficit at 12 days
	L8	Differentially expressed miRNA Pavon vs Yecora Rojo well-watered at 12 days
Roots	R1	Differentially expressed miRNA water deficit vs well-watered at 10 days Pavon
	R5	Differentially expressed miRNA Pavon vs Yecora Rojo water deficit at 10 days
	R6	Differentially expressed miRNA Pavon vs Yecora Rojo well-watered at 10 days
	R8	Differentially expressed miRNA Pavon vs Yecora Rojo well-watered at 12 days

Appendix 4

Averages of the normalized count values for the identified miRNAs in the different leaf samples

miRNA ID	Pavon 76				Yecora Rojo			
	10 days		12 days		10 days		12 days	
	Well-watered	Water deficit	Well-watered	Water deficit	Well-watered	Water deficit	Well-watered	Water deficit
AAAGGCAGGUCCCUUAAGGCA_7A:172978078-172978254 _Unknown	45.74	47.77	55.18	65.67	29.97	42.74	52.20	32.6
AAGCUCAGGAGGGAUAGCGCC_5A:649565390-649565502_miR390a	87.50	42.50	66.60	33	77.26	89.70	88.80	74
AGAAUCUUGAUGAUGCUGCAU_6A:607452425-607452584_miR172a	272.75	302.7	192.3	347.7	422.8	334	163	365
AGUUGAAGAUGAGAUAUUGAA_3B:247570338-247570497_Unknown	29.63	86.71	17.80	150.20	29.72	54.17	16.68	67.7
AUAGCAUCAUCCAUCCUACCA_4D:507427639-507427750_Unknown	992.13	1209.36	868.51	1293.91	810.73	1047.8	740.8	999
AUAGCAUCAUCCAUUCUACCA_4B:654094050-654094305_miR9674b-like	413.7	925.3	759.5	840.5	553.22	714.06	713.2	816
AUCAGGAGAGAUGACACCGA_2B:210258065-210258294_miR1432	45.68	25.11	121.11	27.66	44.91	23.91	49.93	22.7
AUGUAGAAGCACCAGGGUAAG_3A:373156062-373156257_Unknown	27.52	47.95	24.74	60.57	20.24	57.64	25.87	36.7
AUGUCGAAGGUAGUAGCUCGA_7B:737957461-737957552_miR7714-like	7.29	0.00	7.28	0.00	6.96	10.55	10.11	10.4
AUUGAACUAAGGAGGGGUGGA_2B:720801630-720801720_Unknown	128.54	177.35	181.62	198.74	119.78	215.1	124.4	209
AUUGUGUACAGAGGGAGUAGU_3B:587564205-587564383_Unknown	25.80	24.97	22.06	16.44	19.38	18.10	24.49	27.9
CAGAACCAGAAUGAGUAGCUC_2D:537495834-537495917_Unknown	8.83	15.11	9.36	20.50	6.75	6.95	8.10	13.4
CAGCCAAGGAUGACUUGCCGA_2A:683724053-683724303_miR169a	151.9	95.1	141.7	101	148.2	57.14	127.58	35.2
CAUCCUAACAUAAGUGUCUCA_3B:449283776-449283896_Unknown	4.56	8.58	4.31	19.22	2.75	7.66	3.60	13.5
CUCGCCGGUCGCGCGUUCUCC_2B:158881602-158881669_Unknown	6.05	11.6	11.4	10.7	3.98	11.3	10.2	4.5
CUGCAUUUGCACCUGCACCUA_2D:428671838-428671983_miR530	7.07	2.75	17.77	0.00	4.17	1.89	10.52	2.62
CUUCUGAUUUACUCGUCGUGG_7A:668530071-668530160_Unknown	18.17	26.60	14.78	31.93	7.87	30.82	10.86	19.3
CUUGAACUUCUCCAUAGCAUC_4D:507476510-507476746_miR9674b-like	212.32	226.51	182.97	235.70	200.88	205.7	195.9	211
CUUGCGAGCAACGGAUGAAUC_6B:665410920-665411161_Unknown	9.81	8.75	12.84	14.69	5.47	8.40	13.42	5.98
GGGAAAAUAAGGAGUAACGAC_4A:104238549-104238615_Unknown	45.67	53.57	39.82	43.17	33.61	33.47	28.78	27.1
GGUGGGUCUUCUUGGCUAAC_4A:494638262-494638440_Unknown	50.36	0.88	4.40	0.00	22.31	12.47	28.84	0.82
UACGGCCUGAUGACAUCCACG_3A:163805974-163806100_Unknown	85.00	115.44	128.84	124.02	77.50	94.50	90.46	81.4
UAGAAUGGCUGGUGCUAUGGA_4B:654095124-654095247_Unknown	228.14	399.61	251.30	338.98	261.37	238	235.1	262
UAGCCAAGAAUGACUUGCCUG_2A:759843144-759843282_miR169	57.4	15.35	20.12	8.9	71.89	15	23.42	5.8
UAGCCAAGGAUGACUUGCCUG_2A:759835624-759835894_miR169h	7.5	5.06	4. 1	1.1	10.32	3.38	6.40	0.00
UCCACAGGCUUUCUUGAACUG_2A:758894508-758894652_miR396e	2518.50	3334.4	2382.41	4705.2	2854.08	2929	2197	3238
UCCGUCCCAUACUAUAAGAGC_6A:212801503-212801721_Unknown	6.22	9.55	4.25	3.50	4.99	1.70	6.40	6.81
UCGGACCAGGCUUCAAUCCCU_5A:450881162-450881313_miR5168	2255.92	736.98	1757.43	716.46	1190.62	1197	2466	1189
UCGGACCAGGCUUCAUUCCCC_1A:326329166-326329435_miR166	594.40	573.15	720.9	655.5	484.66	643	909.33	791
UGAAGCUGCCAGCAUGAUCUA_5A:580934114-580934281_miR167	226.6	170.45	138.3	188.30	242.14	226	200	243
UGAAGUAGAGCAGAGACCUCA_5B:694314328-694314558_miR9661	1161.20	193.30	180.05	173.12	1632.47	1826.3	995.5	1478
UGAAUUUGUCCAUAGCAUCAG_4D:507434580-507434746_Unknown	209.9	250.8	106	292.38	252.44	223.32	125.85	199
UGAGACGAGAUCUCCCCAUAC_4A:609557174-609557261_Unknown	4.2	3.9	10.1	0.00	1.92	2.2	4.00	3.3
UGAGAUGAGAUUACCCCAUAC_5B:661123811-661123991_miR9772	324.2	186.8	219.50	230.81	269.2	163.4	182.05	123
UGAUUGAGCCGCGCCAAUAUC_1A:366114102-366114228_miR171	43.6	41.1	72.18	47.3	63.6	58.87	47.85	47.2
UGCCAAAGGAGAGUUGCCCUG_2A:652328872-652328990_miR399	10.07	11.39	10.73	10	4.6	2	7.3	3.1
UGCCUGGCUCCCUGUAUGCCA_5B:598031772-598031867_miR160a	79.68	60.2	68.49	25.3	71.9	38.3	71.1	44
UGGAAGGGGCAUGCAGAGGAG_4B:613386160-613386335_miR528	3.37	172.3	2.7	147.98	2.17	47.5	7.26	52.4

UGGACGAGGAUGUGCAGCUGC_2A:707747459-707747572_miR9776	47.3	43.3	45.62	35.26	38.9	63.6	49.0	81.3
UGGAGAAGCAGGGCACGUGCA_1A:403039768-403039869_miR164a	317.2	302.9	416.1	230.4	362.8	251.2	368	277
UGUGUUCUCAGGUCACCCCUU_5D:400242419-400242534_miR398-ath	7	57	4	105.50	4	62	113	21.4
UGUGUUCUCAGGUCGCCCCCG_3D:523327166-523327320_miR398- tae	7.88	103.88	2.41	77.25	4.06	34.88	6.62	35.6
UUAAUUUGUCCAUAGCAUCCG_3D:524000757-524000864_Unknown	93.00	112.12	66.98	116.17	88.14	75.57	65.3	82
UUAGAUGACCAUCAGCAAACA_2A:593354662-593354949_miR827	1370.8	1665.8	1233.5	1517.25	1385.5	1134.7	1073.3	1047
UUCCAAAGGGAUCGCAUUGAU_2D:650221328-650221423_miR393	931.6	1051.3	787.59	901	807.8	770	717.7	689
UUCCACAGCUUUCUUGAACUU_6A:595988565-595988729_miR396d	3482.74	2507.4	1308.89	3845.6	1923.14	2650	1661	2610
UUCGCCGGAGAAGCAUGCUGC_7A:38967196-38967285_Unknown	7.85	0.00	0.00	2.50	14.53	1.98	25.89	0.00
UUCGCCGGAGAAGCUUACUGC_3B:755705205-755705387_Unknown	5.4	21.5	18	26.6	13.5	8.4	11.47	5.4
UUCGCCGGCUGCGCGUUCCCC_7D:208677262-208677523_Unknown	4.5	17	18	8.92	9.5	16.75	20	12
UUCGCCGGUCGCGCGUUCCCCU_4D:234082939-234082999_Unknown	2.5	3.3	10.5	15.6	5	4	3.1	3.24
UUGAGACGAAAACAGACCAAC_5D:83923035-83923151_Unknown	684.31	946.01	403.29	1571.4	486.64	714	367.0	863
UUGAGCCGUGCCAAUAUCACG_2A:712289716-712289801_miR171b	44.87	33.38	31.91	17.78	41.55	39.1	57.19	80.7
UUGAUUCCCAUUUCACUAGCU_3B:527232708-527232942_Unknown	43.97	7.27	1.71	0.00	19.67	43.2	20.42	58.6
UUGGACUGAAGGGUGCUCCCU_3D:330699493-330699744_miR319	4.12	1.18	2.97	0.00	4.08	1.21	4.16	2.63
UUGGCAUUGAGGGAGUCAAGC_7B:164100211-164100386_miR160	110.5	101.7	118.66	67.3	126.3	109.4	133	113
UUUCUGAAGGGUUUAUCGAGG_6A:29948423-29948504_Unknown	220.44	191.35	139.02	177.69	147.42	197.2	133.9	136
UUUGAGACGAACACUGACCAA_5B:91006912-91007179_Unknown	164.90	342.08	134.20	417.88	138.85	207.2	132.3	221
UUUGGCUUGAAGGGAGCUCUG_5D:553838500-553838672_miR159	24.3	36.18	42.7	21.13	22	25	38.3	24.3
UUUUUGGAUGUGCUCCUCUAG_6B:712109341-712109470_Unknown	83.33	112.20	142.68	146.98	69.88	92.33	146.0	59.7

Appendix 5

Averages of the normalized count values for the identified miRNAs in the different root samples

miRNA ID	Pavon 76				Yecora Rojo			
	10 days		12 days		10 days		12 days	
	Well-watered	Water deficit	Well-watered	Water deficit	Well-watered	Water deficit	Well-watered	Water deficit
AAAGGCAGGUCCCUUAAGGCA_7A:172978078-172978254_Unknown	12.30	12.13	18.26	20.30	8.65	12.98	18.68	18.43
AAGCUCAGGAGGGAUAGCGCC_5A:649565390-649565502_miR390a	87.05	70.02	85.33	81.48	94.38	82.46	85.07	59.32
AAGCUCAGGAGGGAUAGCGCC_5B:657146566-657146732_miR390a	96.94	65.83	85.45	76.06	103.83	83.24	84.03	79.45
AAGCUCAGGAGGGAUAGCGCC_5D:521092217-521092343_miR390a	92.59	66.23	78.94	89.05	95.73	79.05	90.77	95.13
AGAAUCUUGAUGAUGCUGCAU_6A:607452425-607452584_miR172a	11.72	13.22	17.67	21.86	16.51	15.81	10.39	7.69
AGAAUCUUGAUGAUGCUGCAU_6B:702432996-702433283_miR172a	14.91	15.70	19.05	14.00	23.09	16.70	16.34	0.00
AGAAUCUUGAUGAUGCUGCAU_6D:460873256-460873481_miR172a	17.76	14.04	18.76	6.58	11.81	16.39	13.25	28.25
AGUUGAAGAUGAGAUAUUGAA_3B:247570338-247570497_Unknown	11.55	18.46	24.60	24.52	22.73	13.84	16.42	3.27
AUAGCAUCAUCCAUCCUACCA_4D:507427639-507427750_Unknown	306.39	348.84	312.89	240.92	264.88	240.20	332.67	274.1
AUAGCAUCAUCCAUCCUACCC_4B:654902301-654902423_miR9674b-like	342.51	319.76	307.97	340.80	326.33	337.54	312.26	370.4
AUAGCAUCAUCCAUCCUACCC_5A:692390195-692390476_miR9674b-like	317.84	317.90	316.73	341.35	342.04	337.17	329.36	392.4
AUAGCAUCAUCCAUUCUACCA_4B:654094050-654094305_miR9674b-like	71.67	75.35	82.75	82.32	94.41	120.24	124.88	82.94
AUCAGGAGAGAUGACACCGA_2B:210258065-210258294_miR1432	84.00	33.49	44.37	24.01	43.21	22.08	10.04	8.71
AUGUAGAAGCACCAGGGUAAG_3A:373156062-373156257_Unknown	10.21	7.44	7.39	9.79	5.01	7.24	6.01	10.07
AUGUCGAAGGUAGUAGCUCGA_7B:737957461-737957552_miR7714-like	12.00	6.96	4.49	7.08	6.00	3.24	11.05	0.00
AUUGAACUAAGGAGGGGUGGA_2B:720801630-720801720_Unknown	0.00	11.90	1.79	5.57	32.24	16.46	20.49	51.33
AUUGAUUAAGACGCAUGGGCC_Un:263357724-263357991_Unknown	24.37	11.93	14.79	15.08	12.30	3.54	6.86	0.00
AUUGUGUACAGAGGGAGUAGU_3B:587564205-587564383_Unknown	13.94	9.57	9.00	11.29	6.52	8.38	13.63	0.00
CAGAACCAGAAUGAGUAGCUC_2D:537495834-537495917_Unknown	1.86	1.70	2.63	0.00	4.51	0.87	1.34	0.00
CAGCCAAGGAUGACUUGCCGA_2A:683724053-683724303_miR169a	26.56	19.71	19.27	20.01	25.92	18.32	13.39	12.52
CAGCCAAGGAUGACUUGCCGA_2B:645654934-645655187_miR169a	24.51	18.71	14.59	23.02	19.37	22.41	13.24	13.13
CAGCCAAGGAUGACUUGCCGA_2D:540313472-540313759_miR169a	28.41	17.14	12.66	25.15	19.95	22.46	14.16	9.87
CAGCCAAGGAUGACUUGCCGA_3A:202585447-202585575_miR169a	13.07	10.91	8.88	10.37	11.24	12.22	11.05	5.71
CAGCCAAGGAUGACUUGCCGA_3B:242751068-242751196_miR169a	18.43	11.85	11.39	22.86	21.99	16.34	16.27	7.01
CUCGCCGGUCGCGCGUUCUCC_2B:158881602-158881669_Unknown	24.41	18.92	14.57	18.73	30.54	23.16	21.60	16.06
CUCGCCGGUCGCGCGUUCUCC_4B:579888737-579888998_Unknown	23.69	18.24	17.69	30.95	21.22	24.30	20.04	39.78
CUCGCCGGUCGCGCGUUCUCC_7A:699802351-699802463_Unknown	13.84	15.75	16.76	23.87	12.71	17.95	13.25	26.66
CUCGCCGGUCGCGCGUUCUCC_7D:421468136-421468314_Unknown	24.24	20.31	19.88	21.09	20.83	23.01	22.64	17.08
CUGCAUUUGCACCUGCACCUA_2D:428671838-428671983_miR530	1.68	0.50	0.00	0.00	0.00	0.00	1.86	0.00
CUUCUGAUUUACUCGUCGUGG_7A:668530071-668530160_Unknown	1.26	0.52	2.63	4.29	0.00	0.00	0.00	0.00
CUUGAACUUCUCCAUAGCAUC_4D:507476510-507476746_miR9674b-like	49.74	46.38	34.80	41.95	51.43	49.44	36.81	25.72
CUUGCGAGCAACGGAUGAAUC_6B:665410920-665411161_Unknown	2.38	1.68	0.72	4.22	5.94	0.78	0.00	0.00
GGUGGGUCUUCUUGGCUAAC_4A:494638262-494638440_Unknown	2.02	1.24	3.01	0.00	2.77	2.21	1.14	3.54
UACGGCCUGAUGACAUCCACG_3A:163805974-163806100_Unknown	19.24	21.52	28.22	9.94	23.10	15.10	17.53	15.58
UAGAAUGGCUGGUGCUAUGGA_4B:654095124-654095247_Unknown	68.18	68.34	62.79	71.61	71.42	85.40	90.86	94.47
UAGCCAAGAAUGACUUGCCUG_2A:759843144-759843282_miR169	7.68	7.42	1.34	7.08	9.01	4.04	2.98	0.00

UAGCCAAGAAUGACUUGCCUG_Un:31860924-31861098_miR169	7.38	6.23	2.16	12.72	9.15	6.78	1.45	2.18
UAGCCAAGGAUGACUUGCCUG_2A:759835624-759835894_miR169h	0.68	0.99	0.82	5.57	1.60	0.88	0.00	0.00
UAGCCAAGGAUGACUUGCCUG_5A:502440252-502440396_miR169h	0.67	1.34	0.00	0.00	1.49	1.15	0.00	0.00
UAGCCAAGGAUGACUUGCCUG_5B:477355303-477355458_miR169h	0.00	0.96	0.00	4.22	1.46	1.03	0.00	0.00
UAGCCAAGGAUGACUUGCCUG_5D:396877526-396877694_miR169h	0.69	1.24	1.24	8.36	4.95	1.12	1.45	0.00
UAGCCAAGGAUGACUUGCCUG_7B:221265370-221265570_miR169h	1.64	1.45	0.72	0.00	1.46	1.54	1.45	0.00
UAGCCAAGGAUGACUUGCCUG_7D:245335473-245335608_miR169h	1.71	1.45	0.93	2.79	0.76	0.77	0.00	0.00
UAGCCAAGGAUGACUUGCCUG_Un:31765997-31766131_miR169h	1.60	0.99	0.00	2.79	1.06	0.72	0.00	0.00
UCCAAAGGGAUCGCAUUGAUC_3A:33260337-33260467_miR393	31.25	29.19	30.17	38.30	32.28	39.52	58.41	53.33
UCCAAAGGGAUCGCAUUGAUC_3B:41449053-41449273_miR393	30.06	24.41	29.03	55.24	20.99	40.48	55.18	51.26
UCCAAAGGGAUCGCAUUGAUC_3D:23964618-23964728_miR393	31.69	22.99	37.68	40.31	36.46	38.26	58.48	40.61
UCCACAGGCUUUCUUGAACUG_2A:758894508-758894652_miR396e	382.90	347.17	503.78	381.62	377.33	487.86	615.02	443.4
UCCACAGGCUUUCUUGAACUG_2B:772062246-772062493_miR396e	371.92	363.24	506.59	444.65	363.54	521.91	631.94	467.4
UCCACAGGCUUUCUUGAACUG_2D:629186110-629186326_miR396e	370.30	357.10	484.29	462.18	365.02	517.78	610.80	459.41
UCCACAGGCUUUCUUGAACUG_6A:614538981-614539249_miR396e	372.31	388.62	495.59	459.75	389.24	525.87	638.51	489.19
UCCGUCCCAUACUAUAAGAGC_6A:212801503-212801721_Unknown	0.68	2.00	0.00	1.43	3.82	0.00	0.00	0.00
UCGGACCAGGCUUCAAUCCCU_5B:411091733-411091849_miR5168	0.00	0.00	0.00	0.00	0.00	0.00	1.86	0.00
UCGGACCAGGCUUCAAUCCCU_5D:350379776-350379922_miR5168	0.00	0.50	0.00	0.00	0.00	1.24	0.00	0.00
UCGGACCAGGCUUCAUUCCCC_1A:326329166-326329435_miR166m	511.67	490.65	353.43	367.19	466.64	381.20	272.57	278.7
UCGGACCAGGCUUCAUUCCCC_1B:357916180-357916415_miR166m	512.42	487.83	338.34	410.71	418.65	361.76	261.21	274.5
UCGGACCAGGCUUCAUUCCCC_1D:254662233-254662359_miR166m	511.67	495.34	329.92	405.59	449.26	362.24	278.16	255.29
UCGGACCAGGCUUCAUUCCCC_4A:16475404-16475504_miR166m	431.19	415.73	307.61	314.96	374.01	351.57	289.59	305.49
UCGGACCAGGCUUCAUUCCCC_4D:450196303-450196438_miR166m	415.50	428.22	310.32	363.62	369.99	350.83	291.61	293.34
UCGGACCAGGCUUCAUUCCCC_7A:561152153-561152408_miR166m	464.78	403.89	314.82	322.12	420.62	368.52	291.58	282.14
UCGGACCAGGCUUCAUUCCCU_6D:418216628-418216757_miR166m	31.11	28.60	24.15	16.86	21.78	12.44	17.87	10.34
UCGGACCAGGCUUCAUUCCUU_6A:560691652-560691733_miR166c	6.77	1.66	7.12	1.43	3.13	4.83	10.12	8.10
UCGGACCAGGCUUCAUUCCUU_6D:418216451-418216534_miR166c	4.88	4.11	9.97	6.15	2.38	6.85	7.93	3.54
UGAAGCUGCCAGCAUGAUCUA_5A:580934114-580934281_miR167	156.89	171.09	146.13	142.99	168.45	154.96	119.87	93.48
UGAAGCUGCCAGCAUGAUCUA_5B:614463466-614463583_miR167	200.95	198.29	156.64	164.15	201.65	172.29	136.85	147.69
UGAAGCUGCCAGCAUGAUCUA_5D:460745713-460745886_miR167	180.22	187.30	160.00	162.45	183.71	173.81	143.70	121.69
UGAAGUAGAGCAGAGACCUCA_5B:694314328-694314558_miR9661	5.51	10.35	5.19	15.79	22.80	16.72	11.80	5.30
UGAAGUAGAGCAGGGACCUCA_4A:613581149-613581230_miR9661	1.38	2.91	2.76	0.00	2.88	1.26	1.48	0.00
UGAAUUUGUCCAUAGCAUCAG_4B:654902505-654902619_Unknown	24.20	28.50	30.19	41.66	31.63	33.46	49.74	9.25
UGAAUUUGUCCAUAGCAUCAG_4D:507434580-507434746_Unknown	18.99	27.14	32.52	43.74	30.02	28.59	38.10	22.85
UGAGACGAGAUCUCCCCAUAC_4A:609557174-609557261_Unknown	23.62	11.30	13.84	19.16	10.63	19.24	9.34	15.31
UGAGACGAGAUCUCCCCAUAC_5D:557480963-557481067_Unknown	25.85	18.47	18.30	16.87	13.84	19.48	18.69	16.20
UGAGAUGAGAUUACCCCAUAC_5B:661123811-661123991_miR9772	50.92	41.01	37.69	67.54	36.59	61.30	48.27	65.80
UGAGAUGAGAUUACCCCAUAC_5D:526002236-526002324_miR9772	48.14	36.08	38.75	49.75	40.68	57.71	52.27	50.95
UGAUUGAGCCGCGCCAAUAUC_1A:366114102-366114228_miR171	0.00	0.00	0.00	0.00	0.00	0.00	1.48	0.00
UGAUUGAGCCGCGCCAAUAUC_6B:240396028-240396116_miR171	1.35	0.50	0.00	1.43	0.00	0.45	0.00	0.00
UGAUUGAGCCGUGCCAAUAUC_4A:562607854-562608024_miR171	97.48	88.49	58.66	52.17	59.16	54.89	37.03	37.75
UGAUUGAGCCGUGCCAAUAUC_4B:55643553-55643777_miR171	64.90	56.08	44.74	41.23	36.52	38.52	27.79	13.20
UGAUUGAGCCGUGCCAAUAUC_4D:487088256-487088352_miR171	62.91	59.52	39.12	61.46	40.08	39.25	33.13	33.81
UGAUUGAGCCGUGCCAAUAUC_5A:668149291-668149396_miR171	63.18	57.27	41.49	32.73	35.11	40.86	29.85	20.00
UGAUUGAGCCGUGCCAAUAUC_7A:564253177-564253270_miR171	90.46	86.65	64.46	47.38	63.76	67.53	53.03	44.35
UGAUUGAGCCGUGCCAAUAUC_7B:526810769-526810855_miR171	87.97	89.51	58.64	60.89	68.58	68.36	53.76	63.48

UGAUUGAGCCGUGCCAAUAUC_7D:498482452-498482537_miR171	93.41	93.79	60.27	68.47	61.10	61.80	54.67	56.56
UGCCAAAGGAGAGUUGCCCUG_2A:652328872-652328990_miR399	1.02	1.93	0.00	5.57	0.80	0.61	0.00	0.00
UGCCAAAGGAGAGUUGCCCUG_2B:596648887-596648984_miR399	1.37	1.09	0.00	0.00	0.69	0.55	0.00	0.00
UGCCAAAGGAGAGUUGCCCUG_2D:507824577-507824655_miR399	0.00	1.45	0.00	2.79	0.00	0.50	0.00	0.00
UGCCUGGCUCCCUGUAUGCCA_5B:598031772-598031867_miR160a	23.03	23.09	30.54	33.31	26.48	29.45	28.54	49.28
UGCCUGGCUCCCUGUAUGCCA_5D:486487581-486487676_miR160a	24.92	20.05	28.49	36.02	26.72	32.64	27.07	19.12
UGCCUGGCUCCCUGUAUGCCA_6A:113538445-113538531_miR160a	26.38	18.08	24.75	30.45	31.12	33.76	25.47	25.10
UGCCUGGCUCCCUGUAUGCCA_6B:177276137-177276224_miR160a	25.15	18.26	20.73	40.66	39.75	31.57	29.63	30.42
UGCCUGGCUCCCUGUAUGCCA_6D:95719039-95719260_miR160a	25.79	20.05	28.54	47.81	38.66	32.01	24.90	27.49
UGCCUGGCUCCCUGUAUGCCA_7A:438858691-438858782_miR160a	43.04	37.86	48.64	42.17	43.17	45.94	52.43	71.15
UGCCUGGCUCCCUGUAUGCCA_7B:383734710-383734802_miR160a	37.11	29.30	53.71	57.53	41.36	51.19	46.15	61.50
UGCCUGGCUCCCUGUAUGCCA_7D:388698517-388698696_miR160a	34.73	28.42	50.28	63.68	61.48	49.04	54.19	71.67
UGGACGAGGAUGUGCAGCUGC_2A:707747459-707747572_miR528	28.18	26.59	36.18	39.16	38.03	30.92	52.50	58.33
UGGACGAGGAUGUGCAGCUGC_2B:680766693-680766982_miR528	28.81	25.67	40.00	31.38	46.30	43.82	46.93	42.72
UGGACGAGGAUGUGCAGCUGC_2D:568229069-568229354_miR528	29.21	26.93	35.40	39.31	66.81	38.84	52.73	36.67
UGGAGAAGCAGGGCACGUGCA_1A:403039768-403039869_miR164a	25.72	37.93	47.15	22.87	36.60	38.45	32.53	47.78
UGGAGAAGCAGGGCACGUGCA_1B:432758102-432758204_miR164a	33.78	34.99	43.03	33.73	40.07	28.23	21.29	52.68
UGGAGAAGCAGGGCACGUGCA_1D:319916820-319916921_miR164a	28.00	34.98	39.85	35.95	39.60	25.47	31.28	29.87
UGGAGAAGCAGGGCACGUGCA_2A:62129298-62129500_miR164a	34.99	37.45	49.43	41.17	34.45	27.84	34.55	26.54
UGGAGAAGCAGGGCACGUGCA_2B:96381861-96381997_miR164a	33.15	39.34	39.64	31.66	38.23	34.04	37.59	57.40
UGGAGAAGCAGGGCACGUGCA_2D:61773259-61773441_miR164a	38.00	37.71	46.35	35.95	44.94	28.77	31.77	28.37
UGGAGAAGCAGGGCACGUGCU_6A:574411488-574411628_miR164d	46.67	52.71	49.18	52.03	54.92	50.29	42.45	90.84
UGGAGAAGCAGGGCACGUGCU_6B:646613368-646613503_miR164d	43.90	46.99	48.96	37.96	61.54	48.97	47.95	81.46
UGGAGAAGCAGGGCACGUGCU_6D:429212150-429212291_miR164d	47.64	48.89	46.11	30.02	61.17	53.65	49.66	73.44
UGUGUUCUCAGGUCACCCCUU_5A:505600425-505600630_miR398-ath	4.89	9.15	0.93	5.72	6.32	1.59	0.00	0.00
UGUGUUCUCAGGUCACCCCUU_5B:480241438-480241607_miR398-ath	5.39	8.72	0.72	2.86	0.69	1.44	0.00	0.00
UGUGUUCUCAGGUCACCCCUU_5D:400242419-400242534_miR398-ath	4.41	9.15	0.00	7.08	1.98	1.46	0.00	0.00
UGUGUUCUCAGGUCGCCCCCG_3A:658244028-658244161_miR398- tae	5.34	5.74	1.44	4.22	6.72	3.32	0.00	0.00
UGUGUUCUCAGGUCGCCCCCG_3D:523327166-523327320_miR398- tae	5.67	3.14	2.16	4.22	6.05	3.09	0.00	2.18
UUAAUUUGUCCAUAGCAUCCG_3D:524000757-524000864_Unknown	19.19	15.11	30.34	22.51	23.59	24.90	30.81	27.83
UUAGAUGACCAUCAGCAAACA_2A:593354662-593354949_miR827	14.18	21.15	17.79	31.37	26.89	19.00	6.72	2.65
UUAGAUGACCAUCAGCAAACA_2B:528817711-528817847_miR827	22.20	24.48	13.40	8.36	18.84	18.49	5.16	5.71
UUCCAAAGGGAUCGCAUUGAU_2A:774802387-774802485_miR393	31.79	28.43	44.94	58.39	40.90	36.67	59.08	66.79
UUCCAAAGGGAUCGCAUUGAU_2B:786139772-786139861_miR393	26.83	28.54	38.71	35.09	19.10	34.18	45.80	32.46
UUCCAAAGGGAUCGCAUUGAU_2D:650221328-650221423_miR393	25.99	26.65	37.28	31.81	28.10	37.42	43.31	23.68
UUCCACAGCUUUCUUGAACUG_6A:596076902-596077022_miR396a	298.44	267.56	188.51	230.54	330.16	241.13	145.07	137.5
UUCCACAGCUUUCUUGAACUG_6B:684140731-684140852_miR396a	293.23	260.46	173.75	280.93	293.86	250.27	148.11	117.2
UUCCACAGCUUUCUUGAACUG_6D:450551313-450551434_miR396a	297.89	257.49	190.19	250.82	297.36	236.64	152.08	102.4
UUCCACAGCUUUCUUGAACUU_6A:595988565-595988729_miR396d	48.57	53.16	40.72	56.67	69.23	46.04	42.25	70.23
UUCCACAGCUUUCUUGAACUU_6D:450527450-450527618_miR396d	46.10	57.30	38.27	43.82	44.69	38.59	48.10	45.60
UUCGCCGGAGAAGCAUGCUGC_7A:38967196-38967285_Unknown	1.00	1.24	0.00	0.00	2.14	1.28	0.00	0.00
UUCGCCGGAGAAGCUUACUGC_3B:755705205-755705387_Unknown	16.16	13.75	12.75	20.23	8.05	6.19	8.83	25.44
UUCGCCGGAGAAGCUUACUGC_4B:654033954-654034154_Unknown	19.18	11.01	15.09	18.02	7.61	9.18	11.48	5.45
UUCGCCGGAGAAGCUUACUGC_5A:534566645-534566883_Unknown	23.65	6.99	14.12	15.08	11.66	10.87	6.77	6.54
UUCGCCGGAGAAGCUUACUGC_5B:272446283-272446353_Unknown	22.32	11.78	11.23	20.15	12.74	11.38	6.94	8.10
UUCGCCGGAGAAGCUUACUGC_7B:706680829-706680989_Unknown	14.49	11.80	13.74	8.94	19.14	7.95	8.29	2.18

UUCGCCGGCUGCGCGUUCCCC_3D:453160413-453160571_Unknown	14.98	17.83	15.65	18.73	16.24	17.22	17.75	16.88
UUCGCCGGCUGCGCGUUCCCC_4D:10922261-10922317_Unknown	16.81	17.82	12.98	13.65	15.86	15.86	24.05	18.23
UUCGCCGGCUGCGCGUUCCCC_5D:275557431-275557488_Unknown	14.25	16.19	16.82	19.22	23.62	16.94	17.75	14.49
UUCGCCGGCUGCGCGUUCCCC_7D:208677262-208677523_Unknown	15.45	15.90	17.30	18.80	20.80	15.83	19.48	15.38
UUCGCCGGUCGCGCGUUCCCCU_2B:408083876-408083976_Unknown	12.69	13.35	11.20	10.37	11.57	10.81	13.75	23.62
UUCGCCGGUCGCGCGUUCCCCU_3D:308784783-308784971_Unknown	11.45	15.71	10.75	6.58	14.40	9.88	8.55	6.80
UUCGCCGGUCGCGCGUUCCCCU_4D:234082939-234082999_Unknown	11.27	11.74	7.72	12.22	9.93	11.63	7.61	10.28
UUCGCCGGUCGCGCGUUCCCUU_3D:549067868-549068015_Unknown	3.96	5.75	3.29	9.86	6.78	7.37	7.34	0.00
UUGAGACGAAAACAGACCAAC_5D:83923035-83923151_Unknown	26.67	36.11	34.27	25.02	24.55	27.32	27.59	31.32
UUGAGCCGUGCCAAUAUCACG_2A:712289716-712289801_miR171b	1.31	0.94	0.00	2.79	0.00	0.00	0.00	0.00
UUGAUUCCCAUUUCACUAGCU_3B:527232708-527232942_Unknown	0.00	8.71	3.29	11.14	18.54	27.77	27.93	8.84
UUGGACUGAAGGGUGCUCCCU_3A:446540933-446541107_miR319b	54.68	35.76	65.84	39.38	72.32	54.03	59.63	35.65
UUGGACUGAAGGGUGCUCCCU_3B:429318600-429318775_miR319b	58.64	39.00	54.40	37.31	60.76	56.18	63.48	67.73
UUGGACUGAAGGGUGCUCCCU_3D:330699493-330699744_miR319b	54.43	34.32	59.70	39.66	67.52	51.25	64.17	66.38
UUUCUGAAGGGUUUAUCGAGG_6A:29948423-29948504_Unknown	100.77	109.34	157.30	146.51	135.46	100.96	105.34	95.18
UUUGAGACGAACACUGACCAA_5B:91006912-91007179_Unknown	50.26	56.34	63.55	52.82	62.12	57.91	83.13	59.83
UUUGGCUUGAAGGGAGCUCUG_5D:553838500-553838672_miR159	13.74	16.09	6.92	4.64	16.09	12.53	6.34	0.00
UUUGGUUCGAAGGGAGCUCUG_5B:696962881-696963069_miR159	26.11	27.59	10.79	25.22	24.23	17.26	9.19	2.18
UUUGGUUUGAAGGGAGCUCUG_4A:614704120-614704296_miR159	36.64	40.06	17.30	14.93	23.33	18.98	14.82	12.05
UUUGGUUUGAAGGGAGCUCUG_5D:553856802-553856978_miR159	32.39	33.66	15.91	20.30	26.90	15.66	10.86	7.69

Abbreviation

ABA	Abscisic acid
AGO	Argonaute
APS	Adenosine-5’-phosphosulphate
ATP	Adenosine Triphosphate
cDNA	Complementary DNA
DCL	Dicer-Like
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
ds RNA	Double-strand RNA
DTT	DithiothreitoI
EDTA	Ethylenediamine tetraacetic acid
Gb	large genome size (Gb for bread wheat)
HEN1	Hen Enhancer 1
HYL1	HYPONASTIC LEAVES 1
miRNA	MicroRNA
mRNA	Messenger RNA
ncRNA	Non-coding RNA
NGS	Next generation sequencing
nt	Nucleotides
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PNK T4	polynucleotide kinase

Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
RACE	Rapid Amplification of cDNA Ends
RCF	Relative centrifugal force
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNA-pol	RNA polymerase
RNase	Ribonuclease
RPM	Revolutions per minute of rotor
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
sRNA	Small RNA
SSC	Sodium chloride- sodium citrate buffer
TBE	Tris-Borate-EDTA
TEMED	Tetramethlethlethylenediamine
UTR	Untranslated Region

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