Differential expression of microRNAs in drought-stressed sorghum roots

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Dissertation submitted in fulfilment of the requirements in respect of the Master's Degree qualification in the Department of Plant Sciences, Faculty of Natural and Agricultural Sciences at the University of the Free State, Qwa-Qwa Campus.

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Declaration

I, Ntumeleng Malefa Mamokoakoa Selinah Hlakotsa, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification in Botany at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

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Dedication

This Master’s dissertation is dedicated to my father Joseph Hlakotsa and mother Emma Moloi.

Acknowledgements

I would like to express my gratitude to the Almighty God, you have been my unfailing source of strength and perseverance. Thank you, God, for giving me the wisdom and grace to finish my degree. My sincere appreciation goes to my family, my father Joseph Hlakotsa, my sister Nteboiseng Hlakotsa, my mother Emma Moloi and grandmother Nfaladi Moloi. Your love, support, and patience have carried me through the most challenging moments of my Master's journey. Your encouragement means a lot to me. To my friends, thank you for the memorable times we shared and also for always being there when I needed someone to talk to. I extend my gratitude to my supervisor, Dr Rudo Ngara for allowing me the opportunity to pursue this degree. Thank you for the guidance, dedication and efforts in helping me overcome the challenges of this journey. To Dr Dirk Swanevelder, I am grateful to you for introducing me to Next Generation Sequencing technologies and the related bioinformatics workflows and training me on the data analyses. I am grateful to the National Research Foundation for financial support. Finally, to the Plant Biotechnology group, especially Sellwane Moloi and Samkelisiwe Ngwenya, thank you for your encouragement and support. Sellwane, thank you for assisting me with my experiments and my chapter write-ups, as well as investing time and effort into my Master's degree. Samkelisiwe, thank you for being with me throughout the CLC Genomics Workbench learning journey. Your help is deeply appreciated.

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List of Abbreviations

ABA	Abscisic acid
AGO	Argonaute
ARC	Agricultural Research Council
ARFs	Auxin response factors
circRNA	Circular RNA
DCL1	Dicer-like RNAse III endonuclease 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNB	DNA nanoballs
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
GCI	Grain Crops Institute
GO	Gene Ontology
HEN1	Hua enhancer 1
HYL1	Hyponastic leaves 1
IAA	Indole-3-Acetic Acid
lncRNA	Long non-coding RNA
MIR	microRNA genes
MOPS	3-(n-morpholino) propanesulfonic acid
mRNA	Messenger RNA
miRNA	microRNA
MYB	Myeloblastosis
NAC	No apical meristem and cup-shaped cotyledon transcription factors

NCBI	National Center for Biotechnology Information
NFY	Nuclear Factor Y
NGS	Next-generation sequencing
ncRNA	non-coding RNA
PEG	Polyethylene glycol
piRNA	Piwi-interacting RNA
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PTGS	Post-transcriptional gene silencing
QC	Quality control
qRT-PCR	Quantitative real-time polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
ROS	Reaction oxygen species
rasiRNA	Repeat-associated short interfering RNA
rRNA	Ribosomal RNA
RWC	Relative water content
RT	Reverse transcription
SE	Serrate
siRNA	small interfering RNA
snRNA	small nuclear RNA
sRNA	small RNA
TGS	Transcriptional gene silencing
TFs	Transcription factors

tasiRNA	Trans-acting siRNA
tRNA	Transfer RNA
tsRNA	tRNA-derived small RNA
Tween20	Polyoxyethylene (20) sorbitan monolaurate
TW	Turgid weight
vasiRNA	Virus-activated small interfering RNA

Research Outputs From This Study

Conference presentations

1. **Hlakotsa N.M.M.S**., and Ngara R. Physiological responses of sorghum seedlings exposed to mild-drought stress. Poster presentation delivered by Ntumeleng M.M.S Hlakotsa at the South African Association of Botanists (SAAB), 48th Annual Conference, University of Limpopo, South Africa, 17-20 January 2023.

2. **Hlakotsa, N.M.M.S**., and Ngara, R. Physiological and small RNA responses of sorghum seedlings under mild drought stress. Oral presentation delivered by Ntumeleng M.M.S Hlakotsa at the 2024 Qwa-Qwa Campus Research Conference. Science, Social Innovation and the Future of Local Societies: keeping pace in a changing knowledge and political landscape, South Africa, 9-10 October 2024.

3. **Hlakotsa, N.M.M.S**., and Ngara, R. Identification of drought-responsive small RNAs in sorghum roots. Oral presentations delivered by Ntumeleng M.M.S Hlakotsa at the University of Johannesburg Postgraduate Symposium (UJ-PGS), Department of Botany and Plant Biotechnology, University of Johannesburg, South Africa, 21 October 2024.

Abstract

Sorghum (*Sorghum bicolor*) is an important source of food, fibre, and fuel. While it is a drought-tolerant crop, its yield is still affected by drought stress, threatening food security. Therefore, there is a need to study the drought responses of plants to develop more drought-resilient crops. This study aimed to identify drought-responsive microRNAs in sorghum roots to understand the gene regulatory processes in drought stressed sorghum. ICSB 338 (drought-susceptible) and SA 1441 (drought-tolerant) sorghum seeds were germinated and grown for three weeks with adequate watering. Subsequently, the plants were divided into two groups: A drought-stressed group, where water was withheld for 15 and 28 days to induce mild and moderate drought stress, respectively, and a control group that continued to receive adequate watering. The results showed a significant decrease in pot weight, soil moisture content, and stomatal conductance for both sorghum varieties under drought stress conditions. The leaf relative water content of ICSB 338, significantly declined following the 15 and 28 days of drought stress treatment compared to the controls. The physiological responses of the two sorghum varieties differed, with ICSB 338 being more affected by drought stress than the SA 1441. The molecular responses of sorghum to drought stress were investigated using small RNA sequencing performed on the watered controls and drought-stressed root samples of both varieties. The MGI DNBSEQ-G400 sequencing technology was used to identify the differentially expressed microRNAs. A total of 81 and 83 constitutively expressed miRNAs were identified in the watered control samples of ICSB 338 and SA 1441, respectively. Among these constitutively expressed miRNAs, 73 were common in both sorghum varieties, while eight and 10 miRNAs were unique to ICSB 338 and SA 1441, respectively. The analysis also revealed that four of the constitutively expressed microRNAs were differentially expressed between ICSB 338 and SA 1441 plants ($p \leq 0.05$). The target genes of the constitutively

expressed sorghum root microRNAs were predicted using the psRNATarget database, which also revealed that most of the miRNA inhibited their target genes through messenger RNA cleavage. Gene Ontology analysis of the target genes revealed that the constitutively expressed microRNAs regulate a wide range of genes with diverse cellular locations, molecular functions and biological processes in sorghum roots. Drought-responsive microRNAs ($p \leq 0.05$) were identified using the CLC Genomics Workbench software (Qiagen) by comparing the watered controls and drought-stressed miRNAs using the Differential Expression for RNA-seq tool. Out of the 111 identified miRNAs in both varieties, only four and nine miRNAs were differentially expressed in ICSB 338 and SA 1441, respectively. miRNAs sbi-miR6233-3p and sbi-miR821a were up-regulated, while sbi-miR5566 and sbi-miR6224a-5p were down-regulated in ICSB 338 sorghum roots. Bioinformatics analyses predicted a *Putative clathrin assembly protein At5g57200* gene as a potential target for sbi-miR6233-3p, while sbi-miR821a targeted an unknown gene. The targets for sbi-miR5566 and sbi-miR6224a-5p were an *Uncharacterized LOC8057912* and *Uncharacterized LOC8055016*, respectively. For SA 1441, two microRNAs (sbi-miR5564c-5p and sbi-miR6232b-3p) were up-regulated while seven (sbi-miR168, sbi-miR2118-5p, sbi-miR395a, sbi-miR5387b, sbi-miR5568c-3p, sbi-miR6229-5p, sbi-miR6235-5p) were down-regulated. The descriptions of the predicted target genes of SA 1441 included *NAC domain-containing protein 83*, *Low affinity sulfate transporter 3*, *Leaf rust 10 disease resistance locus receptor like protein kinase like 2.1*, *Cell division cycle protein 123 homolog*, and *Indole-3-acetaldehyde oxidase*. Overall, the findings of the current study contribute to our knowledge of the mechanisms sorghum uses to cope with drought stress.

Keywords: Sorghum, drought stress, transcriptomics, small RNAs, microRNAs, constitutive expression, differential expression, MGI DNBSEQ-G400 sequencing technology, miRNA target genes.

CHAPTER 1

General Introduction

1.1 Causes of food insecurity

A global aim is to ensure that there is sufficient and diverse food supply for the world population (Misselhorn *et al*., 2012; IPCC, 2019). However, the agricultural sector is struggling to keep up with the increasing demand for food due to the ever-increasing population (McCarthy *et al*., 2018). According to Kousar and colleagues (2021), over 850 million people are food insecure globally, which affects their ability to live a healthy lifestyle. Food insecurity mostly affects disadvantaged people in developing countries, such as in sub-Saharan Africa and Asia (Nelson *et al*., 2009; Wudil *et al*., 2022). Additionally, in these developing countries, nearly 60% of the calories consumed come from cereals, which can be as high as 80% in the poorest regions (Awika, 2011). This shows that people depend on cereals as a primary source of nutrition.

Within the Poaceae family, cereals such as rice *(Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor* L.), and millet (*Pennisetum glaucum* L.) are widely cultivated globally and immensely contribute to the world's food supply (Olugbire *et al*., 2021). Indeed, these cereals contribute immensely as the primary source of stable foods for disadvantaged people in developing countries (Olugbire *et al*., 2021; Noort *et al*., 2022). However, the cultivation of these crops will need to increase to meet the high food demand resulting from the increasing population growth i.e., according to the Food and Agriculture Organisation (FAO), food production must increase by 60% by 2050 (FAO, 2018).

Unfortunately, crop production is negatively affected by a range of abiotic stresses, further decreasing global food security (Rivero *et al*., 2022; Zandalinas *et al*., 2022). Examples of abiotic stresses that plants encounter include high and low light intensities, UV radiation, high and low temperatures, drought, flooding, heavy metals, and salt stress (Verslues *et al*., 2006; Pereira, 2016). Of these environmental stresses, drought is regarded as the main limiting factor to crop production, and it mainly affects the arid and semi-arid regions (Golla, 2021). Drought stress occurs when the soil water content decreases, resulting in plants having insufficient water supply for proper growth and development (Chandra *et al*., 2021). When the water requirements of a plant are not met, cellular metabolism and growth processes are disrupted (Zlatev and Lidon, 2012). For example, cellular dehydration may result in oxidative stress, membrane damage of affected tissues, and inhibition of enzyme activities, ultimately reducing plant growth and productivity (Havrlentova *et al*., 2021).

In response to drought stress, plants activate complex mechanisms to mitigate the effects of the stress (Farooq *et al*., 2009). These mechanisms include a series of physiological, morphological, biochemical, and molecular responses that help plants cope with conditions of limited water supply (Farooq *et al*., 2009; Murtaza *et al*., 2016; Yang *et al*., 2021). When exposed to drought conditions, plants synthesise and accumulate the phytohormone abscisic acid (ABA) in roots, which signals various plant parts to respond to the prevailing water-limiting conditions (Abhilasha and Choudhury, 2021). An increased ABA level reduces the turgor pressure of guard cells, which results in stomatal closure that reduces excessive water loss by transpiration (Chaves *et al*., 2003; Fang and Xiong, 2015). In addition, increased ABA accumulation promotes root elongation to facilitate water uptake from the soil (Shanker and Maheswari, 2017) and induces the expression of drought-responsive genes (Yang *et al*., 2021). The products of drought-responsive genes are divided into functional and regulatory proteins

(Seki *et al*., 2003). Functional proteins maintain cellular osmotic balance and protect cells from oxidative stress during drought stress and examples are the late embryogenesis abundant proteins, proline biosynthesis enzymes and catalase (Shinozaki and Yamaguchi-Shinozaki, 1997; Umezawa *et al*., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Regulatory proteins manage how plant cells respond to drought stress by regulating signal transduction and gene expression; they include transcription factors, protein kinases and protein phosphatases (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007). The current study focused on non-coding miRNAs and their roles in plant responses to drought stress. These miRNAs are considered an important class of plant molecules since they regulate biological processes, such as gene expression, developmental processes, and responses to biotic and abiotic stresses.

1.2 Coding and non-coding RNAs in plants

According to the central dogma of molecular biology, genes encode the genetic information required to synthesise proteins via a messenger RNA (mRNA) intermediate (Mattick, 2003; Szymanski *et al*., 2003). As such, coding RNAs have always been the centre of research in molecular biology (Zhang *et al*., 2019). However, plants also possess non-coding RNAs (ncRNAs) (Waititu *et al*., 2020). Although these ncRNAs are not translated into proteins, they are equally important in plants (Karlik *et al*., 2019). Figure 1.1 illustrates the ncRNAs grouped into housekeeping and regulatory forms (Karlik *et al*., 2019).

Housekeeping RNAs are involved in cellular functions such as translation and splicing and examples include ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) (Zhang *et al*., 2019; Chao *et al*., 2022). On the other hand, regulatory ncRNAs regulate and silence gene expression (Chao *et al*., 2022).

Examples of ncRNAs include long non-coding RNA (lncRNA), tRNA-derived small RNA (tsRNA), circular RNA (circRNA), microRNA (miRNA) and small interfering RNA (siRNA) (Bhogireddy *et al*., 2021; Chao *et al*., 2022).

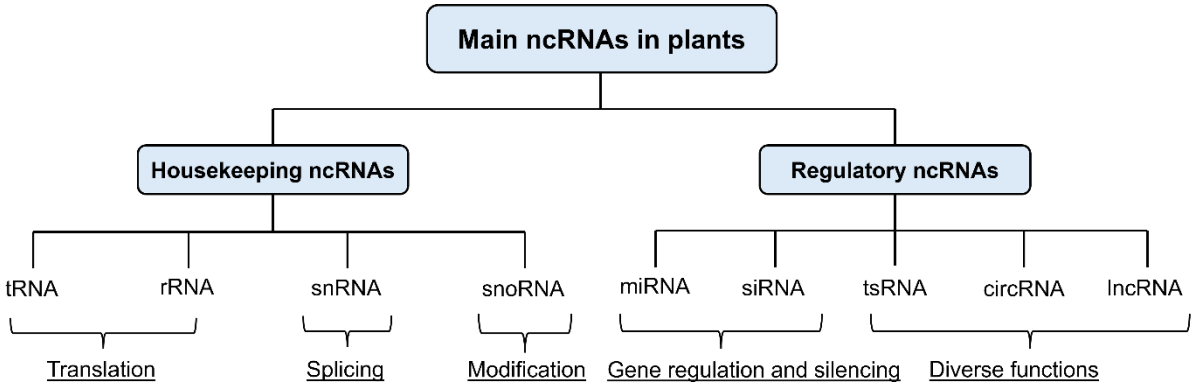

Figure 1.1. The main classes and roles of non-coding RNAs in plants. The non-coding RNAs are diverse and are grouped into housekeeping and regulatory non-coding RNAs. Housekeeping non-coding RNAs include transfer RNA, ribosomal RNA, small nuclear RNA, and small nucleolar RNA. On the other hand, regulatory non-coding RNAs include microRNA, small interfering RNA, tRNA-derived small RNA, circular RNA, and long non-coding RNA. These regulatory non-coding RNAs have various functions that regulate gene expression, through suppression of translation and cleavage of target mRNAs (Adapted from Chao *et al*., 2022).

1.3 Problem statement

Climate change is a major challenge to global food production as it increases the incidences of abiotic stresses (Gomez-Zavaglia *et al*., 2020; Chaudhry and Sidhu, 2022). Among these abiotic stresses, drought severely affects plant growth and development and limits crop yield (Shao *et al*., 2009). Additionally, the ever-increasing global population is causing a serious concern about whether food production will be sufficient in the future (Abhinandan *et al*., 2018; Tian *et al*., 2021). To ensure adequate food provision, it is important to study how plants respond to drought stress. By understanding these responses, specific traits that make plants more resistant to drought can be identified. Additionally, investigating the gene regulatory

networks involved in drought stress responses will provide insights into how plants cope with drought stress (Bhaskarla *et al*., 2020), i.e., by switching genes on and off under stressful conditions (Shinozaki *et al*., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007). The knowledge obtained will allow researchers and plant breeders to identify important molecular pathways that can be targeted for breeding programs to develop improved crop varieties. Specifically, the identification of drought-responsive plant miRNAs will provide insight into how plants regulate gene expression under limited water conditions.

This study used ICSB 338, a drought-susceptible, and SA 1441, a drought-tolerant sorghum line. The selection of the two sorghum lines was based on a previous physiological and proteomics study conducted by Goche *et al*. (2020). In the Goche *et al.* (2020) study, four-day-old sorghum seedlings growing in 2.5 L pots (22 cm in depth) containing soil were deprived of water for two weeks. After two weeks with no water supplied for growth, ICSB 338 plants showed signs of wilting, and these plants eventually died after three weeks, whereas SA 1441 endured the water limiting conditions (Goche *et al*., 2020). These results demonstrated the contrasting level of drought tolerance of the sorghum lines in that greenhouse experiment. Furthermore, the study analysed the comparative physiological, biochemical, and root proteome responses of the sorghum lines to water limitation in a separate experimental set-up. Drought stress was induced from the sorghum seedlings by withholding water in 10 cm deep pots over 12 days. The results showed that the relative water content of ICSB 338 decreased more than that of SA 1441. Additionally, SA 1441 showed an increase in both proline and glycine betaine contents compared to ICSB 338. Furthermore, the root proteome of both ICSB 338 and SA 1441 showed unique and common proteins in response to drought stress.

In the current study, the root tissue was also used. Being the first organs to sense water changes in the soil (Amtmann *et al*., 2022), roots have been observed to express miRNA that enables

plants to adapt during drought stress periods (Seeve *et al*., 2019; Tang *et al*., 2022). Compared to cereals such as rice, maize and wheat, studies on microRNAs analyses in sorghum are scarce (Katiyar *et al*., 2015). Therefore, knowledge obtained will help identify drought-responsive root miRNA analyses present in the tolerant sorghum variety to develop resilient lines to contribute meaningfully towards food security.

1.4 Aim and objectives

The main aim of this study was to isolate and identify drought-responsive microRNAs in ICSB 338 (drought-susceptible) and SA 1441 (drought-tolerant) sorghum root tissue exposed to limited watering. This was achieved through the following objectives:

I. Identify the morphological and physiological responses of two sorghum varieties in response to drought stress,
II. Isolate and quantify constitutively expressed and drought-responsive root microRNAs from two different sorghum varieties using next-generation RNA sequencing,
III. Identify differentially expressed sorghum root microRNAs in response to drought stress and identify the microRNA target genes using bioinformatic tools.

CHAPTER 2
Literature Review

2.1 Importance of sorghum as a cereal crop

Sorghum (Figure 2.1) is one of the most important cereal crops in the world (Reddy, 2017). Globally, sorghum is the fifth most important crop, following rice, wheat, maize and barley and the third most produced crop in Africa (FAO, 2021). In many developing countries, especially those located in arid and semi-arid areas, sorghum is a staple food for millions of people and is also used for fibre and fuel (Deng-feng *et al*., 2019).

This cereal crop is originally from Sub-Saharan Africa and has been cultivated for centuries in Africa and Asia (Mutava *et al*., 2011), where it is often grown in areas with high temperatures and low rainfall. Sorghum production in wetter parts of a country is low compared to crops such as rice and maize (Mundia *et al*., 2019), suggesting that it requires less water for growth than other grain crops. Moreover, sorghum is a C_4 photosynthesising plant that is more drought-tolerant than maize and wheat (Ogbaga *et al*., 2018). Therefore, it serves as a potential model system to study the physiological and molecular mechanisms of drought tolerance in cereals (Krupta *et al*., 2017). Although sorghum is considered a drought-tolerant crop, drought stress still affects its production (Abreha *et al*., 2022).

Figure 2.1. A field of *Sorghum bicolor* plants producing seeds (Russell, 2021).

2.2 Drought stress and its effects on plant growth

In plants, drought stress affects growth and development at different stages. The first phase of a plant life cycle is seed germination and is important for seedling emergence (Lamichhane *et al*., 2018). However, under drought conditions, seed germination is reduced or delayed, due to a low osmotic potential that affects the seeds' ability to absorb water during imbibition (Queiroz *et al*., 2019). Sajjad *et al*. (2023) investigated the effect of different polyethylene glycol (PEG) concentrations on the germination of drought-tolerant Sargodha-202 and drought-susceptible OPV-3 maize accessions. Polyethylene Glycol concentrations of 10%, 20% and 30% were used to induce drought. The control group yielded germination rates of 96.6% for Sargodha-202 and 93.3% for OPV-3. The 10% PEG treatment resulted in germination rates of 93.3% and 90% for Sargodha-202 and OPV-3, respectively, while 20% PEG showed rates of 90% for Sargodha-202 and 76.6% for OPV-3. In the 30% PEG treatment, germination rates were 86.6% for Sargodha-202 and 73.3% for OPV-3. The authors concluded that increasing the PEG concentration led to a decrease in the germination percentage of both varieties.

Drought stress also inhibits radicle emergence and growth by impairing cell division due to reduced turgor pressure (Abreha *et al*., 2022). As a result, seedlings may not have enough access to nutrients and water from the soil, hindering not only seedling emergence but also the development of the above-ground tissues (Ahmad *et al*., 2018). Furthermore, ABA accumulation in the roots is increased to signal the reduction of shoot growth to conserve water (Cutler *et al*., 2010). Drought also causes a reduction in the leaf relative water content, causing stomatal closure (Farooq *et al*., 2009). Subsequently, photosynthesis is reduced due to stomatal closure that decreases carbon dioxide uptake by the leaves (Zlatev and Lidon, 2012). This will result in a decrease in the production of assimilates needed for plant growth and yield.

The limited energy supply impairs normal plant growth, causing an increase in the reactive oxygen species, which may result in tissue necrosis (Miller *et al*., 2010). Furthermore, the accumulation of photosynthetic electron transport components increases molecular oxygen levels, leading to the generation of reactive oxygen species (Foyer, 2018). Figure 2.2 summarises various drought effects on plants, including those of a physiological, morphological, and biochemical nature (Nezhadahmadi *et al*., 2013).

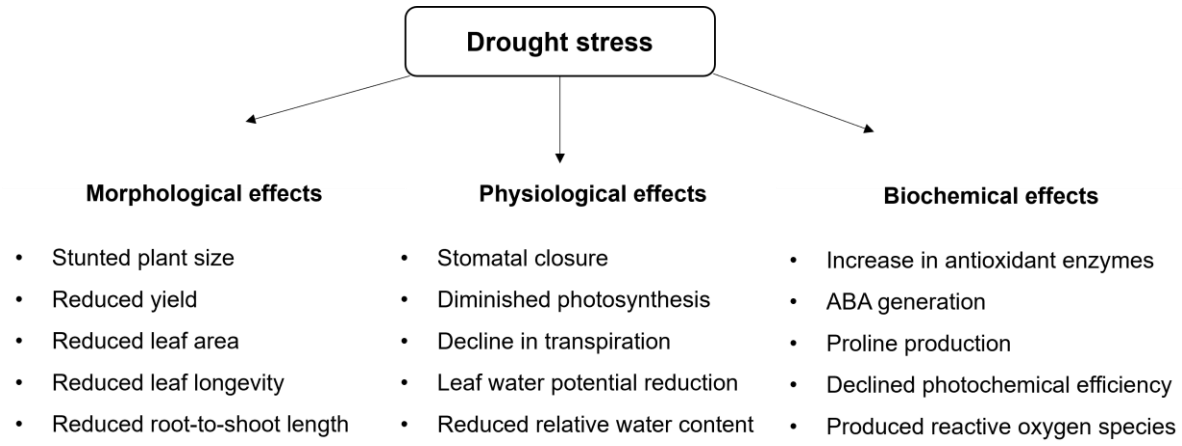

Figure 2.2. The morphological, physiological, and biochemical effects of drought stress on plants (Adapted from Nezhadahmadi *et al*., 2013).

2.2.1 Plant morpho-physiological adaptations to drought stress

Plants use several coping mechanisms to mitigate the effects of drought stress. One such mechanism is drought escape, where plants often complete their life cycle before the onset of severe water shortage (Ahmed *et al*., 2019; Yahaya and Shimelis, 2021). On the other hand, drought avoidance is the ability of plants to sustain high water status to withstand periods of limited water availability (Verma *et al*., 2018). This occurs when plants develop deep root systems to increase water and nutrient uptake (Hong *et al*., 2013). Additionally, the roots produce hormones and organic acids required for development, differentiation, and growth (Ahmed *et al*., 2019). This leads to an increase in the root biomass relative to that of the shoots (Azarbad *et al*., 2018). Some plants roll their leaves, develop thicker cuticles, and others cover their leaves with trichomes to reduce water loss (Su *et al*., 2020; Yang et al., 2021; Hura *et al*., 2022). Plants under drought stress also reduce the leaf number and leaf size to conserve energy and redistribute resources to the rest of the plant (Bertolini *et al*., 2013; Nezhadahmadi *et al*., 2013). Other drought avoidance mechanisms used by plants include increasing the cross-

section of vessels, developing thick leaf veins, and forming shorter internodes to decrease the distance of transporting water, resulting in improved water conduction (Hura *et al*., 2022).

Lastly, drought tolerance refers to the ability of plants to maintain enough cell turgor, allowing metabolic processes to continue even under water limitations (Zlatev and Lidon, 2012). This is achieved through maintaining turgor through osmotic adjustment by accumulating compatible solutes, increasing cell elasticity and reducing cell size (Turner, 2018). Additionally, plants accumulate dehydrins and late-embryogenesis abundant proteins in response to decreasing water content (Bray, 1997). These proteins serve as chaperones that protect membrane structures and proteins from dehydration (Yadav and Sharma, 2016). As a result, crop adaptation to drought must have a balance between mechanisms of drought escape, avoidance and tolerance while ensuring optimum crop yield.

2.2.2 Plant biochemical adaptations to drought stress

Plants can detoxify reactive oxygen species by producing different enzymatic or non-enzymatic antioxidants (De Carvalho, 2008; Moucheshi *et al*., 2014). The antioxidants indirectly combat ROS by chelating transition metals and preventing their involvement in harmful reactions (Dumanovic *et al*., 2021). They also combat ROS directly by donating or receiving electrons, thus scavenging free radicals and preventing them from damaging biological molecules and structures (Dumanovic *et al*., 2021). Enzymatic antioxidants include catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase, and polyphenol oxidase (Das and Roychoudhury, 2014). Non-enzymatic antioxidants include ascorbate, glutathione, tocopherol, carotenoids, and flavonoids (Chakraborty and Pradhan, 2012).

Osmotic adjustment is the ability of plant cells to accumulate solutes in response to limited water supply to maintain turgor pressure (Rane *et al*., 2021). These solutes, also known as osmoprotectants, have a low molecular weight and are highly soluble molecules that are often harmless at high cellular concentrations (Ashraf and Foolad, 2007). The accumulated compatible solutes comprise organic solutes such as glycine betaine, amino acids (proline, aspartic acid, and glutamic acid), sugars (fructose and sucrose), cyclitols (mannitol and pinitol), and inorganic ions such as potassium, calcium, and silicon (Ahmad *et al*., 2018). For example, glycine betaine protects cells from drought stress by regulating intracellular osmotic potential and stabilizes plant cellular structure (Sakamoto and Murata, 2002; Ahmad *et al.*, 2018). Proline is the most commonly produced amino acid in cereals under drought stress (Ahmad *et al*., 2018). It stabilizes sub-cellular structures and scavenge free radicals in the cell, while potassium functions in stomatal regulation (Ashraf and Foolad, 2007).

Phytohormones also regulate other plant processes involved with water deficit response (Chhaya *et al*., 2020). They work together to control stress signalling pathways and adaptation to drought stress (Xiong *et al*., 2002). For instance, hormones like ABA and jasmonic acid are regulators of stomatal closure (Chaves *et al*., 2003). ABA is also regarded as a signalling molecule under drought stress (Salvi *et al*., 2021). It is produced in the roots and translocated to the shoots, where it sends the signal of the soil water status (Schachtman and Goodger, 2008). The level of ABA increases in plants under drought stress, resulting in stomatal closure and drought-responsive gene expression changes (Sah *et al*., 2016). The above-mentioned adaptations to drought stress are at least partially dependent on changes in gene expression.

2.2.3 Molecular responses to drought stress

To withstand drought stress, plants have developed various molecular responses (Bashir *et al*., 2021). These responses include the perception of the stress, signal transduction processes and regulation of gene expression (Ni *et al*., 2009; Yoshida *et al*., 2014) and are summarised in Figure 2.3. Under conditions of limited water availability, the expression of genes changes through various signalling pathways (Fang and Xiong, 2015; Abobatta, 2019). However, a gene being expressed during drought stress does not indicate that its product contributes to the plants ability to cope with the stress (Bray, 1993; Ramanjulu and Bartels, 2002; Haghpanah *et al*., 2024). The expression of some genes can be due to injury during a period of drought, while other genes are important for adaptation during stress (Bray, 1993; Haghpanah *et al*., 2024). The protective genes may be up-regulated, while those that have negative effects on plants under drought conditions may be down-regulated (Shriram *et al*., 2016).

It has been reported that although ABA is one of the primary signals during drought, it does not control all genes induced by water limitation (Aslam *et al*., 2022). For instance, transcription factors such as dehydration-responsive element-binding proteins also regulate drought-responsive genes (Singh and Laxmi, 2015). This shows that responses to drought stress are regulated by both ABA-dependent and ABA-independent signal transduction pathways (Soma *et al*., 2021). The signal transduction pathway starts with signal detection, which is followed by the generation of secondary messengers (Xiong *et al*., 2002). Specific secondary messengers regulate the calcium ion levels, leading to a protein phosphorylation cascade that targets transcription factors that control specific stress-regulated genes (Xiong *et al*., 2002). Another signal transduction mechanism involves mitogen-activated kinases that relay drought stress signals from receptors to the nucleus, where gene expression is

regulated (Bhargava and Sawant, 2013). Research suggests that small RNAs also participate in regulating gene expression post-transcriptionally to enhance plants response to drought stress (Wang and Chekanova, 2016).

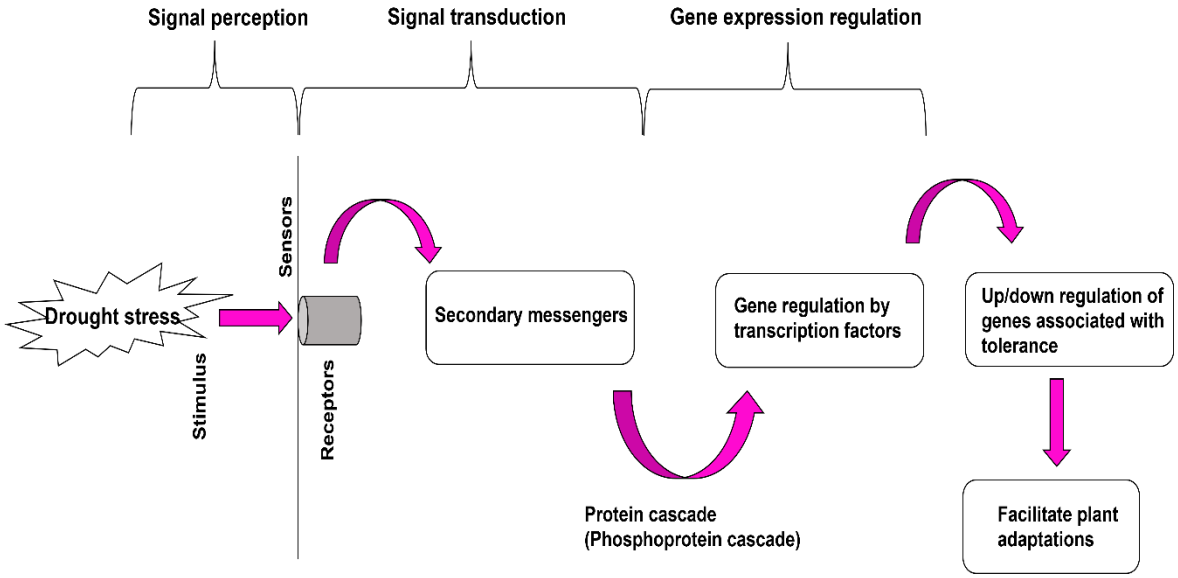

Figure 2.3. Plant signalling networks involved in response to drought stress. Drought triggers the sensors to send signals to receptors which activate secondary messenger molecules to send signals to the nucleus. Transcription factors regulate gene expression involved in drought tolerance. The regulated gene expression results in the plant's ability to respond to drought stress. (Adapted from Pamungkas *et al*., 2022).

2.3 Introduction to small RNAs

In biological systems, deoxyribonucleic acid (DNA) is regarded as the foundation of heredity (Vaucheret, 2006). Conversely, ribonucleic acid (RNA) is a biomolecule that is involved in genetic information processing in all organisms, acting as an intermediate between DNA and the synthesis of proteins (Brown, 2016). Ribonucleic acid molecules are diverse in nature, consisting of coding and non-coding sequences (Szymanski *et al.*, 2003; Karlik *et al*., 2019).

Coding RNA sequences consist of mRNA (Fernandes *et al*., 2019). On the other hand, non-coding sequences include tRNA, rRNA, sRNA, snRNA, miRNA, siRNA, tsRNA, circRNA, and lncRNA (Guleria *et al*., 2011; Chao *et al*., 2022). mRNA carries the genetic information obtained from DNA and is translated into a polypeptide sequence, whereas tRNAs and rRNAs are both involved in protein synthesis (Garrett and Grisham, 2010).

As discussed in Chapter 1, non-coding RNAs are grouped into housekeeping and regulatory forms (Karlik *et al*., 2019). Figure 2.4 illustrates a summary of various types of coding and non-coding RNAs (Guleria *et al*., 2011). Small RNAs (sRNAs) are RNA molecules of about 18-30 nucleotides in length (Shanker and Maheswari, 2017) and play crucial roles in regulating gene expression (Movahedi *et al*., 2018). Small RNAs are divided into sub-classes such as microRNAs (miRNAs), short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), trans-acting small interfering RNA (tasiRNAs), repeat-associated short interfering RNA (rasiRNAs) and virus-activated small interfering RNA (v(a)siRNA) (Guleria *et al*., 2011). Small RNAs are known to act via two main mechanisms. In transcriptional gene silencing (TGS), sRNAs guide DNA or histone methylation, resulting in heterochromatin formation (Won *et al*., 2014). In post-transcriptional gene silencing (PTGS), sRNAs guide the cleavage or translational inhibition of their target mRNAs (Won *et al*., 2014). The current study focused on miRNAs and their roles in sorghum responses to drought stress.

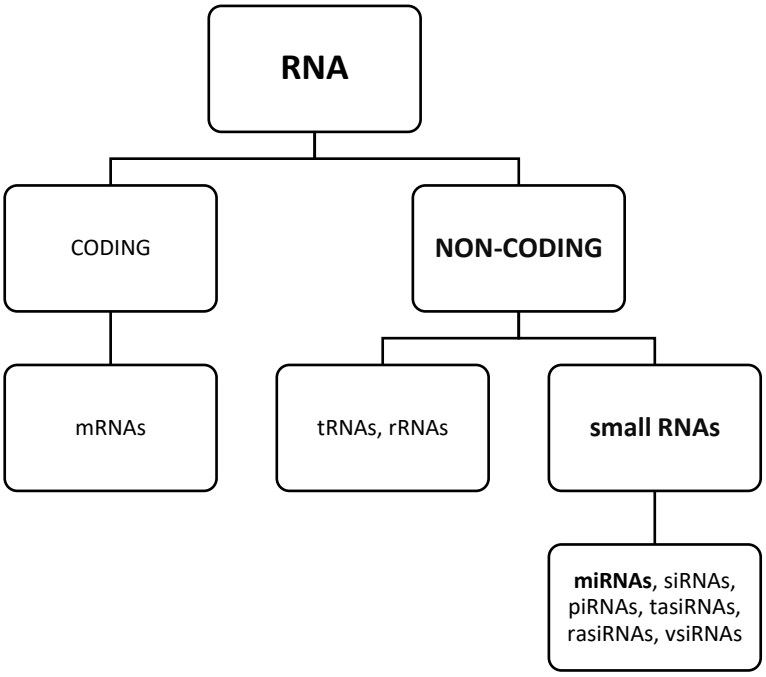

Figure 2.4. Different types of RNAs in plant cells. mRNA, messenger RNA; tRNAs, transfer RNAs; rRNAs, ribosomal RNAs; sRNAs, small RNAs; miRNAs, microRNAs; siRNAs, short interfering RNAs; piRNAs, piwi-interacting RNAs; tasiRNAs, trans-acting small interfering RNAs; rasiRNAs, repeat-associated short interfering RNA; and v(a)siRNA, virus-activated small interfering RNA (Adapted from Guleria *et al*., 2011).

2.3.1 Biogenesis, structure and function of plant microRNAs

2.3.1.1 MicroRNAs canonical and non-canonical biogenesis

MicroRNA biogenesis occurs in the nucleus through a network of enzyme catalysed reactions (Figure 2.5). Briefly, in canonical biogenesis, most miRNA genes (MIRs) are intergenic and are transcribed individually from their genomic loci (Wang *et al*., 2019; Hajieghrari and Farrokhi, 2022). Plant MIRs are transcribed by RNA polymerase II, resulting in long primary miRNA transcripts (pri-miRNA) (Budak and Akpinar, 2015). These imperfect stem-loop structures of pri-miRNAs are recognised and processed by Dicer-like RNAse III endonuclease 1 (DCL1) with the assistance of the dsRNA-binding protein Hyponastic leaves 1 (HYL1) and zinc finger protein Serrate (SE). The process results in the formation of precursor miRNAs

(pre-miRNAs) which are further processed by DCL1, leading to the formation of miRNA/miRNA* duplexes (Budak and Akpinar, 2015; Djami-Tchatchou *et al*., 2017; Li and Yu, 2021). Thereafter, the miRNA-miRNA* duplex is methylated at the 3´ ends by Hua enhancer 1 (HEN1) to inhibit the functions of enzymes that target the hydroxyl groups in the last nucleotide such as the ligases, terminal nucleotidyl transferases or polymerases (Chen, 2005; Yu *et al*., 2017). Subsequently, the miRNA-miRNA* duplex is transported out of the nucleus by the exportin protein HASTY into the cytoplasm.

When in the cytoplasm, the miRNA/miRNA* duplex is incorporated into the RNA-induced silencing complex (RISC) through binding with the GC (AGO) proteins and unwinds (Budak and Akpinar, 2015). The miRNA* is then ejected and degraded. Finally, the AGO directs miRNA-RISC towards targeted genes based on sequence complementarity for the regulation of gene expression. It is this miRNA-mRNA sequence complementarity that allows for gene regulation via cleavage of target mRNA or by repressing the translation of target mRNA under drought conditions (Djami-Tchatchou *et al*., 2017; Wang *et al*., 2019; Pagano *et al*., 2021).

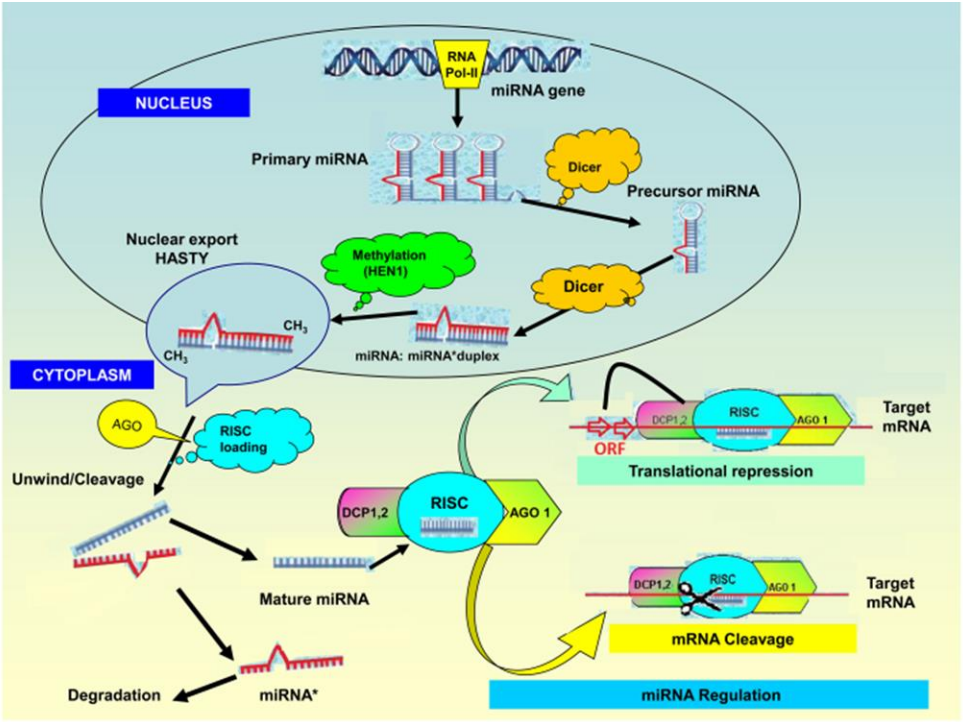

Figure 2.5. microRNA (miRNA) biogenesis in plants. RNA pol-II, RNA polymerase II; Dicer, Dicer-like RNA III; HY1, Hyponastic leaves 1; SE, serrate; pre-miRNA, precursor miRNA; HEN1, Hua enhancer 1; and AGO, Argonaute (Adapted from Djami-Tchatchou *et al*., 2017).

2.3.1.2 Structure and general functions of microRNAs in plants

Since the first discovery of miRNAs in plants over two decades ago, they have become the most studied small RNAs (Szwacka *et al*., 2018). This class of single-stranded RNA molecules range from 20 to 24 nucleotides in length (Voinnet, 2009; Wu, 2013). MicroRNA molecules interact with targeted mRNAs at the post-transcriptional level to control the expression of the protein-coding genes either by cleaving or repressing the translation of the target mRNA (Kaur *et al*., 2023). The level of complementarity between miRNAs and mRNAs sequences determines their regulatory functions (Du and Zamore, 2005). When the miRNA-mRNAs bind is perfect or near perfect, it facilitates mRNA cleavage, thereby repressing gene expression. Conversely, when the miRNA-mRNA bind is imperfect, it results in translational inhibition (Du and Zamore, 2005; Sun, 2012).

A single miRNA sequence can bind to a limited number of targets as they require perfect or near-perfect complementarity (Naqvi *et al*., 2012). The expression of miRNAs influences the expression of genes that encode transcription factors, stress-responsive proteins, and other proteins that contribute to plant growth and development (Rogers and Chen, 2013). Consequently, interactions between miRNAs and their targets allow miRNAs to regulate physiological and developmental processes as well as stress responses (Chen, 2005). The regulatory mechanisms between transcription factors and miRNAs in response to biotic and abiotic stresses are illustrated in Figure 2.6. MicroRNAs regulate plant developmental changes such as leaf morphogenesis, shoot and root formation, floral development and vegetative phase changes (Zhang *et al*., 2006; Wang and Li, 2007; Yang *et al*., 2007). Additionally, miRNAs are involved in signal transduction and responses to environmental stress conditions (Sun, 2012). Nadarajah and Kumar (2019) stated that miRNAs regulate various targets, making them master regulators of gene expression.

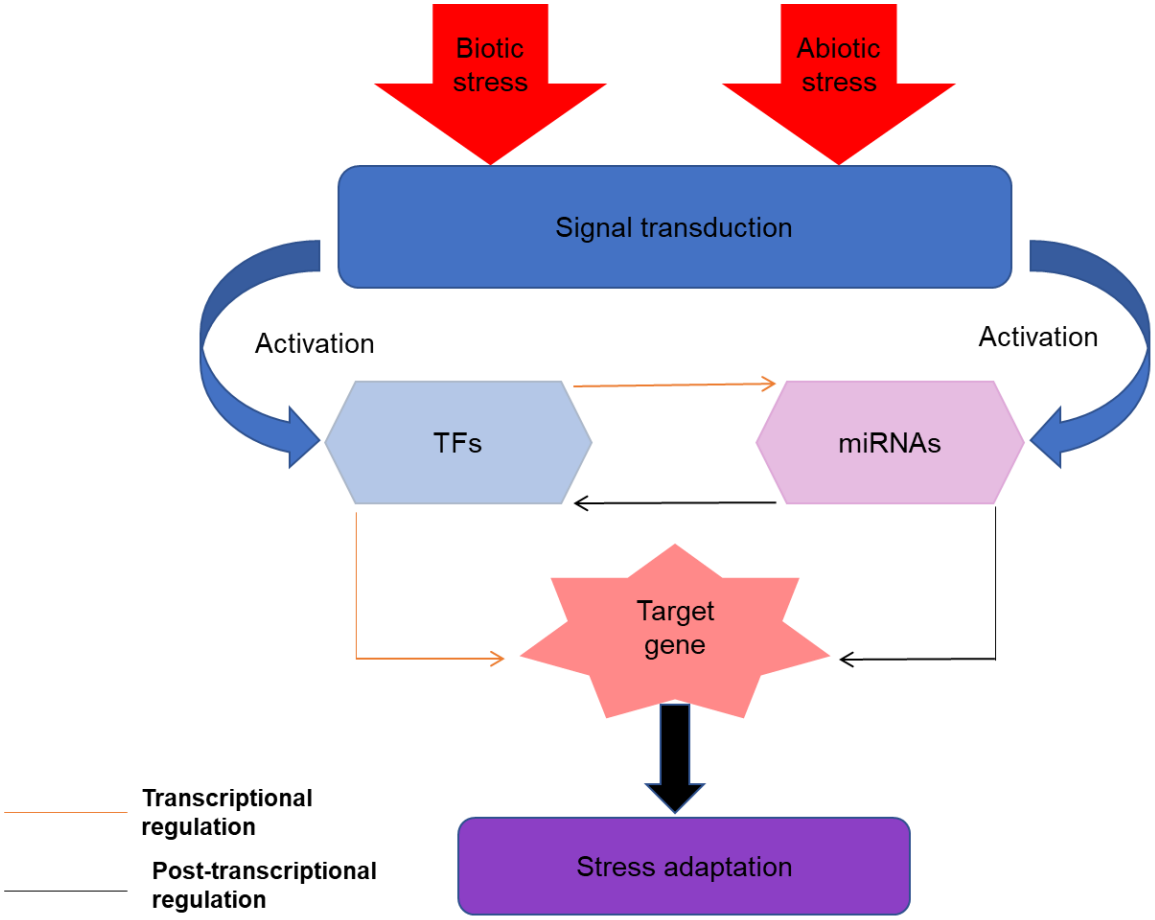

Figure 2.6. The crosstalk between microRNAs (miRNAs) and transcriptional factors (TFs) induced during abiotic and biotic stresses in plants. miRNAs can regulate target gene post-transcriptionally as shown with the solid line or through TF as shown with the dotted line (Adapted from Samad *et al*., 2017).

MicroRNA families are classified into two groups based on variation across species (Axtell and Bowman, 2008; Tang, 2010). The first group consists of well-conserved miRNA families with high expression levels (Djami-Tchatchou *et al*., 2017). The conserved miRNAs regulate developmental processes in plants and some examples include miR156, miR159, miR160, miRNA165/166, miR319 and miR444 (Sunkar and Jagadeeswaran, 2008). The second group is that of non-conserved miRNAs, which are only expressed under specific environmental conditions or specific tissues (Chakraborty *et al*., 2020) and include miR163, miR472, miR820,

miR858, miR5200 and miR6019 (Qin *et al*., 2014).

2.3.1.3 Techniques used for studying microRNAs

Identifying miRNAs is the first step in understanding their function (Shriram *et al*., 2016). Experimental and computational approaches have been widely used in plants to identify miRNAs (Zhang and Wang, 2015). Previously, miRNAs were identified using genetic screening and genetic cloning technologies (Sun, 2012). However, a limited number of miRNAs have been identified due to their short sequence length and methylation status (Tripathi *et al*., 2015). Consequently, these technologies are being replaced by next-generation sequencing (NGS) technology, which is a powerful tool for enhancing the discovery of novel miRNAs and target identification (Djami-Tchatchou *et al*., 2017). The advantage of NGS technology is that it can generate many sequences per run, unlike genetic screening and genetic cloning technologies, which can only analyse a single miRNA per run, making them costly and time-consuming (Raza and Ahmad, 2019).

Furthermore, the expression levels for each identified miRNA can also be assessed using NGS, northern blotting, quantitative real-time PCR (qRT-PCR), and miRNA microarrays (Schmittgen *et al*., 2008; Raza and Ahmad, 2019). Generally, miRNA microarrays are less expensive compared to other profiling methods (Pritchard *et al*., 2015). Pritchard *et al*. (2015) discussed miRNA microarray limitations and suggested using qRT-PCR or northern blotting techniques to validate miRNAs. Since the above-mentioned experimental approaches generate large data sets, computational methods are required to interpret the data (Gasparis *et al*., 2017).

Computational approaches are increasingly used to identify miRNAs due to their speed, relative accuracy, and cost-effectiveness (Pandey *et al*., 2013). There are two computational approaches for miRNA identification, namely, comparative and non-comparative approaches (Gomes *et al*., 2013). These two approaches have their advantages and disadvantages. Comparative methods, such as the homology-based approach, depend on preserving genetic information in closely related species genomes (Kleftogiannis *et al*., 2013). This method uses sequence and structural homology to identify miRNAs (Li *et al*., 2010). However, the disadvantage of comparative methods is that they cannot detect novel miRNAs that lack sequence homology to known miRNAs (Gomes *et al*., 2013). On the other hand, non-comparative methods make use of machine learning techniques to categorize miRNAs based on sequence variation and structural and thermodynamic features (Kleftogiannis *et al*., 2013; Alptekin *et al*., 2017).

Several public databases have been established in response to the discovery of various miRNAs and their target genes (Liao *et al*., 2018). The databases are repositories that contain comprehensive and organised information about miRNAs found in various species (Griffiths-Jones *et al*., 2006). In addition, some include experimental evidence about miRNA, computational predictions, and expression profiles. Table2.1 shows some of the miRNA databases available for identifying miRNAs and their targets.

Table 2.1. Examples of miRNA databases.

Database name	Description	Link	References
MicroRNA database (miRBase)	miRbase offers access to extensive miRNA sequences data, annotations and predicted gene targets. It is also the main repository for miRNA sequences and associated annotations.	http://www.mirbase.org/	Griffiths-Jones *et al*., 2006
Plant miRNA target Database (TarDB)	TarDB uses cross species conservation filters, degradome and analysis of small RNA-seq data. Its gathers newly discovered miRNA targets and phasiRNA data, specifically those that are associated with lineage specific or species-specific miRNAs.	http://www.biosequencing.cn/TarDB/	Liu *et al*., 2021
Plant miRNA database (PMRD)	PMRD combines extensive data on plant miRNAs such as miRNA sequences, targets, genes, secondary structure information, expression patterns and a genome browser.	http://bioinformatics.cau.edu.cn/PMRD/	Zhang *et al*., 2009
miRNEST	miRNEST has a collection of plant, animal and virus miRNAs. The database includes miRNA predictions from deep sequencing libraries, data from degradome analyses, information on miRNA splice sites and pre-miRNA classification with HuntMi.	http://mirnest.amu.edu.pl/	Szczesniak and Makalowska, 2014
Plant small RNA Target (psRNATarget)	psRNATarget identifies transcripts targeted by small RNAs. The database determines the small RNAs and their targets complementarity.	https://www.zhaolab.org/psRNATarget/	Dai *et al*., 2018
Plant Transcription Factor database (PlantTFDB)	PlantTFDB provides information on transcription factors, supporting the identification of miRNA-transcription factor regulatory interactions from different species.	http://planttfdb.gao-lab.org/	Jin *et al*., 2017
RNACentral	RNACentral has more than 18 million non-coding RNA sequences from different organisms. The database has been integrated into many RNACentral member databases such as Rfam and miRbase. To enable a detailed category of RNA types and subtypes, the sequences in RNACentral have been annotated with sequence ontology.	https://rnacentral.org/	RNACentral Consortium, 2020
Plant miRNA Encyclopedia (PmiREN)	PmiREN is a functional plant miRNA with small RNA library sequences used to quantify miRNA expression patterns and Parallel Analysis of RNA Ends sequencing (PARE-Seq) libraries used to confirm the predicted miRNA target pairs.	https://www.pmiren.com/	Guo *et al*., 2019

2.3.1.4 Plant microRNAs in response to drought stress

Under drought stress, plant growth and developmental processes are also controlled by stress-responsive miRNAs that regulate drought-responsive genes (Ferdous *et al*., 2015). The miRNA-based stress regulation involves direct targeting of specific gene transcripts (Alptekin *et al*., 2017). Hamza *et al*. (2016) investigated the effect of drought stress on the expression of selected miRNAs in leaf tissue across 11 sorghum genotypes using the stem-loop RT-PCR. Eleven sorghum genotypes, showing different performances under drought stress were compared in the study. The eight selected miRNAs were sbi-miR160, sbi-miR166, sbi-miR167, sbi-miR168, sbi-miR393, sbi-miR396, sbi-miR397-5p and sbi-miR398. These selected miRNAs target various genes, including *auxin-response factor, homeobox-leucine zipper family protein, protein of unknown function, stabiliser of iron transporter, poly nucleotidyl transferase, auxin signalling F-box proteins, growth regulating protein, factor and laccase,* and *copper/zinc superoxide dismutase* genes, respectively (Hamza *et al*., 2016). In the study, the miRNAs were selected for profiling due to their conservation and known down-regulation under abiotic stress, as documented in the miRbase release 20. The expression levels of sbi-mi396 and sbi-mi389 were the highest in all genotypes under drought stress. At the same time, there was a down-regulation of sbi-mi393, sbi-mi397-5p, sbi-miR166, sbi-miR167, and sbi-miR168. The authors concluded that their findings suggest that miRNAs play a significant role in regulating plant growth in vegetative tissues under abiotic stress (Hamza *et al*., 2016).

In a study by Katiyar *et al*. (2015), novel drought-responsive miRNAs and trans-acting siRNAs were identified in leaves of two contrasting sorghum varieties using high-throughput sequencing analysis. A drought-tolerant M35-1 and drought-susceptible C43 sorghum genotypes were grown under well-watered and drought stress conditions. Water was

withheld from the drought-stressed plants until the leaf relative water content was approximately 60-65% in both varieties. In the study, 96 unique miRNAs, belonging to 8 known and 88 novel families, were identified under drought stress. Furthermore, out of the 96 drought responsive miRNAs, 23 were up, and 9 were down-regulated in both genotypes. Forty-four miRNAs under drought stress conditions were up-regulated and down-regulated for M35-1 (drought-tolerant) and C43 (drought susceptible) genotypes, respectively. On the other hand, 19 miRNAs were downregulated in M35-1 and they were up-regulated in C43 genotype under drought stress. Lastly a novel miRNA sbi-miR259 was undetectable in the drought-tolerant but down-regulated in the drought-susceptible variety. In conclusion, the authors suggest their findings will be useful for further functional characterization of miRNAs in sorghum in response to drought stress (Katiyar *et al*., 2015).

CHAPTER 3

Morphological and Physiological Responses of ICSB 338 and SA 1441 Sorghum Seedlings to Drought Stress

3.1 Introduction

Drought stress induces morphological, physiological, and biochemical changes that ultimately affect plant growth and development (Amoah and Antwi-Berko, 2020; Oguz *et al*., 2022). However, the extent of these changes may vary within and between different plant species, the stages of growth at which the plant experienced the stress, and the intensity and duration of the stress (Sallam *et al*., 2019; Seleiman *et al*., 2021). Nevertheless, when plants experience water deficit, they synthesise a hormone called ABA in the roots; this hormone is transported to various parts of the plant, including leaves, to initiate responses to the stress (Basu *et al*., 2016). Consequently, roots may continue to grow while shoot growth is inhibited (Pandey and Shukla, 2015; Kurepa and Smalle, 2022). This increased root growth enhances water uptake from deeper layers of the soil (McAdam *et al*., 2016; Azarbad *et al*., 2018), while reduced shoot growth helps plants to redirect photosynthates to the roots, thus conserving energy (Bashir *et al*., 2021). Although root growth is generally thought to increase during drought stress, in some instances, both shoot and root growth stagnate (Bibi *et al*., 2010).

Other studies have reported various growth, physiological and biochemical responses of plants to drought (Efeoglu *et al*., 2009; Fadoul *et al*., 2018). Fadoul *et al*. (2018) conducted a morphological study of drought-tolerant EL9 and drought-sensitive Tabat sorghum lines in response to drought stress. The plants were subjected to drought stress by withholding water for seven days at the seedling stage. Results showed a reduction in both shoot and root length for the drought-sensitive Tabat cultivar and an increase in both measurements for the drought-

tolerant EL9 sorghum variety. The authors concluded that the reduction in root and shoot growth of the drought-sensitive line indicates the susceptibility of this variety to water limitation and the harmful effects of drought on plant development of some varieties (Fadoul *et al*., 2018). Due to reduced cell water content and low turgor, leaves may wilt and close stomatal openings during drought (Ahanger *et al*., 2021). As a result of stomatal closure, carbon dioxide uptake is reduced, thus negatively affecting photosynthesis (Pandey and Shukla, 2015). Furthermore, drought stress causes a reduction in photosynthetic assimilates and energy available in plants, which contributes to impaired growth (Bibi *et al*., 2012).

Chlorophyll content may decrease under drought stress, possibly due to increased chlorophyll degradation caused by oxidative stress (Anjum *et al*., 2011) or reduced chlorophyll biosynthesis due to chloroplast damage (Dalal and Tripathy, 2012). Efeoglu *et al*. (2009) investigated the physiological responses of three maize accessions, Doge, Vero, and Luce, to drought stress. The three maize accessions showed different levels of drought tolerance, with Doge being identified as drought sensitive. To induce drought stress on the plants, water was withheld for 12 days. The authors observed a significant reduction in chlorophyll a, b and a + b content of all maize varieties under water limiting conditions compared to the control. They attributed this pigment reduction to a loss of photosynthetic reaction centres I and II due to drought stress (Efeoglu *et al*., 2009).

Plants also respond to drought stress by accumulating various antioxidants, which reduce oxidative damage, and osmoprotectants, which stabilise cell structure (Ilyas *et al*., 2021). These response mechanisms can vary among varieties of the same species, highlighting the importance of comparative studies in plant stress biology (Bashir *et al*., 2021). Furthermore, the genetic differences within species contribute to the genetic diversity required for

developing new crop varieties with enhanced resilience towards harsh environmental conditions (Rosero *et al*., 2020; Salgotra and Chauhan, 2023). Therefore, this chapter aimed to evaluate various morpho-physiological and biochemical changes of drought-susceptible ICSB 338 and drought-tolerant SA 1441 sorghum seedlings under drought conditions.

3.2 Materials and methods

3.2.1 Plant material

Two sorghum varieties, the drought-susceptible ICSB 338, and drought-tolerant SA 1441, were obtained from Dr Nemera Shargie at the Agricultural Research Council (ARC)-Grain Crops Institute (GCI), Potchefstroom, South Africa. The two sorghum varieties were selected based on their contrasting drought phenotypes and the results of a sorghum root proteomic study conducted by Goche *et al*. (2020).

3.2.2 Seed surface sterilization

Seed sterilization was conducted in a laminar airflow cabinet. A total of a 100 ICSB 338 and SA 1441 sorghum seeds each were placed into Falcon tubes containing 40 ml of 70% (v/v) ethanol. The Falcon tubes were shaken for a minute, and the ethanol waste was discarded. Subsequently, 40 ml of absolute commercial bleach (3.5% (w/v) sodium hypochlorite) containing 0.1% (v/v) Tween 20 was added, and the mixture was shaken occasionally for 30 minutes. The seeds were rinsed three times with sterile distilled water and briefly air-dried on sterile filter paper.

3.2.3 Seed germination and seedling growth conditions

The surface sterilised sorghum seeds were germinated in Petri-dishes lined with a sterile, moist paper towel and incubated in the growth chamber (Model: GC-539DH, Already

Enterprise Inc., Taipei, Taiwan) at temperatures of 27 °C/19 °C (day/night) under dark conditions for 5 days. Thereafter, the seedlings were transplanted into a 10 cm top diameter, 8.2 cm height, and 6.5 cm bottom diameter plastic pots with 10 plants in each pot. The growth medium contained potting soil (Culterra, Muldersdrif, South Africa), cocopeat (Bio-leaf Technologies, Olifantsfontein, South Africa), and vermiculite (Sigma-Aldrich, Gosforth Park Germiston, South Africa) at a ratio of 1:1:1. Each pot was filled with 300 ml of the growth medium. This growth medium was chosen after preliminary experiments were conducted to determine a suitable growth medium and ratios for use in the study. The growth medium was fully saturated with diluted Nitrosol Nutrient Solution (Fleuron, Germiston, South Africa) as per manufacturer's recommendations and left to drain for three hours. Thereafter, the pots were incubated in the growth chamber at temperatures of 27 °C/19 °C (day/night) with a light cycle of 16/8 hours (day/night). The potted plants were watered with sterile distilled water every second day for three weeks, before being subjected to drought stress treatment.

3.2.4 Drought stress treatment

3.2.4.1 Imposition of drought stress

The diluted Nitrosol Nutrient Solution (Fleuron) was applied to the plants when they were 20 days old. Plants were divided into two water treatment groups: The first group was the watered control which received equal volumes of water every second day. The second group was the drought-stressed treatment with a water regime where water was withheld for 15 and 28 days. Each sorghum variety had three biological replicate pots with 10 seedlings each for the control and drought-stressed plants. Stomatal conductance and pot weight measurements were taken daily during the drought stress treatment period. The leaves and roots of the watered control and drought-stressed plants were harvested on days 15 and 28 of the stress treatment and

immediately frozen in liquid nitrogen prior to storage at -80 °C for chlorophyll content assays (Chapter 3) and total RNA extraction (Chapter 4).

3.2.4.2 Pot weight readings

The weight readings of pots containing the growth medium and ICSB 338 and SA 1441 plants were taken daily from day 0 to day 28, with day 0 denoting the beginning of the drought stress treatment. These measurements were taken to establish any difference in pot weight and thus soil water content within and between the control and drought treatment groups with time. Three biological pot weight replicates were measured for both varieties.

3.2.4.3 Soil moisture content measurements

The soil moisture content was estimated using the gravimetric method as described by Vineeth *et al*. (2016) with minor modifications. Plants including the roots were harvested from pots containing the soil growth medium. Then the weight of the soil growth medium of each pot was taken. Thereafter, the soil was oven-dried at 105 ℃ for 48 hours, and the dry soil mixture was weighed. The soil moisture content was calculated using the formula described by Vineeth *et al*. (2016):

$$\text{Soil moisture content (\%)} = [(WW - DW)/DW] \times 100$$

Where WW is the wet weight and DW is the dry weight.

3.2.5 Physiological parameters and growth measurements

3.2.5.1 Stomatal conductance

The stomatal conductance was measured on the third oldest fully expanded leaf of sorghum plants using a SC-1 leaf porometer (Decagon Devices, Inc., Washington, United States of America) during the 15 days of drought stress treatment. The leaf porometer was calibrated

according to the manufacturer's instructions before taking measurements. The measurements were taken daily and at the same time during the 15-day stress treatment period. Three biological replicates were used per treatment. Unfortunately, stomatal conductance readings were not taken on day 28 of drought stress.

3.2.5.2 Leaf relative water content

The leaf relative water content (RWC) was assessed following the method outlined by Barrs and Weatherley (1962). The third oldest leaf was cut from the control and drought-stressed plants and immediately used to determine the fresh weight (FW). Thereafter, the leaf samples were immersed in 50 ml Falcon tubes filled with distilled water and stored at 4 °C for 24 hours to reach full turgor. After the incubation period, each leaf sample was blotted dry on paper towel and measured to determine the turgid weight (TW). To estimate the dry weight (DW), the leaf samples were oven-dried at 60 °C for 48 hours. The RWC was calculated using the formula, described by Barrs and Weatherley (1962):

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Where RWC is the relative water content, FW is the fresh weight, DW is the dry weight, and TW is the turgid weight.

3.2.5.3 Shoot and root length and biomass measurements

Plants were carefully removed from the pots of both the watered control and drought-stressed SA 1441 and ICSB 338. The roots were washed with sterile distilled water to remove any soil that could cover the actual length, then blotted dry using a paper towel. The shoot length was measured from the point of attachment of the shoot to the tip of the longest leaf. Thereafter, the root length was measured from the shoot point of attachment to the longest root. The shoot

and root lengths were measured using a ruler in centimetres. After measuring the lengths, the roots were cut off at the point of attachment with the shoot and separately weighed to determine the fresh weight. The shoot and root samples were then placed in an oven at 60 °C for 48 hours to determine the dry weight. Four biological replicates were used for each treatment per sorghum variety.

3.2.6 Chlorophyll and carotenoids content

The chlorophyll and carotenoids content were estimated as previously described by Wellburn (1994). Fifty milligrams of frozen ground leaf material was mixed with 10 ml of dimethyl sulfoxide (DMSO) in 15 ml Falcon tubes. The samples were incubated in a water bath at 65 °C for 3 hours. Thereafter, 1 ml of the extract was transferred into cuvettes for absorbance measurements at 480, 649, and 665 nm against DMSO as a blank solution. Where absorbance values were greater than 0.7, the extract was diluted to 50% with DMSO. The chlorophyll and carotenoid content were calculated using the equations described by Wellburn (1994):

$$\text{Chlorophyll a (mg/g)} = 12.19(A_{665nm}) - 3.45(A_{649nm})$$

$$\text{Chlorophyll b (mg/g)} = 21.99(A_{649nm}) - 5.32(A_{665nm})$$

$$\text{Carotenoids (mg/g)} = [1000A_{480nm} - 2.14Ca - 70.16Cb]/ 220$$

Where Ca is chlorophyll a and Cb is chlorophyll b.

3.2.7 Statistical analysis

Statistical analysis was done using ANOVA with a Tukey-Kramer post-test to compare the means between treatments and varieties. A Student's *t*-test was done to compare the means between the watered controls and drought stress plants. A probability of $p \leq 0.05$ was set as

the significance level in both ANOVA and Student's *t*-test. Graphpad Prism 8.0.2.263 was used to analyse the data.

3.3 Results

3.3.1 Leaf stomatal conductance measurements

During the 15-day drought stress period, stomatal conductance measurements were taken daily using a leaf porometer. The readings were taken to assess the degree of stomatal opening and closing in both the watered control and drought-stressed plants. In comparison to the control, the stomatal conductance of the drought-susceptible variety, ICSB 338 was statistically lower as early as day 4 of the drought stress treatment, while that of the drought-tolerant variety SA 1441 was statistically low starting on day 8. Figure 3.1 shows a statistically significant decrease in the stomatal conductance of drought-stressed plants on days 4, 5, 11, 14 and 15 for ICSB 338 and on days 8, 13, 14, and 15 for SA 1441.

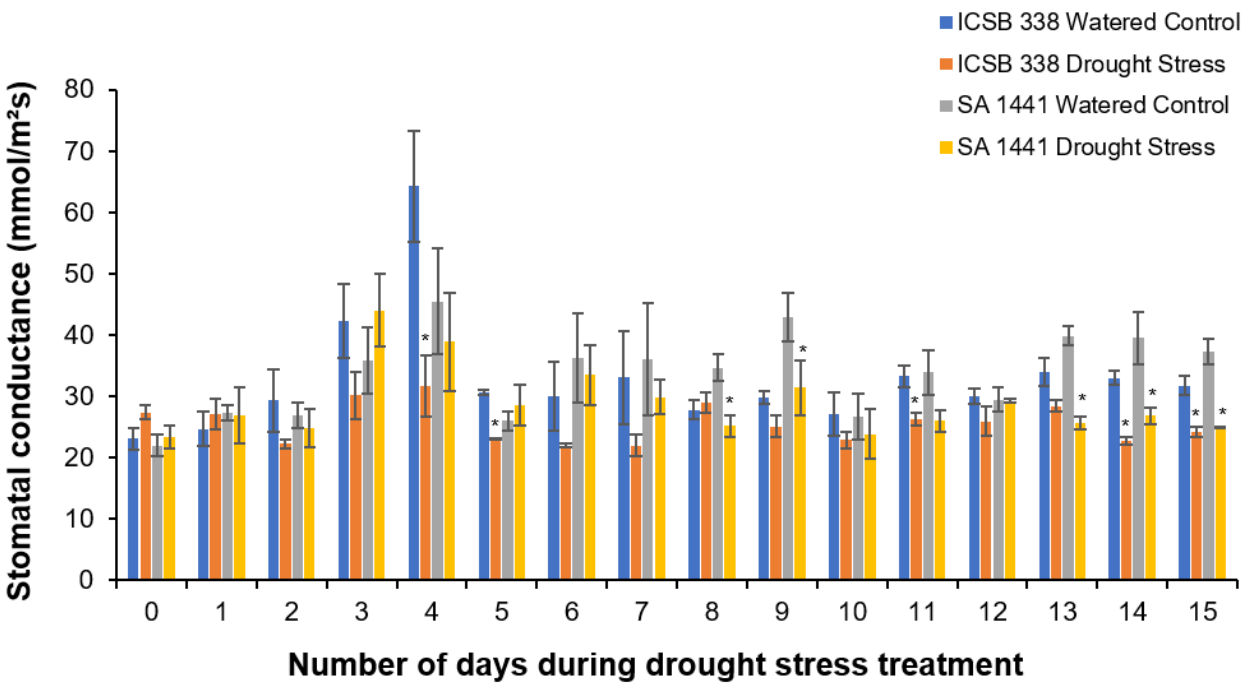

Figure 3.1. The effects of 15-day drought stress treatment on leaf stomatal conductance of both watered control and drought-stressed sorghum plants. The two sorghum varieties, ICSB 338 and SA 1441 were grown for three weeks before the imposition of drought stress treatment on the seedlings. Measurements were taken daily during the 15 days of stress treatment. Data presented as mean ± SE (n = 3). Bars with asterisks are statistically different between the control and treatment of ICSB 338 and SA 1441 at each time point according to the Student's t-test ($p \leq 0.05$).

3.3.2 Pot weight measurements

The weight readings of the pots containing soil and plants were taken daily to establish any weight difference in soil water content over time and between the treatment groups. As expected, a gradual but statistically significant decrease in soil weight was observed from day 3 during the 28 days of drought stress treatment for both ICSB 338 and SA 1441 pots (Figure 3.2A).

Figure 3.2B illustrates the percentage reduction of the soil weight during the 28 days of drought stress treatment. There was a notable reduction in soil weight as the drought stress treatment progressed for 28 days. However, the drought-stressed SA 1441 pots seemed to lose more water than the ICSB 338 pots (Figure 3.2B).

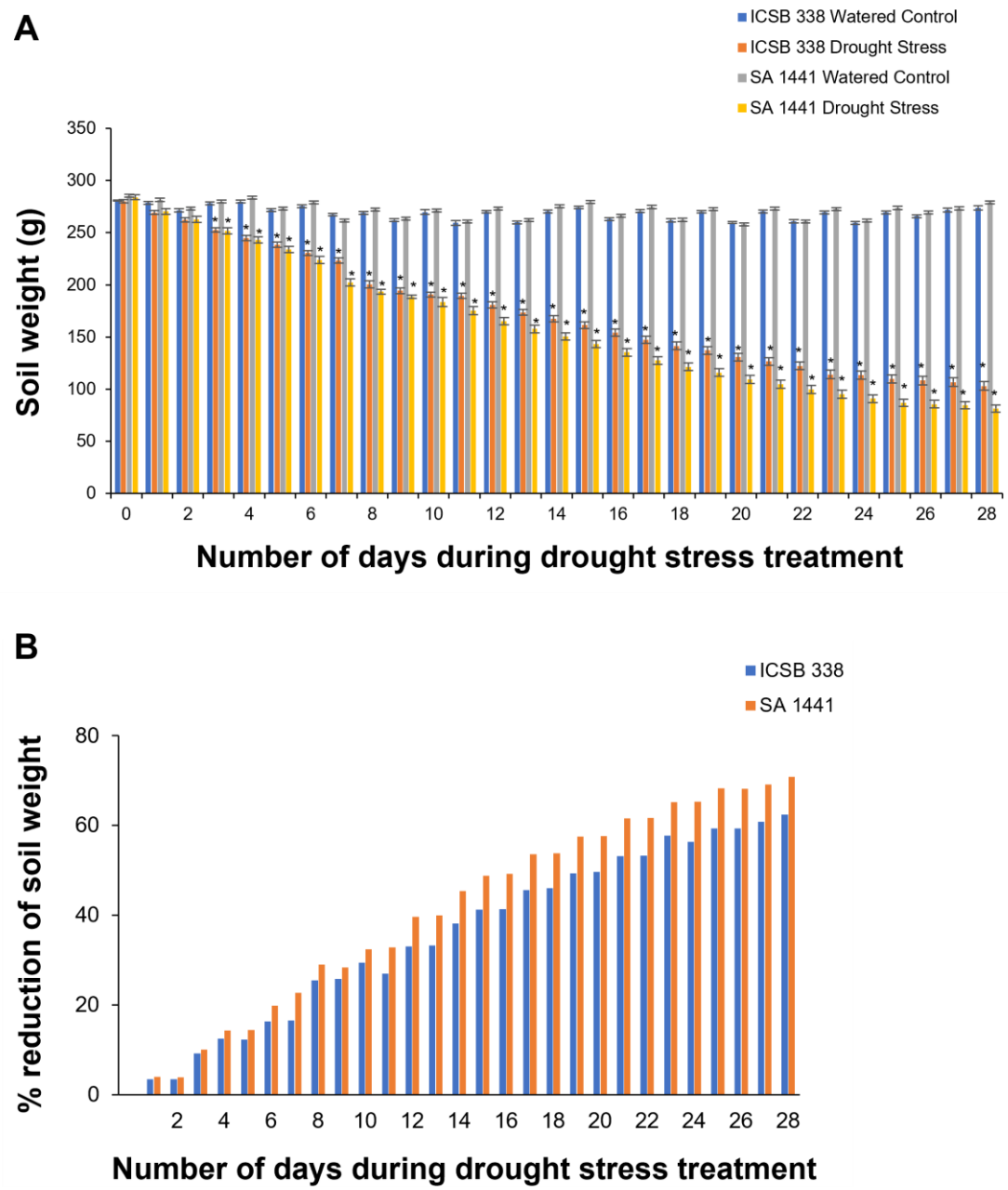

Figure 3.2. Changes in soil weight during the 28 days of drought stress treatment of sorghum potted plants. (A) shows the pot weight readings of ICSB 338 and SA 1441 plants taken daily during the 28 days of drought stress treatment. (B) shows the percentage reduction of soil weight for the 28 days of drought treatment. Data presented as mean ± SE (n = 3). Bars with asterisks are statistically different between the controls and drought stress treatments of ICSB 338 and SA 1441 at each time point according to the Student's t-test ($p \leq 0.05$).

3.3.3 Sorghum seedlings growth phenotype and soil moisture content

The soil moisture content in both the control and drought-stressed pots was estimated using the gravimetric method on days 15 and 28 of the drought stress treatments. Generally, a reduction in soil moisture content was observed at both time points in both sorghum varieties (Figures 3.3A and 3.3B). On day 15, the soil moisture content decreased significantly from 100% to 77% and 71% for ICSB 338 and SA 1441, respectively (Figure 3.3A). By day 28, the soil moisture content further decreased from 100% to 54% for ICSB 338 and 42% for SA 1441 relative to the watered controls (Figure 3.3B).

Drought stress reduced the plant height of stressed plants in both ICSB 338 and SA 1441 compared to the watered controls (Figures 3.3C and 3.3D). Water deficiency caused stunted growth of ICSB 338 plants at both 15 and 28 days of stress (Figures 3.3C and 3.3D). However, for ICSB 338, the reduction in growth was more visible at day 28 (Figure 3.3D). ICSB 338 plants stressed for 28 days had brittle leaves and thinner shoots compared to the watered control. Overall, drought reduced the plant height of ICSB 338 more than that of SA 1441. Additionally, the leaves of both varieties subjected to drought stress for 28 days appeared to be more chlorotic compared to plant leaves stressed for 15 days (Figures 3.3C and 3.3D).

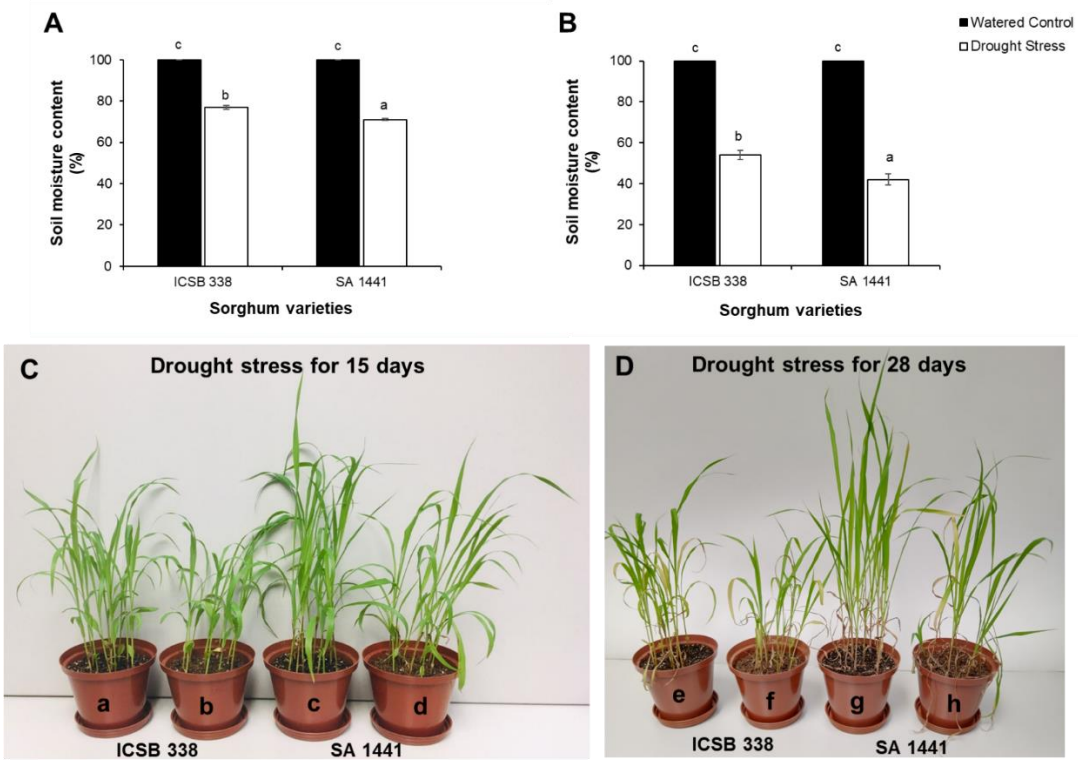

Figure 3.3. Soil moisture content and sorghum growth at different time points of drought stress. Drought stress was imposed on the sorghum seedlings by withholding water. (A) and (B) show the soil moisture content of ICSB 338 and SA 1441 sorghum plants on days 15 and 28 of drought stress treatment, respectively. (C) and (D) show the growth of watered control and drought-stressed plants of ICSB 338 and SA 1441 on days 15 and 28 of drought stress treatment, respectively. Small case letters on pots represent the different treatment groups, with a, c, e and g being watered controls and b, d, f, and h being drought treated samples. Data presented as mean ± SE (n = 3). Bars with different letters are statistically different at $p \leq 0.05$ according to ANOVA using the Tukey-Kramer test.

3.3.4 Leaf relative water content

Leaf RWC of ICSB 338 and SA 1441 plants were taken on days 15 and 28 of the drought stress treatment (Figure 3.4). The RWC of ICSB 338 sorghum seedlings was statistically lower on days 15 and 28 compared to their respective watered controls. The RWC of ICSB 338 decreased from 87% to 68% and from 79% to 58% on days 15 and 28, respectively (Figures 3.4A and 3.4B). In contrast, the decrease in RWC for SA 1441 drought-stressed plants was not statistically significant compared to their respective watered controls at both 15 and 28 days of drought stress.

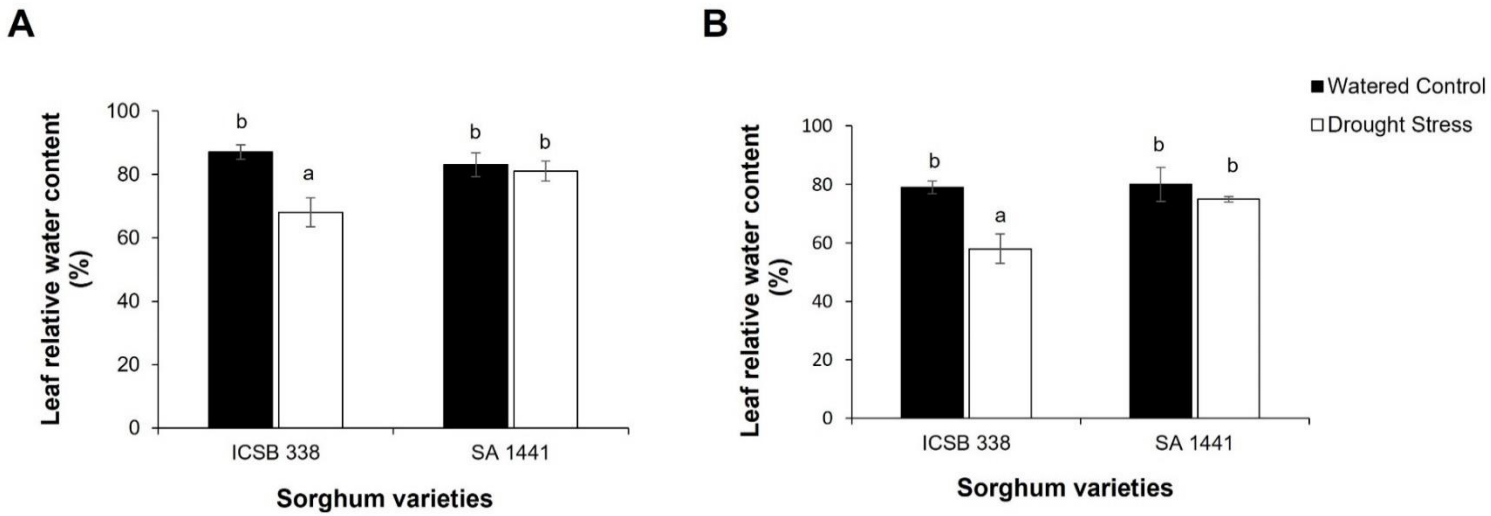

Figure 3.4. The leaf relative water content of two sorghum varieties following drought stress treatment. (A) and (B) shows the relative water content of ICSB 338 and SA 1441 subjected to drought stress for 15 and 28 days, respectively. The third oldest leaf was used to measure the relative water content. Data presented as mean ± SE (n = 3). Bars with different letters are statistically different at $p \leq 0.05$ according to ANOVA using the Tukey-Kramer test.

3.3.5 Chlorophyll and carotenoid content

The changes in chlorophyll a and b, and carotenoid content were also determined in the two sorghum varieties following 15 and 28 days of water limitation (Figure 3.5). The results indicated a general decrease in chlorophyll a and b, and carotenoid content in both ICSB 338 and SA 1441 seedlings following the drought stress treatment. However, there was no statistically significant changes in the pigment content observed in both sorghum varieties (Figure 3.5).

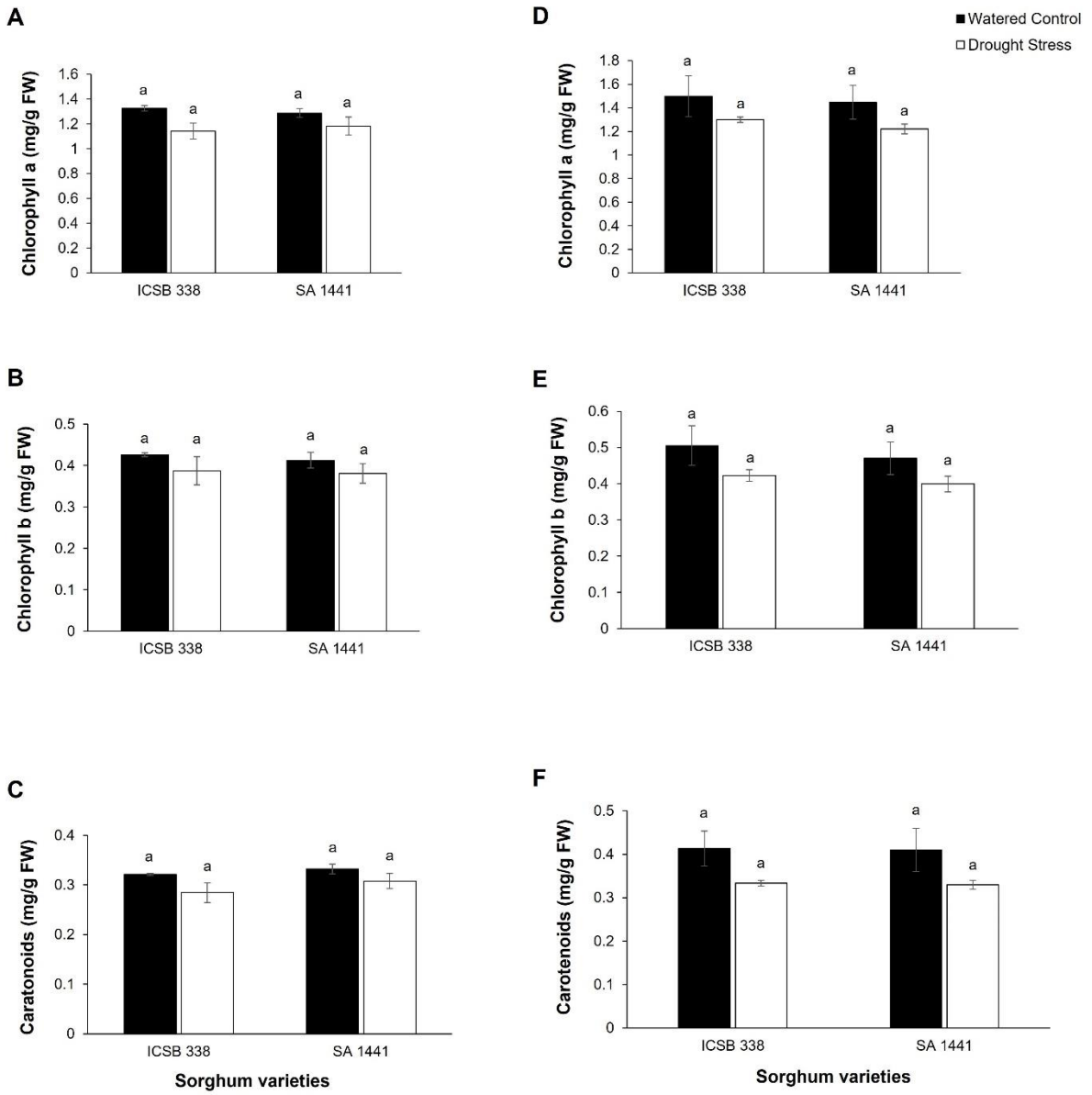

Figure 3.5. The effects of drought stress on chlorophyll and carotenoid content of sorghum seedlings. Drought stress was induced by withholding water. (A-C) and (D-F) show the chlorophyll and carotenoid content of the two sorghum varieties stressed for 15 and 28 days, respectively. Data presented as mean ± SE (n = 3). Bars with different letters are statistically different at $p \leq 0.05$ according to ANOVA using the Tukey-Kramer test.

3.3.6 Shoot and root growth measurements

The effects of the 15 days of drought stress on the shoot and root growth of drought-stressed plants relative to the control in both sorghum varieties were also estimated. The results showed a significant decrease of ~14.6%, ~15.1% and ~23.8% for ICSB 338 shoot length, shoot fresh and dry weight, respectively (Figures 3.6A, C and E). However, for SA 1441 there was no statistically significant difference in the shoot length, shoot dry and weight, and root fresh weight of the drought-stressed relative to the control. Additionally, there was a non-significant increase in the root length of drought-stressed SA 1441 plants (Figure 3.6B).

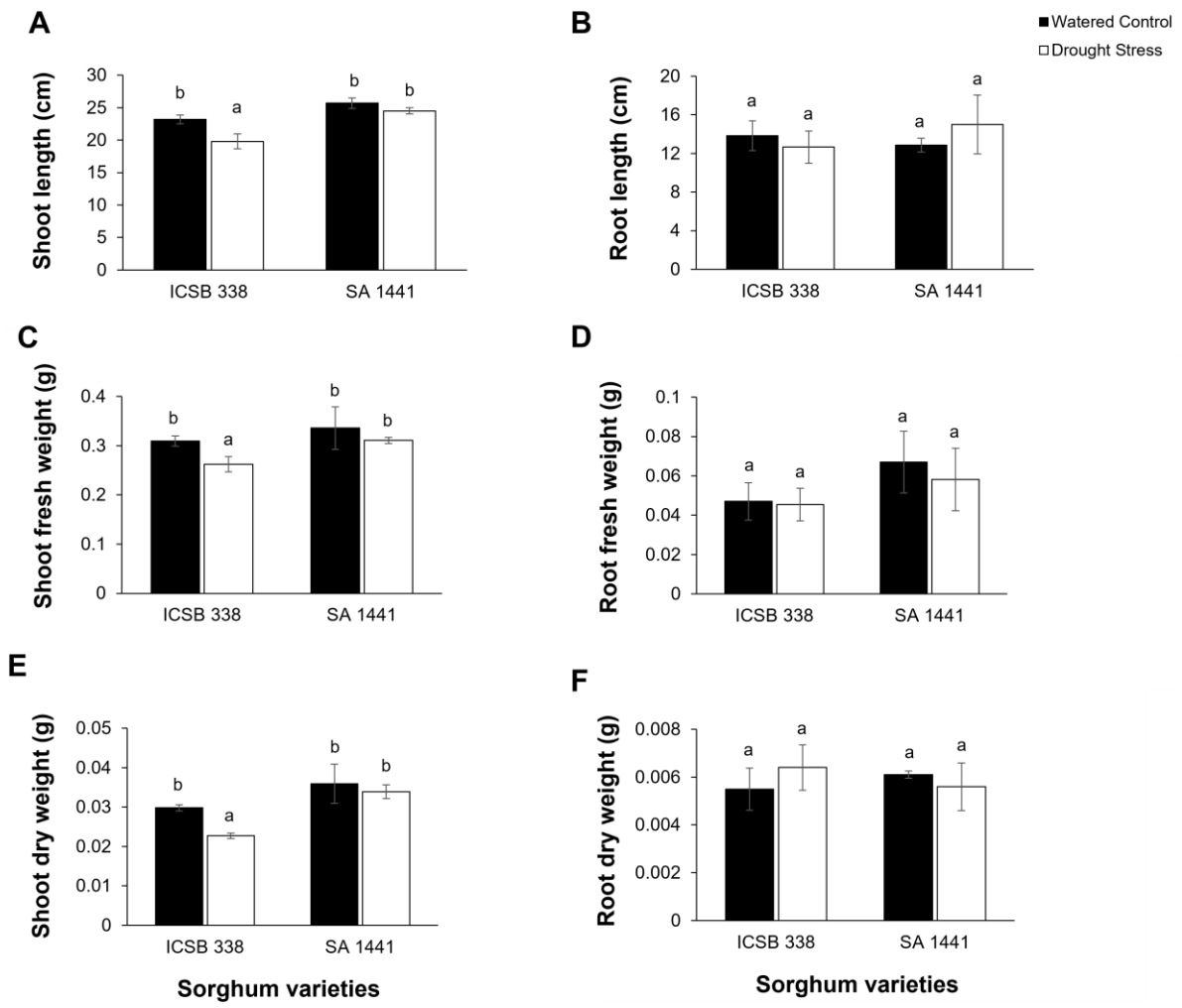

Figure 3.6. Shoot and root growth measurements of two sorghum varieties following the 15 days of drought stress. (A) shows the shoot length, (B) root length, (C) shoot fresh weight, (D) root fresh weight, (E) shoot dry weight, and (F) root dry weight. ICSB 338 and SA 1441 seedlings were grown for three weeks and were subjected to drought stress for 15 days. Data presented as mean ± SE (n = 3). Bars with different letters are statistically different at $p \leq 0.05$ according to ANOVA using the Tukey-Kramer test.

The effects of drought stress on the shoot and root growth of the sorghum varieties were also measured on day 28 of drought stress treatment. For SA 1441, there was a statistically significant decrease of ~10.9%, ~52.7% and ~32.4% in shoot length, shoot fresh and dry weight, respectively (Figures 3.7A, C and E). However, the root length (Figure 3.7B), root fresh and dry weight (Figures 3.7D and F) of SA 1441 showed no significant change from the watered controls following the 28 days of drought stress treatment. For ICSB 338, there was

no significant change across all the growth measurements in the drought-stressed plants relative to the watered controls at 28 days (Figure 3.7).

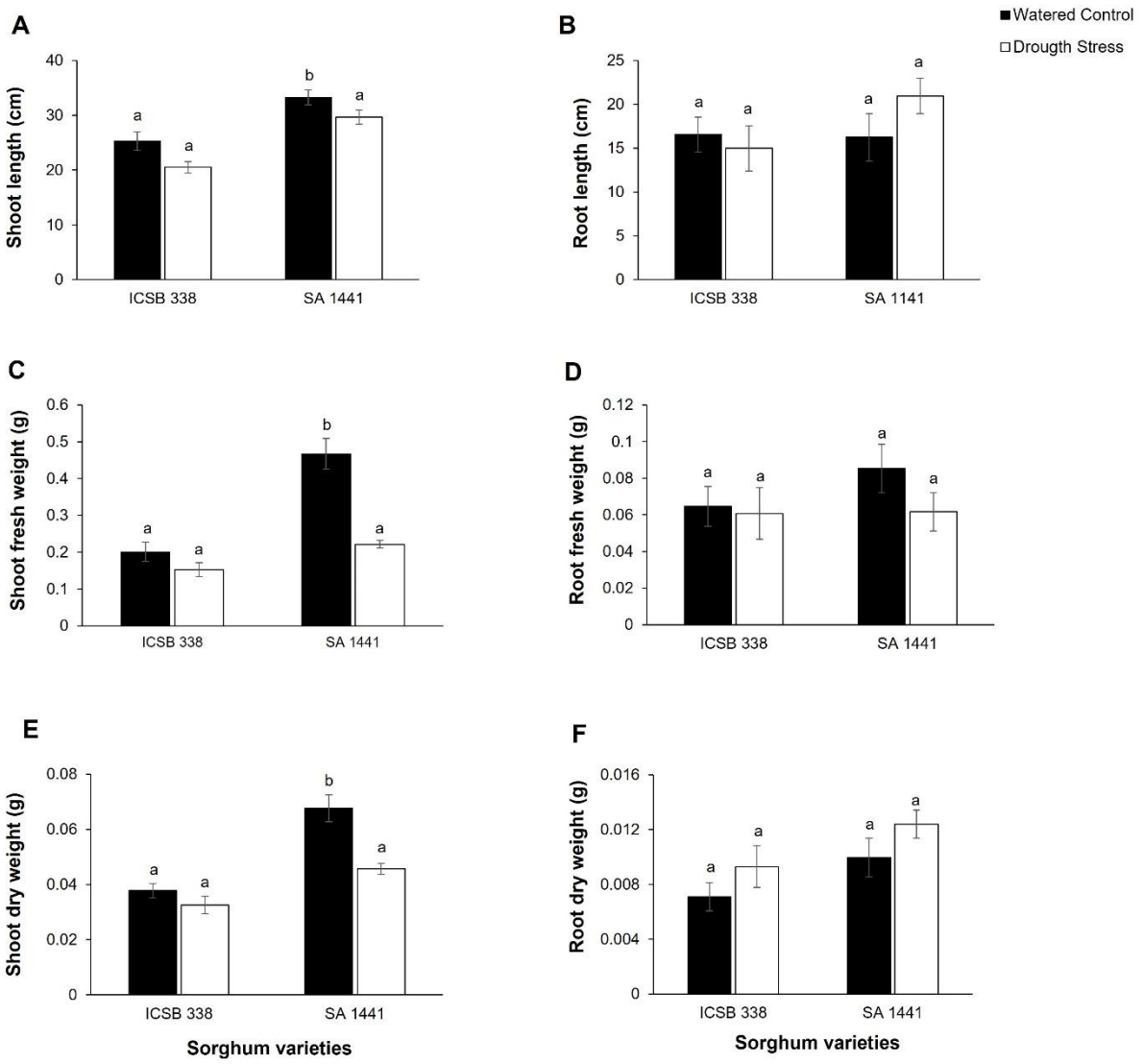

Figure 3.7. Shoot and root growth measurements of two sorghum varieties following 28 days of drought stress. (A) shows the shoot length, (B) root length, (C) shoot fresh weight, (D) root fresh weight, (E) shoot dry weight, and (F) root dry weight. ICSB 338 and SA 1441 seedlings were grown for three weeks and were subjected to drought stress for 28 days. Data presented as mean ± SE (n = 3). Bars with different letters are statistically different at $p \leq 0.05$ according to ANOVA using the Tukey-Kramer test.

3.4 Discussion

Drought stress affects the growth and functioning of plants (Seleiman *et al*., 2021), although the impact depends on the plant species, genotype developmental stage, and duration, and intensity of the stress (Sallam *et al*., 2019). Prolonged drought conditions can reduce crop production, thus threatening food security (He *et al*., 2019). Therefore, it is important to grow drought-tolerant crops to reduce the challenges of food insecurity as climate change is predicted to increase the frequency and severity of drought episodes (McCarthy *et al*., 2018). This study evaluated the morphological and physiological changes of the drought-susceptible ICSB 338 and the drought-tolerant SA 1441 sorghum varieties under drought conditions. These two sorghum varieties were chosen based on their contrasting drought phenotypes and the findings of a root proteomic study under drought stress (Goche *et al*., 2020). The study categorized SA 1441 as drought-tolerant and ICSB 338 as drought-susceptible (Goche *et al*., 2020). Understanding the drought responses of two contrasting varieties of the same plant species provides insight into specific traits that could possibly contribute towards drought resilience for future use in developing drought-resistant crops (Fang and Xiong, 2015; Bandurska, 2022).

The drought-tolerant and susceptible sorghum varieties were grown on soil to investigate the morpho-physiological traits under water limitation stress (Figures 3.3C and 3.3D). When selecting a soil mixture for seedling growth, it is important to consider factors such as water-holding capability and whether there are enough nutrients for seedling growth (Baligar *et al*., 2001; Nagase and Dunnet, 2011). In addition, the growth medium chosen should not damage the roots of the seedlings (Nagase and Dunnet, 2011). In this study, sorghum plants were grown in a growth medium consisting of potting soil, cocopeat and vermiculite with a ratio of 1:1:1. The growth medium was selected from preliminary experiments (results not shown) due to its ease of plant removal from the pots without damaging the roots.

The early stages of sorghum growth, including germination, emergence, and seedling establishment, are sensitive to drought stress (Bayu *et al*., 2005). However, drought is categorized into mild, moderate, and severe levels depending on the intensity of water scarcity (Anjum *et al*., 2017). For example, when compared to the adequately watered controls at field capacity, different levels of drought stress have been described (Anjum *et al*., 2017; Zhao *et al*., 2020). Under mild drought stress, the soil moisture content ranges between 65 - 80% relative to that of the watered controls, 45 - 65% under moderate drought stress, and 25 - 45% under severe drought stress (Anjum *et al*., 2017; Zhao *et al*., 2020). The shift between these levels happens gradually, showing the progression of drought stress and the different degrees of drought intensity (Laxa *et al*., 2019).

In this study, the soil moisture content of the drought-stressed plants ranged between 71% - 77% relative to the watered controls for the 15 days of stress (Figure 3.3A). However, for the 28 days of water limitation, the soil moisture content ranged between 42% - 54% relative to the watered controls (Figure 3.3B). The 71% - 77% range in soil moisture content indicates mild drought stress, while the 42% - 54% range indicates moderate drought stress. In the current study, the drought-tolerant variety, SA 1441 had a lower soil moisture content compared to ICSB 338, following 15 and 28 days of drought stress (Figures 3.3A and 3.3B). This observation could be due to SA 1441 plants being larger than those of ICSB 338 (Figures 3.3C and 3.3D), leading to more water usage. Although the drought-stressed soil of both varieties had similar weight up until day 3 of the stress, from day 4 until day 28, the soil weight of SA 1441 decreased faster than that of ICSB 338 (Figure 3.2B). In this experiment, the changes in pot weight are attributed to both water loss during the progression of the drought stress treatment and changes in plant growth over time. Given the larger size of SA 1441 plants

(Figure 3.3D), these plants possibly absorbed water at a faster rate, resulting in a higher percentage reduction in soil weight (Figure 3.2B).

Under drought stress conditions, plants close their stomata to avoid excessive water loss (Chaves *et al*., 2003). However, this protective mechanism reduces carbon dioxide absorption (Zlatev and Lidon, 2012) and lowers photosynthetic efficiency (Ghotbi-Ravandi *et al*., 2014). In this study, the drought-susceptible ICSB 338 sorghum variety exhibited an early reduction in stomatal conductance compared to the drought-tolerant SA 1441 variety (Figure 3.1). This could be due to the plant's immediate response to save water because of the decreasing soil water levels. The delay in closing the stomata of the drought-tolerant SA 1441 could allow for continued gas exchange even under drought conditions. The prolonged gas exchange could help sustain carbon dioxide uptake, leading to efficient photosynthesis compared to the ICSB 338 variety. The results of this study are similar to findings from Goche *et al*. (2020), where the drought-susceptible ICSB 338 closed the stomata on day 3 of drought stress, which was one day earlier than in SA 1441. Further studies could also measure photosynthesis efficiency, intercellular carbon dioxide concentration and the net photosynthetic rate of these two sorghum varieties under mild, moderate and severe drought conditions.

Exposure of sorghum plants to drought stress for 15 and 28 days reduced plant growth, as evidenced by the shorter plant height of water deprived plants (Figures 3.3C and 3.3D). There was a noticeable reduction in the height of ICSB 338 seedlings compared to SA 1441 for plants stressed for 15 days. This could be attributed to ICSB 338 being more susceptible to drought stress than the tolerant variety SA 1441 (Goche *et al*., 2020). However, the 28 days of stress resulted in a marked height reduction for both ICSB 338 and SA 1441 plants. It could possibly be due to the extended period of water limitation that led to reduced growth irrespective of the

sorghum variety. These results indicate that the drought stress treatment conditions of this study led to moderate stress conditions at day 28 (Figure 3.3D) that negatively affected plant growth and development (Figure 3.3D). Greenhouse and field studies assessing plant yield under similar drought conditions would provide more insight into the long-term impacts of the stress.

SA 1441 root lengths were increased in plants exposed to water limitation for 15 and 28 days (Figures 3.6B and 3.7B). However, these changes between the watered control and drought-treated plants were not statistically significant. The response could be attributed to the plants trying to absorb water in deeper soil layers since soil dries from the top to the bottom layers. Sharp (2002) also stated that root growth is less affected than shoot growth under limited water conditions. Similar results were reported in this study, where drought stress significantly reduced the shoot length, shoot fresh and dry weight of SA 1441 plants following 28 days of stress treatment (Figures 3.7A, C and E). The reduction in shoot growth could help to redirect photosynthates to the roots, enhancing root growth for improved water uptake (Bashir *et al*., 2021).

The leaf RWC is an important measure of the plant water status under conditions of drought (Tiwari *et al*., 2021). Drought stress may lead to a decrease in the RWC measurements of plant leaves (Siddique and Islam, 2000) although this may vary between varieties of the same species with contrasting drought phenotypes (Goche *et al*., 2020). Generally, a drought-induced reduction in RWC is due to reduced water uptake by plant roots, which decreases the overall water content of plant tissues (Wach and Skowron, 2022). In the present study, there was a significant decrease in the leaf RWC of drought-stressed ICSB 338 plants following 15 and 28 days of drought-stress treatment (Figure 3.4A and B) but not for SA 1441. This suggests that the drought-tolerant SA 1441 variety might be able to absorb water more effectively by using

a larger root system under drought conditions (Figure 3.7B) and conserve the water via tighter stomatal closure (Figure 3.1). Additionally, the ability of SA 1441 to maintain a higher RWC could be due to efficient osmotic regulation by proline and glycine betaine, contributing to its resilience under drought conditions (Goche *et al*., 2020). The results of the current study concur with those of Li *et al*. (2019), who observed that a drought-susceptible maize variety Denghai 605, had a significant reduction in the leaf RWC compared to that of a drought-tolerant Liyu 35 maize genotype under drought stress. In that study, drought stress was induced until the soil moisture content was at 40% field capacity (Li *et al*., 2019). Overall, the drought-tolerant SA 1441 maintained a higher relative water content showing its ability to conserve water possibly to continue its metabolic activities under drought conditions.

A plant's ability to maintain normal chlorophyll levels determines its capacity to sustain photosynthetic activity (Chen *et al*., 2016; Abreha *et al*., 2022). According to Chen *et al*. (2016), examining chlorophyll content effectively monitors plant growth. Chlorophyll a and b are susceptible to drying soils (Monteoliva *et al*., 2021) although the extent of these effects varies with the plant species, genotypes and drought severity (Seleiman *et al*., 2021). In this study, there was a reduction in chlorophyll a, b, and carotenoid content under drought conditions, but it was not statistically significant following 15 and 28 days of drought stress treatment in both sorghum varieties (Figure 3.5). These findings are consistent with those of Ayalew *et al*. (2018), who found that drought conditions decreased chlorophyll levels in the drought-tolerant and drought-susceptible sorghum lines. This could result from either chlorophyll degradation and photo-oxidation induced by reactive oxygen species and/or reduced chlorophyll biosynthesis due to increased chloroplast damage (Monteoliva *et al*., 2021).

3.5 Conclusion

In conclusion, this study evaluated morpho-physiological and biochemical changes of drought-susceptible ICSB 338 and drought-tolerant SA 1441 sorghum seedlings under varying levels of drought stress. Two drought levels were used: mild stress that lasted for 15 days and moderate stress for 28 days in a laboratory growth chamber. It was observed that drought effects on the growth of sorghum plants differed with sorghum variety and the levels of drought stress. The observed different drought responses of the two sorghum varieties are possibly due to their contrasting drought phenotypes under water limitations.

CHAPTER 4

Identification of Constitutively Expressed and Drought-Responsive microRNAs in ICSB 338 and SA 1441 Sorghum Roots

4.1 Introduction

Drought stress has a widespread global impact, affecting plant growth and ultimately reducing agricultural production (Kapoor *et al*., 2020). To cope with drought, plants have evolved defence mechanisms such as reprogramming gene expression through the use of microRNAs (miRNAs) (Islam *et al*., 2022; Singh *et al*., 2023). miRNAs are small, endogenous non-coding RNA molecules that regulate gene expression during plant development and in response to environmental stresses (Zhang *et al*., 2006; Wang and Li, 2007; Sun, 2012; Wang *et al*., 2019).

Identifying differentially expressed miRNAs in response to drought stress enhances our understanding of their regulatory roles in plants (Bakhshi and Fard, 2023). Drought-induced miRNAs downregulate their target mRNAs, reducing the expression of targeted genes (Ding *et al*., 2013). Conversely, some miRNAs are downregulated under water-limited supply, leading to increased expression of their targeted mRNAs helping plants adapt to drought conditions (Ding *et al*., 2013).

Bakhshi *et al*. (2014) evaluated the expression levels of miR160, miR164, and miR167 in roots of the IR64 rice (*Oryza sativa*) genotype under drought stress using quantitative real-time polymerase chain reaction (qRT-PCR). The rice seedlings were subjected to drought stress by withholding water for two weeks, while the control group was regularly watered. The study reported a decrease in miR160, miR164, and miR167 expression levels leading to an increase in their target transcription factors, which promoted root growth under drought stress. The computational target prediction results of that study showed that miR160 and miR167 families

target the auxin response factors (ARFs), while miR164 targets F-box proteins, myeloblastosis (MYB) and NAC (no apical meristem and cup-shaped cotyledon) family of transcription factors (Bakhshi *et al*., 2014). According to Nadarajah and Kumar (2019), miR160 targets ARF10 and ARF16, while miR167 regulates ARF6 and ARF8 in rice plants under drought stress. ARFs are a family of transcription factors that regulates plant growth and development, are involved in hormone crosstalk, and either activate or repress target genes during stress response (Chandler, 2016; Kou *et al*., 2022). The authors concluded that an increase in the expression of miRNA target genes such as F-box, ARF and NAC under drought conditions plays an important role in increasing root length (Bakhshi *et al*., 2014).

It is common for miRNA expression levels under drought stress to be species-dependent (Ferdous *et al*., 2015). In some cases, miRNAs are differentially expressed between different tissues or developmental stages and genotypes of the same species under drought stress (Singroha *et al*., 2021). Akdogan *et al*. (2016) investigated the drought-responsive miRNA expression patterns in the leaves and roots of wheat using miRNA array analysis. Ten-day-old wheat seedlings were subjected to drought stress in pots containing solid Murashige and Skoog (MS) medium supplemented with 20% polyethylene glycol (PEG) 6000 for 24 hours. The plants used for control were kept in fresh MS medium without PEG. The study identified 285 and 244 miRNAs in leaf and root tissues, respectively. Of these miRNAs, 23 and 26 were differentially expressed in the leaves and roots, respectively. Some miRNAs in the miR159, miR169, and miR408 families were significantly downregulated in the roots but up-regulated in the leaves. The authors reported that miR159 targets MYB transcription factors that are positive regulators of ABA responses, and miR169 targets NFY transcription factors that are important for plant development and adaptation to stress, whereas miR408 plays a role in drought tolerance (Akdogan *et al*., 2016). These findings highlight the specific roles of certain miRNAs in plant responses to drought through the regulation of transcription factors. This

chapter aimed to identify the drought-responsive miRNAs in ICSB 338 a drought-susceptible and drought-tolerant SA 1441 sorghum seedling subjected to 15 days of drought stress.

4.2 Materials and methods

4.2.1 Plant material and drought stress treatment

Two contrasting sorghum varieties, a drought-susceptible ICSB 338 and a drought-tolerant SA 1441 were selected for study (discussed in Chapter 3, Section 3.2.1). The sorghum varieties were grown, and drought stress was induced as described in Section 3.2.3 (Chapter 3). On day 15 of the drought-stress treatment, root samples were harvested from the watered-control and drought treated groups and immediately flash frozen in liquid nitrogen and stored at -80 °C for total RNA extraction. Three biological replicates were harvested with each replicate consisting of pooled root tissue from 10 plants. This was done to bulk up root tissue for downstream total RNA extractions.

4.2.2 Total RNA extraction

The -80 °C stored root samples were ground in liquid nitrogen using a mortar. Liquid nitrogen was used to keep plant material frozen and avoid RNA degradation during tissue disruption. Total RNA was extracted from root samples using the RNeasy® Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 450 µl RLT buffer was added to 100 mg of ground root tissue and vortexed vigorously. The samples were incubated on a heat block at 56 °C for 3 minutes. The lysate was then transferred to a QIAshredder spin column in a 2 ml collection tube and centrifuged for 2 minutes at 21 000 × *g*. Thereafter, the supernatant was transferred to a new microcentrifuge tube and immediately mixed with 225 µl absolute ethanol. A volume of 650 µl of the mixture was transferred into a RNeasy spin column in a 2 ml collection tube and centrifuged for 15 seconds at 9300 × *g*. After spinning, the flow-through was discarded.

Genomic DNA contamination in the RNA samples was removed with an on-column DNase digestion step using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. Briefly, the spin column membrane was washed with 350 µl buffer RW1 by centrifugation at 21000 × *g* for 15 seconds. The flow through was discarded. Thereafter, 80 µl of DNase set 1 mixture was added to the RNeasy spin column membrane and incubated on the benchtop for 15 minutes. Then the RNeasy spin column was washed with 350 µl buffer RW1 by centrifugation at 9300 × *g* for 15 seconds. The flow-through was discarded.

Thereafter, the RNeasy spin column membrane was washed with 700 µl RW1 buffer by centrifuging at 9300 × *g* for 15 seconds. The flow-through was discarded, and the RNeasy spin column was washed with 500 µl of the RPE buffer by centrifuging for 15 seconds at 9300 × *g*. After discarding the flow-through, another wash step with 500 µl of the RPE buffer was performed by centrifuging the spin column for 2 minutes at 9300 × *g*. Thereafter, the RNeasy spin column was placed into a new 2 ml collection tube and was centrifuged for 1 minute at 21000 × *g* to eliminate any possible carry over of Buffer RPE or residual flow-through before RNA elution. Total RNA was eluted from the RNeasy spin column membrane using 50 µl of the RNAse-free water and a centrifugation step at 9300 × *g* for 1 minute.

4.2.3. RNA quantification and gel electrophoresis

The extracted RNA was quantified using a Spectrophotometer Genova Nano (Jenway, Cole-Parmer Ltd., Staffordshire, United Kingdom) following the manufacturer's instructions. The total RNA quality was visualised using agarose gel electrophoresis, which separates the total RNA molecules according to molecular size. For this procedure, a 1.2% (w/v) agarose gel containing 5% of SafeView™ Classic (Applied Biological Materials Inc, Richmond,

Canada) was prepared in MOPS buffer. The gel solution was mixed gently to avoid bubbles and poured onto trays to solidify. Thereafter, the gels were submerged in 3-(n-morpholino) propanesulfonic acid (MOPS) running buffer [20 mM MOPS (pH 7), 2 mM sodium acetate (pH 7), 1 mM ethylenediamine tetraacetic acid (EDTA) (pH 8)]. RNA samples were prepared by mixing equal volumes of 2× RNA loading dye (New England Biolabs, Massachusetts, USA) and 300 ng of the total RNA and incubating on a heat block at 65 °C for 10 minutes followed by a quick spin. Thereafter, the samples were loaded on the agarose gels and run at 90 V for 45 minutes in 1× MOPS running buffer using the Mini-Sub® Cell GT agarose electrophoresis system (Bio-Rad, Hercules, USA). The gels were imaged using a Molecular imager® Gel Doc™ XR+ with Image Lab™ Software version 5.2.1 (Bio-Rad).

The total RNA of ICSB 338 and SA 1441 sorghum roots were sent frozen on dry ice to the Agricultural Research Council's (ARC) Biotechnology Platform, Onderstepoort, Pretoria, where further quality checks were conducted prior to small RNA sample preparation and sequencing. The RNA-seq reads clean-up, adapter trimming, quality control and data analysis using the CLC Genomics Workbench (Qiagen, Aarhus, Denmark) and bioinformatics were done at the University of the Free State, Qwa-Qwa Campus.

4.2.4 Small RNA library preparation

4.2.4.1 Sample quality assessment using Qubit™ 2.0 Fluorometer

The Qubit™ RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used to quantify the total RNA samples according to the manufacturer's instructions. A working solution was prepared for 12 samples and two standards by mixing 2786 µl (199 µl x 14 samples) Qubit buffer with 14 µl (1 µl x 14 samples) Qubit reagent in a sterile 15 ml Falcon tube. A volume of 190 µl of the Qubit working solution was mixed with 10 µl of the two

standards into separate 0.5 µl Qubit tubes. Thereafter, 2 µl of each total RNA sample was mixed with 198 µl of the working solution in separate Qubit tubes. The tubes were vortexed for 3 seconds and then incubated at room temperature for 2 minutes. The samples were read using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The broad range RNA protocol was used, starting with the two standards followed by the root total RNA samples.

4.2.4.2. Small RNA library construction

Small RNA was isolated from the total RNA samples using the MGIEasy Small RNA Library Prep Kit version 2.0 (MGI Tech Co., Ltd., Shenzhen, China) according to the manufacturer's instructions. Figure 4.1 shows an overview of small RNA library construction process. In short: Adapter ligation was initiated by mixing 500 ng of undiluted total RNA of each sample with 1 µl 3' Adapter to a final volume of 7 µl with nuclease-free water. The 3' adapter sequence used was AAGTCGGATCGTAGCCATGTCGTTCTGTGAGCCAAGGAGTTG and the 5' AAGTCGGAGGCCAAGCGGTCTTAGGAAGACAA and were obtained the ARC Biotechnology Platform, Onderstepoort, Pretoria. The denaturing reaction mixture was performed in a thermocycler at 70 °C for 2 minutes and immediately transferred to ice for at least 1 minute. Then, 13 µl adapter ligation reaction mixture was prepared on ice by mixing 10 µl 3' Ligation Buffer Mix, 1 µl RNAse inhibitor and 2 µl 3' Ligation enzyme. The tube was briefly centrifuged to collect the solution at the bottom of the tube. The solution was added to the denaturing reaction mixture and mixed by pipetting, prior to incubation in a thermocycler at 25 °C for 2 hours, and then held at 4 °C. The 3' adapter digestion reaction mixture (6 µl) was prepared using 2.5 µl Adapter depletion Enzyme I, 1.7 µl Adapter depletion enzyme II, 1 µl RNAse inhibitor and 0.8 µl Adapter depletion buffer and incubated in a thermocycler (30 °C for 30 min, 37 °C for 30 min, 70 °C for 20 minutes and 4 °C hold). Thereafter, the 5' adapter ligation was immediately performed.

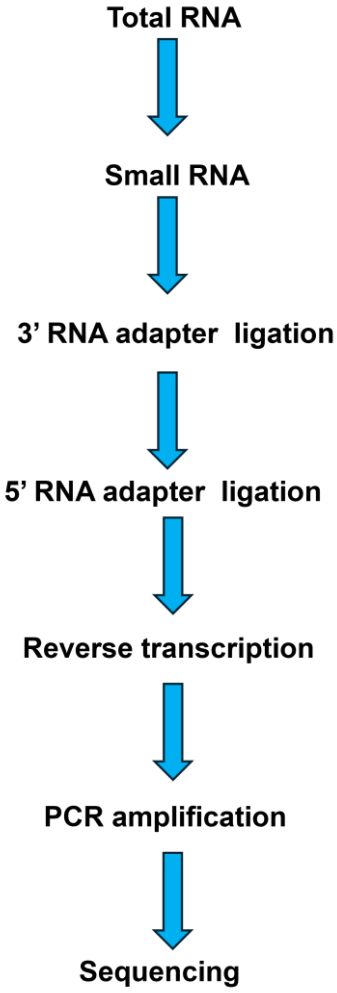

Figure 4.1. An overview of small RNA library construction as described in the small RNA library prep kit (MGI Tech Co., Ltd.).

Given the starting total RNA amount of 500 ng, an undiluted 5' Adapter volume of 1 µl was added to a reaction tube and incubated at 70 °C for 2 minutes. The 5' adapter ligation mixture (7 µl) was prepared on ice using 4 µl 5' Ligation buffer mix, 1 µl RNAse inhibitor and 2 µl 5' Ligation enzyme and mixed with the denatured 5' Adapter. The reaction mixture was placed in a thermocycler at 25 °C for 1 hour, then held at 4 °C. After the reaction was completed, reverse transcription (RT) was performed immediately. For this, 1 µl RT Primer was added to the 5' adapter ligation reaction mixture, pipetted 10 times, incubated at 65 °C

for 3 minutes, and placed on ice for at least 1 minute. Following this, 15 µl of the RT reaction mixture was prepared using 12.5 µl FS Reaction buffer mix, 1.5 µl RNAse inhibitor and 1 µl RT enzyme, mixed with RT Primer prepared above, and incubated in a thermocycler (42 °C for 60 min, 70 °C for 15 minutes and held at 4 °C).

For PCR amplification, 50 µl reaction mixtures were prepared on ice using 23 µl of the reverse transcription product, 2 µl PCR primer mix (HC) and 25 µl PCR enzyme mix. The reaction mixtures were vortexed 3 times, pulse centrifuged and incubated in a thermocycler and run using the following program: 95 °C for 3 min, followed by 20 cycles of 98 °C for 20 seconds; 56 °C for 15 seconds and 72 °C for 15 seconds. The 20 cycles were followed by a 10-minute incubation step at 72 °C and a 4 °C hold.

4.2.4.3 Size selection of PCR products by using magnetic beads

Magnetic beads included in the MGIEasy DNA Clean Beads Kit were used for size selection of the PCR products. Beads, stored at 4 °C, were normalised to room temperature for 30 minutes prior to being vortexed to ensure thorough mixing before use. The beads (180 µl) were mixed with 50 µl ethanol in a 1.5 µl Eppendorf tube. The PCR reaction (Section 4.2.4.2) was stopped by adding 6 µl Stop Buffer (HC) followed by a thorough mix. A 100 µl of this PCR reaction was added to the beads-ethanol mix in the 1.5 µl tube. The tube was gently pipetted at least 10 times to mix thoroughly, with special attention given to ensure that all the solution and beads were expelled from the tip into the tube at the end. The mix was incubated at room temperature for 10 minutes, with gently pipetting as needed at least 5 times to prevent precipitation of the beads. The tube was briefly centrifuged and placed on the Magnetic Separation Rack for 5 minutes until the liquid became clear. The supernatant was carefully transferred into a new 1.5 ml tube. With the tube on the Magnetic Separation

Rack, 500 µl of freshly prepared 80% (v/v) ethanol was added to the tube without disturbing the beads. The tube was incubated for 30 seconds. The supernatant was carefully removed and discarded, and the washing step repeated. Thereafter, all liquid was removed from the tube without disrupting the beads. The tube was kept on the Magnetic Separation Rack with the lid open to air-dry the beads until no wetness was visible but before the pellet cracked.

The tube was removed from the Magnetic Separation Rack and 16 µl of Tris-EDTA (TE) Buffer was added to elute DNA. Thereafter, the entire volume was gently pipetted up and down 10 times to mix thoroughly or until the beads are fully resuspended. The tube was incubated at room temperature for 10 minutes, briefly centrifuged and placed on the Magnetic Separation Rack for another 5 minutes until the liquid became clear. A volume of 14 µl of the supernatant containing the purified PCR products was transferred to a new 1.5 ml tube and stored at -20 °C.

4.2.5 Sequencing of small RNAs

Quality control (QC) of the selected amplicons was done using the Qubit™ dsDNA HS Assay (Thermo Fisher) following the same volumes as used for QC of the total RNA in the Section 4.2.4.1. The Agilent Bioanalyzer was used to check the size of the library (amplicons) prior to sequencing. Ideal libraries had a size of 105-133 bp and a concentration of 4.16-5.27 ng. To prepare single stranded DNA for making DNA nanoballs (DNB), 48 µl of a 1 pmol concentration library was denatured at 95 °C for 3 minutes and then placed on ice for 2 minutes. This was followed by single strand circularization where the 48 µl denatured library was mixed with 11.6 µl of Splint Buffer and 0.5 µl DNA Rapid Ligase. The reaction was placed in a thermocycler at 37 °C for 30 minutes and kept at 4 °C. Thereafter, enzymatic digestion was done by adding 1.4 µl of Digestion buffer and 2.6 µl of

Digestion Enzyme and incubated in a thermocycler at 37 °C for 30 minutes. After completion, 7.5 µl of stop digestion buffer was added to the reaction mixture. The reaction was purified using MGI Library Purification Beads, followed by quality control using the Qubit ssDNA assay and the Agilent Bioanalyzer. To make DNA nanoballs, a volume equivalent to 40 mol of the single stranded DNA library was adjusted with TE buffer to a total volume of 20 µl, which was mixed with 20 µl Make DNB buffer. The following program was run on the thermocycler: 95 °C for 1 minute, 65 °C for 1 minute, 40 °C for 1 minute, followed by a 4 °C hold.

A second DNB reaction was carried out by adding 40 µl Make DNB enzyme Mix I and 4 µl Make DNB enzyme Mix II (LC) to contents of the previous reaction. This reaction was placed in a thermocycler at 35 °C for 25 minutes. The lid temperature for this reaction was kept as close as possible to 35 °C. After this, 20 µl of Stop DNB reaction buffer was added, and the resulting DNB were quantified using Qubit ssDNA assay. To load a flow cell for sequencing, 30 µl DNBs were mixed with 8 µl of DNB load buffer II and 0.25 µl Make DNB enzyme Mix II (LC). Thereafter, 30 µl of this mix was added to a flow cell and sequenced on the MGI DNBSEQ-G400 following the Paired-Ends Flow Cell (PE150 FLC) sequencing chemistry.

4.2.6 Bioinformatic analysis of miRNA sequences

CLC Genomics Workbench version 23.0.4 (Qiagen) was used for miRNA data analysis using a workflow summarised in Figure 4.2. Quality control was performed on the raw reads generated by the MGI DNBSEQ-G400 sequencing technology to ensure high-quality reads for further processing. The raw reads were processed by trimming twice to remove low-quality, ambiguous bases, MGI miRNA adapter sequences (forward sequence: AAGTCGGAGGCCAAGCGGTCTTAGGAAGACAA and reverse sequence:

AAGTCGGATCGTAGCCATGTCGTTCTGTGAGCCAAGGAGTTG), reads shorter than 18 nucleotides and those longer than 25 nucleotides. Reads before and after trimming were assessed using the FASTQC tool on the CLC Genomics Workbench (Qiagen). The reads were mapped to the *Sorghum_bicolor*_NCBIv3 reference genome (Genbank: ABX00000000.1) (Paterson *et al*., 2009) using the miRBase database (http://miRbase.org/) (Griffiths-Jones *et al*., 2006). The Differential Expression for RNA-seq tool in CLC Genomics Workbench was used to identify miRNAs that are drought-responsive (Section 4.2.8).

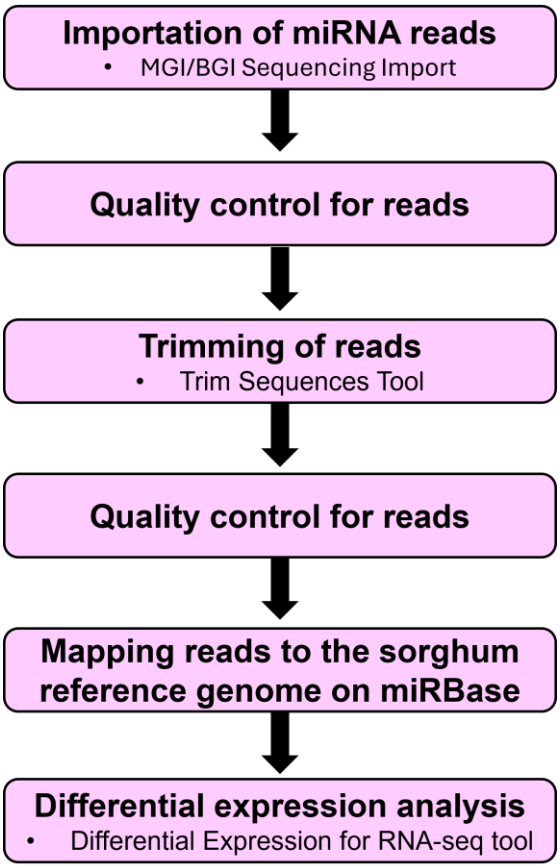

Figure 4.2. Workflow for miRNA data analysis using CLC Genomics Workbench. The *Sorghum_bicolor*_NCBIv3 reference genome (Genbank: ABX00000000.1) (Paterson *et al*., 2009) was used for mapping the miRNA reads.

4.2.7 Bioinformatics analysis of constitutively expressed miRNA

The miRNA IDs obtained from the mapping exercise using the CLC Genomics Workbench (Qiagen) were used to obtain the nucleotide sequences of the miRNAs from the miRbase (Griffiths-Jones *et al*., 2006), accessed on 25 September 2024. The miRNA nucleotide sequences were subsequently used to identify the miRNA target genes on the psRNATarget database (https://www.zhaolab.org/psRNATarget/) (Dai *et al*., 2018). For this step, the *Sorghum bicolor*, transcript, JGI genomic project, Phytozome 13, 454_v3.1.1 cDNA library was used following default parameters. The putative target gene of each miRNA at the top of the list and the corresponding Expect value were noted for each of the identified miRNAs. The Expect values range from 0 to 5, where 0 represents a perfect alignment. The mode of inhibition of each miRNA on its target was also recorded. Sobic numbers of the target genes were used to search for the latest SORBI gene numbers on the Gramene database (https://www.gramene.org/) (Liang *et al*., 2008). Both psRNATarget and Gramene databases were accessed on 7 October 2024. The SORBI number of each target gene was used to retrieve the Gene Ontology (GO) data and gene descriptions from the Gramene database (https://www.gramene.org/) (Liang *et al*., 2008) and the National Center for Biotechnology Information (NCBI) Gene database (https://www.ncbi.nlm.nih.gov/gene), respectively. Both the Gramene and NCBI databases were accessed on 7 October 2024.

4.2.8 Analyses of differentially expressed miRNA

Differentially expressed miRNA were identified using the Differential Expression for RNA-seq tool in the CLC Genomics Workbench version 23.0.4 (Qiagen) using default settings. In this study, the p-value of ≤ 0.05 and the $\log_2$ fold change were used to identify the drought-responsive miRNAs and no minimum threshold was set for the $\log_2$ fold change.

4.3 Results

4.3.1 Quantification of sorghum root total RNA samples

Table 4.1 shows the concentration of the total RNA extracted from the ICSB 338 and SA 1441 sorghum root samples. The Genova Nano and Qubit quantification techniques were used at different stages of the total RNA processing steps before the construction and sequencing of the small RNA libraries. The results showed that the Genova Nano concentrations for all watered control and drought-stress samples in SA 1441 were higher than those from the Qubit. However, for ICSB 33, Qubit concentrations for all three watered control samples and one drought stress treatment sample were higher than those of the Genova Nano.

Table 4.1. Total RNA quantification using the Genova Nano and Qubit machines.

Sorghum variety	Treatment group	Biological replicates	Total RNA concentration (ng/µl)	
			Nano	Qubit
ICSB 338	Watered control	1	276	451
		2	383	411
		3	176	400
	Drought stress	1	523	382
		2	487	510
		3	418	411
SA 1441	Watered control	1	421	280
		2	558	245
		3	341	320
	Drought stress	1	457	301
		2	422	315
		3	409	354

4.3.2 Gel electrophoresis of sorghum root total RNA samples

In this study, each biological replicate consisted of root samples pooled from 10 plants. This was done to bulk up root tissue for RNA extraction and subsequent analyses. Approximately 300 ng of each of the total RNA samples was visualised on a 1.2 % (w/v) agarose gel (Figure 4.3). All the total RNA samples were of good quality, with the total RNA, including mRNAs,

rRNAs, and small RNAs, observed on the gel (Figure 4.3). In these profiles, the small RNAs of each root sample were located near the bottom of the gel. Unfortunately, the Genova Nano machine used at the University of the Free State (UFS), Qwa-Qwa Campus did not provide the RNA integrity and purity values.

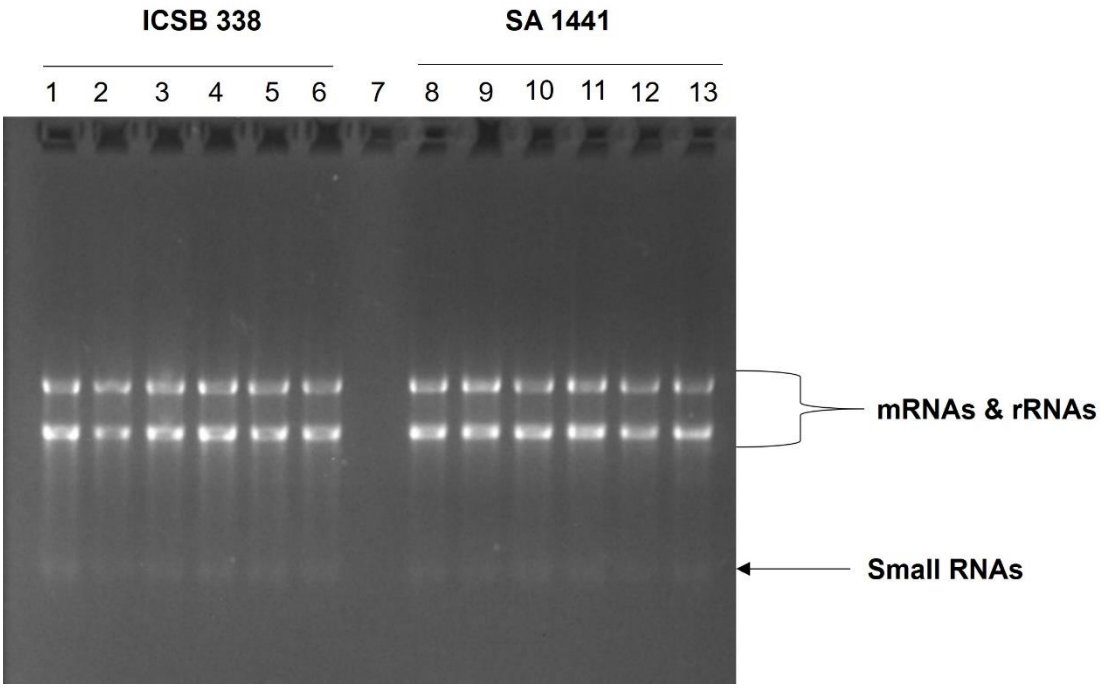

Figure 4.3. Agarose gel electrophoresis of total RNA extracted from the roots of two sorghum varieties. The two sorghum varieties, ICSB 338 and SA 1441, were grown for three weeks before the imposition of drought stress treatment on the seedlings. Lanes 1-3 and 8-10 shows the total RNA extracts of the watered control samples of ICSB 338 and SA 1441 sorghum root samples, respectively. Lanes 4-6 and 11-13 shows the total RNA extracts of the drought stressed samples of ICSB 338 and SA 1441 sorghum root samples, respectively. Approximately 300 ng of each total RNA sample was loaded on the 1.2% (w/v) agarose gel.

4.3.3 Small RNA library analysis

Twelve small RNA libraries were constructed using total RNA extracted from the watered controls and drought-stressed sorghum root samples to identify the miRNAs present, each treatment in triplicate. The small RNA libraries were sequenced on a MGI DNBSEQ-G400

using PE150 FLC, generating a total of 340 660 184 raw reads across all 12 samples (Table 4.2). The quality of the reads was evaluated using the Phred quality score, which was greater than 30 for all 12 samples (Table 4.2). These results indicate good quality data for downstream analysis. The adapters and low-quality reads were subsequently removed from the data using the CLC Genomics Workbench software's Trimming tool resulting in a total of 96 118 089 clean reads for all samples. A large portion of the reads were trimmed since 150 bp paired-end sequencing was performed on the libraries. A summary of the reads obtained before and after trimming off the adapters, and removal of the low-quality reads is shown in Table 4.2.

Table 4.2. Summary of sequence results from the miRNA libraries obtained from the watered controls and drought-stressed sorghum root samples, with total number of reads obtained, their PHRED scores, GC % and reads mapped to the sorghum genome.

Sorghum variety	Treatment groups	Biological replicates	Raw reads	Number of reads after trimming	PHRED Score range	GC %	Mapped* reads	Unmapped reads
ICSB 338	Watered control	1	41 752 488	41 625 832	25 - 38	52.23	23 074 522	17 996 355
		2	30 296 388	30 211 949	25 - 38	52.04	16 979 216	12 851 098
		3	30 014 654	29 907 072	25 - 38	52.51	16 093 586	13 439 565
	Drought stress	1	29 543 578	29 433 810	25 - 38	52.11	16 607 182	12 399 515
		2	39 786 272	39 625 264	25 - 38	51.73	21 324 700	17 717 334
		3	21 618 810	21 532 439	25 - 38	52.47	11 411 484	9 818 778
SA 1441	Watered control	1	29 893 210	29 763 687	25 - 38	52. 17	15 111 398	14 222 078
		2	15 041 512	14 950 247	25 - 38	51.60	7 400 310	7 327 914
		3	33 652 578	33 520 980	25 - 38	51.53	18 666 490	14 414 213
	Drought stress	1	26 215 370	26 119 988	25 - 38	51.79	13 680 050	12 057 899
		2	19 336 844	19 254 246	25 - 38	51.47	9 865 948	9 096 922
		3	23 508 480	23 414 867	25 - 38	51.76	12 231 314	10 877 637

*The reads were mapped to the *Sorghum_bicolor*_NCBIv3 reference genome (Genbank: ABX00000000.1) (Paterson *et al*., 2009) using the miRBase database (http://miRbase.org/) (Griffiths-Jones *et al*., 2006).

4.3.4 Constitutively expressed miRNAs in sorghum roots

A total of 81 and 83 miRNAs were positively identified in the well-watered controls of ICSB 338 and SA 1441 root tissues, respectively. Among these constitutively expressed miRNAs, 73 were common between the two varieties, while 8 and 10 were unique to ICSB 338 and SA 1441, respectively (Figure 4.4). The psRNATarget database (https://www.zhaolab.org/psRNATarget/) (Dai *et al*., 2018) was used to identify the putative target genes and mode of inhibition of each of the constitutively expressed miRNAs. Thereafter, Gene Ontology (GO) data for each of these putative target genes were retrieved from the Gramene database (Liang *et al*., 2008) to assist in characterising the miRNA target in terms of molecular function, biological processes and cellular component.

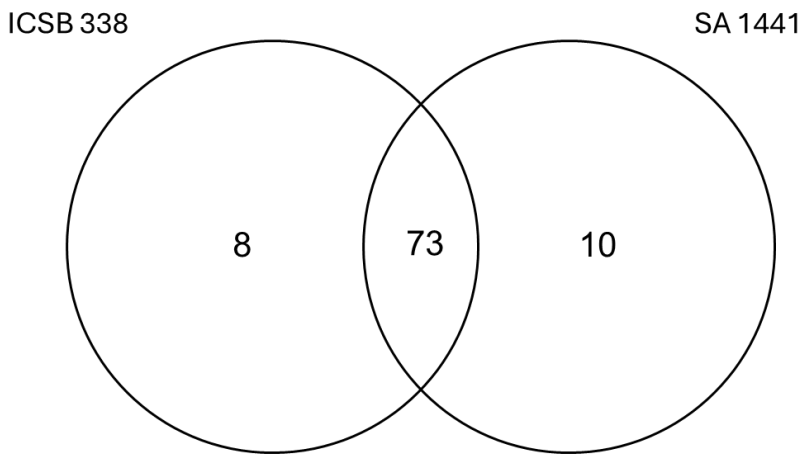

Figure 4.4. Venn diagram of common and unique constitutively expressed miRNAs in ICSB 338 and SA 1441 sorghum roots as obtained from CLC Genomics Workbench (Qiagen) analyses.

4.3.4.1 Constitutively expressed miRNAs shared by both ICSB 338 and SA 1441

Table 4.3 summarises the 73 constitutively expressed root miRNAs common to both ICSB 338 and SA 1441 sorghum varieties obtained from the CLC Genomics Workbench bioinformatics data analyses. A total of 47 miRNA families were identified, including sbi-miR1435, sbi-miR159, sbi-miR160, sbi-miR166, sbi-miR167, sbi-miR168, sbi-miR169, sbi-miR171, sbi-miR172 and sbi-miR2118. Some miRNAs identified were predicted to have more than one target gene with perfect alignment as measured by Expect values of 0 (Table 4.3). These miRNAs include sbi-miR160a, sbi-miR172f, sbi-miR5565e, sbi-miR5565g-5p, sbi-miR5568g-5p and sbi-miR5568g-3p (highlighted with asterisks in Table 4.3), while a complete list of their putative targets is given in the Appendix Table A1. It was noted that some of the multiple target genes of these miRNAs are from the same gene families. For example, sbi-miR160a targets ARF18 and ARF8, while sbi-miR172f targets Floral homeotic protein APETALA 2 (Appendix Table A1).

The levels of the constitutively expressed miRNAs were also compared between ICSB 338 and SA 1441 sorghum varieties using the Differential Expression for RNA-seq tool on the CLC Genomics Workbench (Qiagen) (Appendix Table A2). The results revealed that four miRNAs, namely sbi-miR6224a-5p, sbi-miR6233-3p, sbi-miR394a, and sbi-miR395a were differentially expressed between the control samples of ICSB 338 relative to those of SA 1441 ($p \leq 0.05$) (Appendix Table A3). Table 4.3 also shows that the predicted targets of the differentially expressed sbi-miR6233-3p, sbi-miR394a and sbi-miR395a were *Putative clathrin assembly protein Atg57200*, *F-box-only protein 6,* and *Low affinity sulfate transporter 3*, respectively. However, the target of sbi-miR6224a-5p is an *Uncharacterized LOC8055016* gene.

Table 4.3. List of 73 constitutively expressed miRNAs commonly identified in ICSB 338 and SA 1441 sorghum roots.

miRNA ID[a]	miRNA accession[b]	Target ID[c]	Target description[d]	Expect value[e]	Inhibition[f]	GO analysis[g]		
						P	F	C
sbi-miR1435a	MI0010931	*Sobic.002G392500/ SORBI_3002G392500*	*Heat stress transcription factor B-4b*	1.5	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
sbi-miR159a	MI0001572	*Sobic.003G013900/ SORBI_3003G013900*	None	0.5	Cleavage	None	None	None
*sbi-miR160a	MI0001508	*Sobic.001G217300/ SORBI_3001G217300*	*Auxin response factor 22*	0	Cleavage	Response to hormone	DNA binding	Nucleus
sbi-miR166a	MI0001500	*Sobic.001G157400/ SORBI_3001G157400*	*Homeobox-leucine zipper protein HOX32*	1.0	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
sbi-miR167a	MI0001513	*Sobic.004G055400/ SORBI_3004G055400*	None	0	Cleavage	None	None	None
sbi-miR168	MI0001556	*Sobic.003G409800/ SORBI_3003G409800*	*NAC domain-containing protein 83*	2.5	Cleavage	Regulation of DNA-templated transcription	DNA-binding	Nucleus
sbi-miR169d-3p	MIMAT0026431	*Sobic.004G167700/ SORBI_3004G167700*	*Pectinesterase inhibitor 8*	2.0	Cleavage	None	Enzyme inhibitor activity	Apoplast
sbi-miR171e	MI0001570	*Sobic.006G181800/ SORBI_3006G181800*	*Uncharacterized LOC8057596*	0	Cleavage	Microtubule cytoskeleton organization	Gamma tubulin binding	Gamma-tubulin complex
sbi-miR172a	MI0001503	*Sobic.009G024600/ SORBI_3009G024600*	*Ethylene-responsive transcription factor RAP2-7*	0.5	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
*sbi-miR172f	MI0013256	*Sobic.009G024600/ SORBI_3009G024600*	*Ethylene-responsive transcription factor RAP2-7*	0	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
sbi-miR2118-3p	MIMAT0026427	*Sobic.006G084701/ SORBI_3006G084701*	*B3 domain-containing protein*	1.0	Cleavage	None	None	None
sbi-miR2118-5p	MIMAT0026426	*Sobic.006G084701/ SORBI_3006G084701*	None	1.0	Cleavage	None	None	None
sbi-miR319a	MI0001573	*Sobic.001G066100/ SORBI_3001G066100*	*Transcription factor PCF6*	1.5	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus

sbi-miR390	MI0010887	*Sobic.006G160000/ SORBI_3006G16000*	None	1.0	Cleavage	Protein phosphorylation	Protein kinase activity	Membrane
**sbi-miR394a	MI0001531	*Sobic.003G407900/ SORBI_3003G407900*	*F-box only protein 6*	0	Cleavage	SCF dependent proteasomal ubiquitin dependent protein catabolic process	Ubiquitin protein transferase activity	None
**sbi-miR395a	MI0001534	*Sobic.001G470800/ SORBI_3001G470800*	*Low affinity sulfate transporter 3*	1.0	Cleavage	Inorganic anion transport	Anion transmembrane transporter activity	Membrane
sbi-miR396a	MI0001539	*Sobic.004G335200/ SORBI_3004G335200*	None	0	Cleavage	None	None	None
sbi-miR397-3p	MIMAT0020940	*Sobic.002G027166/ SORBI_3002G027166*	None	2.5	Cleavage	None	None	None
sbi-miR408	MI0010901	*Sobic.007G227300/ SORBI_3007G227300*	*Basic blue protein-like*	1.0	Cleavage	Electron transport chain	Electron transfer activity	None
sbi-miR437x-3p	MIMAT0026407	*Sobic.002G195000/ SORBI_3002G195000*	None	1.5	Cleavage	Metabolic process	Carbon sulfur lyase activity	None
sbi-miR528	MI0013259	*Sobic.001G519700/ SORBI_3001G519700*	*Uncharacterized protein At4g15970*	0	Cleavage	Cell wall organisation	Glycosyltransferase activity	Golgi membrane
sbi-miR5382	MI0018689	*None*	None	None	None	None	None	None
sbi-miR5384	MI0018691	*None*	None	None	None	None	None	None
sbi-miR5385	MI0018692	*Sobic.001G412400/ SORBI_3001G412400*	*Uncharacterized LOC8084893*	2.0	Cleavage	None	None	None
sbi-miR5386	MI0018693	*Sobic.003G024100/ SORBI_3003G024100*	*Protein male discoverer 2*	1.5	Cleavage	Protein phosphorylation	Protein kinase activity	Membrane
sbi-miR5387b	MI0019104	*Sobic.003G080000/ SORBI_3003G080000*	*leaf rust 10 disease-resistance locus receptor-like protein kinase-like 2.1*	1.5	Translation	Protein phosphorylation	Protein kinase activity	Membrane
sbi-miR5388	MI0018695	*Sobic.009G079950/ SORBI_3009G079950*	*Uncharacterised LOC8065943*	1.5	Cleavage	None	None	None
sbi-miR5564a	MI0019096	*Sobic.002G057050/ SORBI_3002G057050*	*Putative pentatricopeptide repeat-containing protein At5g59900*	1.0	Cleavage	None	Protein binding	None

sbi-miR5564c-5p	MIMAT0026390	*Sobic.005G027680/ SORBI_3005G027680*	None	1.5	Cleavage	None	Protein binding	None
sbi-miR5564c-3p	MIMAT0026391	*Sobic.009G207100/ SORBI_3009G207100*	*Enoyl-CoA delta isomerase 2, peroxisomal*	2.5	Cleavage	Metabolic process	Catalytic activity	None
sbi-miR5565a	MI0019107	*Sobic.004G101100/ SORBI_3004G101100*	*Stigma-specific STIG1-like protein 1*	0	Cleavage	None	None	None
sbi-miR5565d	MI0019109	*Sobic.002G311300/ SORBI_3002G311300*	*UDP-glycosyltransferase 88B1*	0.5	Cleavage	Metabolic process	UDP-glycosyltransferase activity	Intracellular membrane-bounded organelle
*sbi-miR5565e	MI0019098	*Sobic.003G224900/ SORBI_3003G224900.1*	None	0	Cleavage	None	None	None
*sbi-miR5565g-5p	MIMAT0026398	*Sobic.009G208700/ SORBI_3009G208700*	*Non-classical arabinogalactan protein 31*	0	Cleavage	None	None	None
sbi-miR5566	MI0019100	*Sobic.002G113400/ SORBI_3002G113400*	*Uncharacterized LOC8057912*	1.5	Cleavage	None	None	Endoplasmic reticulum
sbi-miR5567	MI0019101	*Sobic.001G470900/ SORBI_3001G470900*	*Low affinity sulfate transporter 3*	1.0	Cleavage	Sulfate transport	Secondary active sulfate transmembrane transporter activity	Membrane
sbi-miR5568b-5p	MIMAT0026388	*Sobic.001G222400/ SORBI_3001G222400*	*Protein NPGR2*	0	Cleavage	None	Protein binding	Plastid
sbi-miR5568b-3p	MIMAT0026389	*Sobic.001G222400/ SORBI_3001G222400*	*Protein NPGR2*	0.5	Cleavage	None	Protein binding	Plastid
sbi-miR5568c-3p	MIMAT0026401	*Sobic.003G098900/ SORBI_3003G098900*	*Cell division cycle protein 123 homolog*	1.5	Cleavage	Regulation of cell cycle	None	Cytoplasm
sbi-miR5568c-5p	MIMAT0026400	*Sobic.007G135301/ SORBI_3007G135301*	*MADS-box transcription factor 23*	0.5	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
sbi-miR5568d-5p	MIMAT0026422	*Sobic.004G117850/ SORBI_3004G117850*	None	0.5	Cleavage	None	None	None
sbi-miR5568e-5p	MIMAT0026438	*Sobic.002G005700/ SORBI_3002G005700*	*RRP15-like protein*	1.0	Cleavage	rRNA processing	None	Nucleolus
sbi-miR5568f-5p	MIMAT0026442	*Sobic.001G342800/*	None	0.5	Cleavage	None	None	None

		SORBI_3001G342800						
sbi-miR5568f-3p	MIMAT0026443	*Sobic.004G237500/ SORBI_3004G237500*	*Uncharacterized LOC8075709*	0.5	Cleavage	Ceramide metabolic process	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	Membrane
*sbi-miR5568g-5p	MIMAT0026465	*Sobic.004G223350/ SORBI_3004G223350*	None	0	Cleavage	None	None	None
*sbi-miR5568g-3p	MIMAT0026466	*Sobic.003G092500/ SORBI_3003G092500*	*E3 ubiquitin-protein ligase SINA-like 7*	0	Cleavage	Protein ubiquitination	Ubiquitin protein ligase activity	Nucleus
sbi-miR6218-3p	MIMAT0026397	*Sobic.003G176500/ SORBI_3003G176500*	None	1.5	Cleavage	None	None	None
sbi-miR6219-5p	MIMAT0026402	*Sobic.005G109900/ SORBI_3005G109900*	*Obtusifoliol 14-alpha demethylase*	1.0	Cleavage	Sterol biosynthetic process	Oxidoreductase activity	None
sbi-miR6219-3p	MIMAT0026403	*Sobic.005G109900/ SORBI_3005G109900*	*Obtusifoliol 14-alpha demethylase*	0.5	Cleavage	Sterol biosynthetic process	Monooxygenase activity	None
sbi-miR6220-5p	MIMAT0026404	*Sobic.006G113100/ SORBI_3002G113100*	*CASP-like protein 1D1*	0	Cleavage	Microtubule based movement	ATP hydrolysis activity	Kinesin complex
sbi-miR6221-3p	MIMAT0026409	*Sobic.002G376300/ SORBI_3002G376300*	*Ankyrin repeat domain-containing protein 54*	3.0	Cleavage	Receptor internalization	Protein binding	Early endosome
sbi-miR6221-5p	MIMAT0026408	*Sobic.009G031700/ SORBI_3009G031700*	*Uncharacterised LOC8071279*	3.0	Cleavage	None	None	None
sbi-miR6222-5p	MIMAT0026410	*Sobic.001G451200/ SORBI_3001G451200*	*Putative pentatricopeptide repeat-containing protein At5g08310, mitochondrial*	2.0	Cleavage	None	mRNA binding	None
sbi-miR6223-3p	MIMAT0026413	*Sobic.001G247200/ SORBI_3001G247200*	None	2.0	Cleavage	None	None	None
sbi-miR6223-5p	MIMAT0026412	*Sobic.005G133900/ SORBI_3005G133900*	*Succinate dehydrogenase assembly factor 2, mitochondrial*	2.0	Cleavage	Tricarboxylic acid cycle	Succinate dehydrogenase (ubiquinone) activity	Mitochondrion

**sbi-miR6224a-5p	MIMAT0026414	*Sobic.005G123400/ SORBI_3005G123400*	*Uncharacterized LOC8055016*	2.0	Cleavage	None	None	Membrane
sbi-miR6224a-3p	MIMAT0026415	*Sobic.002G061600/ SORBI_3002G061600*	*protein nuclear fusion defective 4*	0.5	Cleavage	Transmembrane transport	None	Membrane
sbi-miR6226-3p	MIMAT0026425	*Sobic.003G395600/ SORBI_3003G395600*	*Magnesium transporter MRS2-F*	1.0	Cleavage	Ion transport	Magnesium ion transmembrane transporter activity	Membrane
sbi-miR6226-5p	MIMAT0026424	*Sobic.008G128500/ SORBI_3008G128500*	None	3.0	Cleavage	None	None	None
sbi-miR6227-3p	MIMAT0026429	*Sobic.001G258200/ SORBI_3001G258200*	*Shewanella-like protein phosphatase 1*	0	Cleavage	None	Hydrolase activity	Peroxisome
sbi-miR6227-5p	MIMAT0026428	*Sobic.003G354050/ SORBI_3003G354050*	*Cell division cycle protein 123 homolog*	0	Cleavage	None	None	None
sbi-miR6228-5p	MIMAT0026432	*Sobic.003G034800/ SORBI_3003G034800*	*Transcription factor SRM1*	0.5	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
sbi-miR6228-3p	MIMAT0026433	*Sobic.004G049600/ SORBI_3004G049600*	*Formin-like protein 10*	0	Cleavage	Actin cytoskeleton organization	Actin binding	Membrane
sbi-miR6229-3p	MIMAT0026435	*Sobic.002G049600/ SORBI_3002G049600*	*Protein lingerer*	2.5	Cleavage	None	None	None
sbi-miR6229-5p	MIMAT0026434	*Sobic.006G028200/ SORBI_3006G028200*	*Uncharacterized LOC8067772*	2.0	Cleavage	None	None	Membrane
sbi-miR6230-5p	MIMAT0026436	*Sobic.001G395100/ SORBI_3001G395100*	*DEAD-box ATP-dependent RNA helicase 24*	1.5	Cleavage	RNA secondary structure unwinding	RNA helicase activity	Nucleus
sbi-miR6231-5p	MIMAT0026440	*Sobic.007G140500/ SORBI_3007G140500*	*Probable CCR4-associated factor 1 homolog 6*	2.5	Cleavage	Negative regulation of translation	Nuclease activity	Nucleus
sbi-miR6231-3p	MIMAT0026441	*Sobic.009G084500/ SORBI_3009G084500*	*Transcription factor bHLH68*	3.0	Translation	Regulation of transcription by RNA polymerase II	RNA polymerase II cis-regulatory region sequence specific DNA binding	Nucleus
sbi-miR6232a-5p	MIMAT0026444	*Sobic.004G143550/*	None	0	Cleavage	None	None	None

		SORBI_3004G143550						
sbi-miR6232b-5p	MIMAT0026446	*Sobic.001G058200/ SORBI_3001G058200*	*Pentatricopeptide repeat-containing protein At1g63130, mitochondrial*	2.0	Cleavage	None	mRNA binding	None
sbi-miR6232b-3p	MIMAT0026447	*Sobic.002G043500/ SORBI_3002G043500*	*Indole-3-acetaldehyde oxidase*	1.5	Cleavage	None	Oxidoreductase activity	Cytosol
**sbi-miR6233-3p	MIMAT0026449	*Sobic.010G217700/ SORBI_3010G217700*	*Putative clathrin assembly protein At5g57200*	1.0	Cleavage	Endocytosis	SNARE binding	Cytoplasmic vesicle
sbi-miR821a	MIMAT0011385	*Sobic.003G175050 SORBI_300G175050*	*None*	2.0	Cleavage	Phenylpropanoid biosynthesis process	None	Apoplast

[a]miRNA ID obtained from the miRbase database search against *Sorghum bicolor* accessed on 25 September 2024.
[b]miRNA accession numbers obtained from the miRbase database, accessed on 28 October 2024.
[c]The sobic gene ID represents the target gene ID for each miRNA as obtained from the psRNATarget database. This sobic number was used to search for the latest SORBI gene number of the respective targets on the Gramene database. Both psRNATarget and Gramene databases were accessed on 7 October 2024.
[d]Target descriptions were obtained from the National Centre for Biotechnology Information (NCBI) database, accessed on 7 October 2024.
[e]Expect value assigned to the alignment of each miRNA nucleotide sequence and that of its putative target gene on the psRNATarget database. The Expect values range from 0 to 5, where 0 represents a perfect alignment.
[f]Inhibition represents the type of regulation of each miRNA on its putative target gene as obtained on the psRNATarget database.
[g]Gene Ontology (GO) data of each miRNA target gene retrieved from the Gramene database, accessed on 25 September 2024. P stands for biological process, F stands for molecular function, and C stands for cellular component.
*Denotes miRNAs with more than one target gene with perfect alignment as measured by an Expect value of 0.
**Denotes miRNAs differentially expressed between the control samples of ICSB 338 relative to those of SA 1441.

4.3.4.2 Regulatory mechanisms of the common constitutively expressed miRNAs and GO annotations of their target genes

Nucleotide sequence alignment results obtained from the psRNATarget database (Dai *et al*., 2018) showed that the 73 constitutively expressed miRNAs that are commonly found in both sorghum varieties were complementary to a variety of target genes (Table 4.3). However, the mode of inhibition of the identified miRNAs on their respective targets was either cleavage (94%) or translation inhibition (3%), while 3% of the miRNAs had no predicted mechanism of action (Figure 4.5). These results suggest that the most common mode of inhibition of the shared, constitutively expressed miRNAs on their putative targets is cleavage.

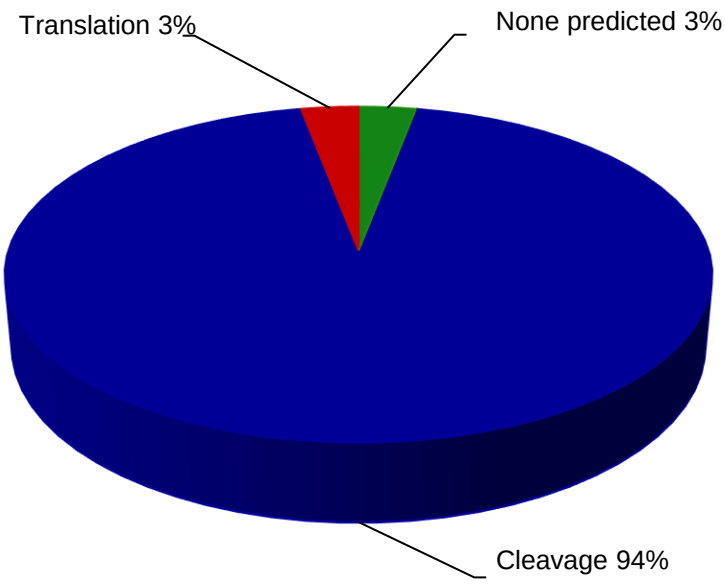

Figure 4.5. Type of inhibition of the constitutively expressed miRNAs identified in control root samples of both sorghum varieties.

The GO data retrieved from the Gramene database (Liang *et al*., 2008) revealed that of the 73 shared, constitutively expressed miRNAs, the majority (30) did not have any predicted molecular function (Figure 4.6). The rest of the target genes were associated with various molecular functions, with DNA binding (9), protein binding (5), protein kinase activity (3) being the top three represented terms (Figure 4.6).

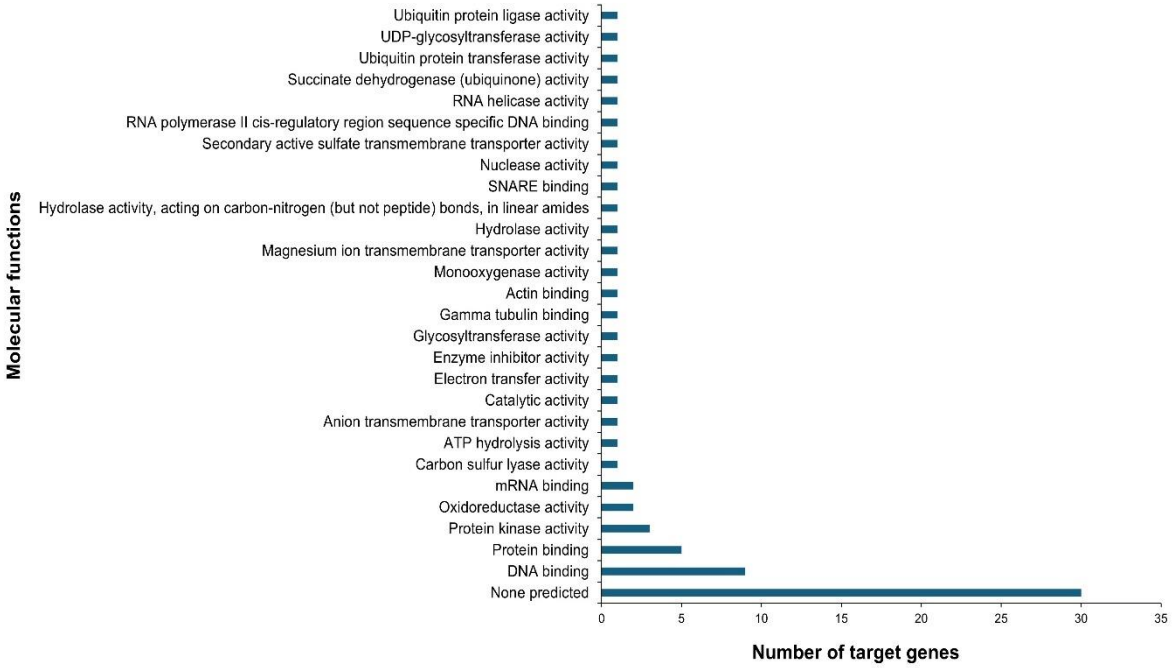

Figure 4.6. Putative molecular functions of the target genes of constitutively expressed root miRNAs identified in both sorghum varieties.

For biological processes, the majority (35) of the target genes of the common, constitutively expressed miRNAs did not have any predicted GO terms (Figure 4.7). However, regulation of DNA-templated transcription (8), metabolic process (3) and protein phosphorylation (3) were the three most enriched terms (Figure 4.7).

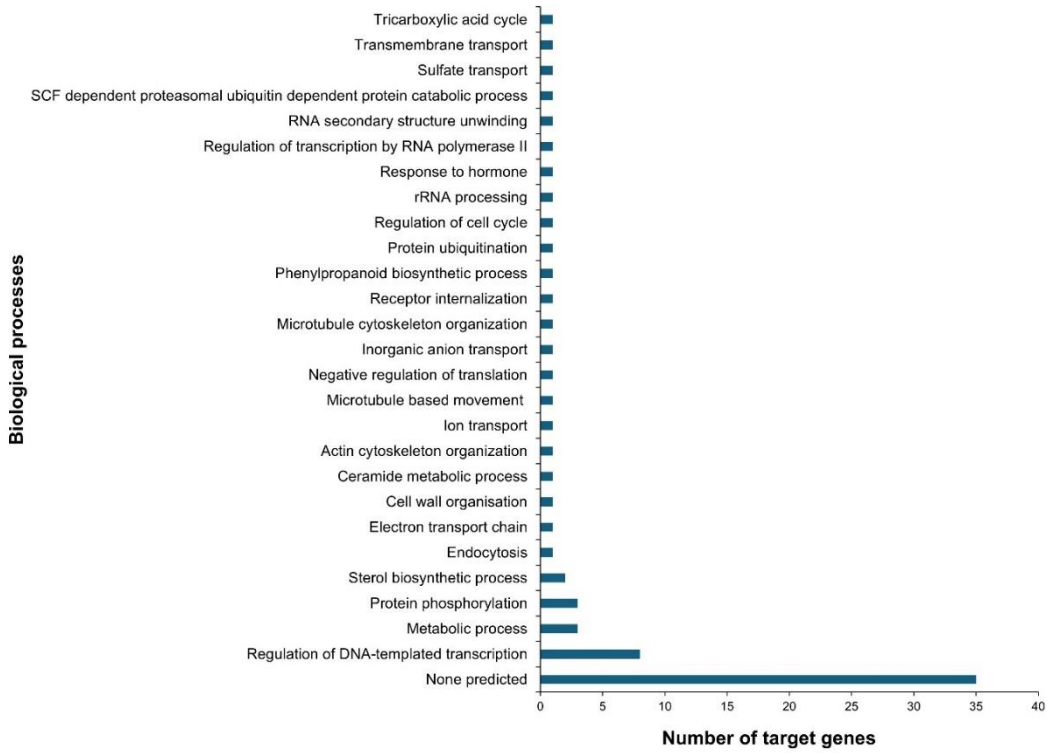

Figure 4.7. Putative biological processes of the target genes of constitutively expressed root miRNAs identified in both sorghum varieties.

The GO analysis also suggests that most (33) of the target genes of the common constitutively expressed miRNAs did not have predicted cellular locations (Figure 4.8). Conversely, the results suggest that the identified constitutively expressed miRNAs present in both sorghum varieties inhibit target genes present in diverse cellular locations, with the nucleus (13), membrane (11), plastid (2) and apoplast (2) being the most represented GO terms (Figure 4.8).

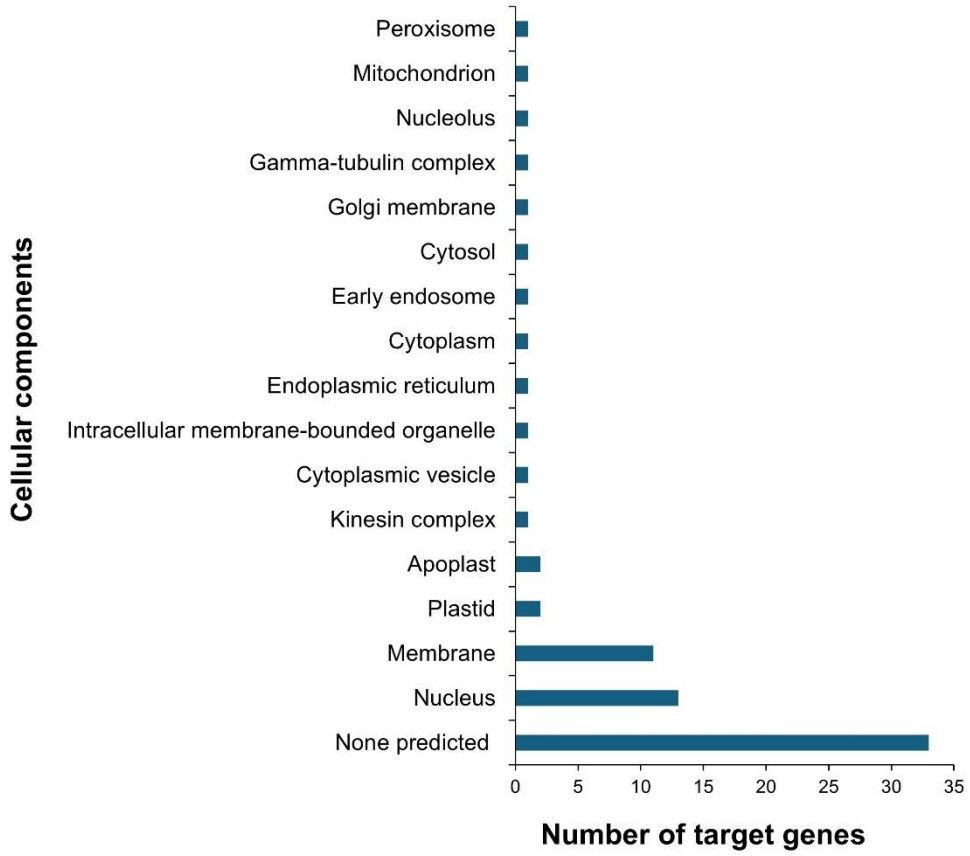

Figure 4.8. Putative cellular components of the target genes of constitutively expressed root miRNAs identified in both sorghum varieties.

4.3.4.3 Gene ontology annotations of the target genes of the unique constitutively expressed miRNAs

Tables 4.4 and 4.5 list the constitutively expressed root miRNAs that are unique to ICSB 338 and SA 1441 sorghum varieties, respectively. The mode of inhibition for all the unique constitutively expressed miRNAs is cleavage for both sorghum varieties. The target genes of

these miRNAs were also subjected to GO analysis and results are summarised in Tables 4.4 and 4.5, and Figure 4.9. The cellular component results showed that some of the target genes of the unique constitutively expressed miRNAs were commonly present in the nucleus and apoplast of both sorghum varieties (Figure 4.9A). However, cellular locations such as the vacuole, peroxisome, SCF ubiquitin ligase complex, and membrane were only present in ICSB 338, while microtubule associated complex and early endosome were only present in SA 1441 (Figure 4.9A).

The GO results showed that most of the target genes for the unique constitutively expressed miRNAs in ICSB 338 and SA 1441 did not have predicted molecular functions (Figure 4.9B). The terms serine-type peptidase activity, oxidoreductase activity, peroxisome matrix targeting signal-2 binding, and isomerase activity were present in ICSB 338 only. In contrast, protein kinase activity, microtubule binding, and RNA binding molecular functions were only present in SA 1441 (Figure 4.9B).

Similarly, the results suggest that most of the target genes of these unique constitutively expressed miRNAs did not have predicted biological processes in both varieties (Figure 4.9C). However, some of the target genes were involved in the regulation of DNA-templated transcription and phenylpropanoid biosynthesis process in both sorghum varieties. Biological process terms such as proteolysis, lignin catabolic process, protein targeting to peroxisome, and SCF dependent proteosomal ubiquitin dependent protein catabolic process were unique to ICSB 338. On the other hand, cytokinesis by cell plate formation and exocytosis were unique to SA 1441 (Figure 4.9C).

Table 4.4. List of constitutively expressed miRNAs identified in ICSB 338 sorghum roots only.

miRNA ID[a]	miRNA accession[b]	Target ID[c]	Target description[d]	Expect value[e]	Inhibition[f]	GO analysis[g]		
						P	F	C
sbi-miR393a	MI0001530	*Sobic.009G045300/ SORBI_3009G045300*	*Transport inhibitor response 1-like protein Os05g0150500*	1.0	Cleavage	SCF dependent proteosomal ubiquitin dependent protein catabolic process	Protein binding	SCF ubiquitin ligase complex
sbi-miR397-5p	MIMAT0011359	*Sobic.003G352800/ SORBI_3003G352800*	*Laccase-13*	0.5	Cleavage	Lignin catabolic process	Oxidoreductase activity	Apoplast
sbi-miR398	MI0013257	*Sobic.002G119900/ SORBI_3002G119900*	*Dirigent protein 22*	2.5	Cleavage	Phenylpropanoid biosynthetic process	Isomerase activity	Apoplast
sbi-miR5383	MI0018690	*Sobic.003G308300/ SORBI_3003G308300*	*Lysosomal Pro-X carboxypeptidase*	2.5	Cleavage	Proteolysis	Serine-type peptidase activity	Vacuole
sbi-miR5565g-3p	MIMAT0026399	*Sobic.003G269800/ SORBI_3003G269800*	*Phosphatidylinositol/phosphatidylcholine transfer protein SFH6*	0	Cleavage	None	None	Membrane
sbi-miR6230-3p	MIMAT0026437	*Sobic.004G317800/ SORBI_3004G317800*	*None*	2.0	Cleavage	None	None	None
sbi-miR6232a-3p	MIMAT0026445	*Sobic.004G106700/ SORBI_3004G106700*	*Peroxisome biogenesis protein 7*	0.5	Cleavage	Protein targeting to peroxisome	Peroxisome matrix targeting signal-2 binding	Peroxisome
sbi-miR6235-3p	MIMAT0026455	*Sobic.003G353700/ SORBI_3003G353700*	*Transcription factor PCL1-like*	0.5	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus

[a]miRNA ID obtained from the miRbase database search against *Sorghum bicolor* accessed on 25 September 2024.
[b]miRNA accession numbers obtained from the miRbase database, accessed on 28 October 2024.
[c]The sobic gene ID represents the target gene ID for each miRNA as obtained from the psRNATarget database. This sobic number was used to search for the latest SORBI gene number of the respective targets on the Gramene database. Both psRNATarget and Gramene databases were accessed on 7 October 2024.
[d]Target descriptions were obtained from the National Center for Biotechnology Information (NCBI) database, accessed on 7 October 2024.
[e]Expect value assigned to the alignment of each miRNA nucleotide sequence and that of its putative target gene on the psRNATarget database. The Expect values range from 0 to 5, where 0 represents a perfect alignment.
[f]Inhibition represents the type of regulation of each miRNA on its putative target gene as obtained on the psRNATarget database.
[g]Gene Ontology (GO) data of each miRNA target gene retrieved from the Gramene database, accessed on 25 September 2024. P stands for biological process, F stands for molecular function, and C stands for cellular component.

Table 4.5. List of constitutively expressed miRNAs identifies in SA 1441 sorghum roots only.

miRNA ID[a]	miRNA accession[b]	Target ID[c]	Target description[d]	Expect value[e]	Inhibition[f]	GO analysis		
						P	F	C
sbi-miR164a	MI0001512	*Sobic.008G071400/ SORBI_3008G071400*	*Protein cup-shaped cotyledon 1*	1	Cleavage	Regulation of DNA-templated transcription	DNA binding	Nucleus
sbi-miR171a	MI0001566	*Sobic.001G513900/ SORBI_3001G513900*	*None*	0	Cleavage	None	None	None
sbi-miR529	MI0010924	*Sobic.004G278700/ SORBI_3004G278700*	*Microtubule-binding protein TANGLED1*	1.5	Cleavage	Cytokinesis by cell plate formation	Microtubule binding	Microtubule associated complex
sbi-miR5389	MI0018696	*Sobic.009G004900/ SORBI_3009G004900*	*Probable serine/threonine-protein kinase PBL28*	0	Cleavage	Protein phosphorylation	Protein kinase activity	None
*sbi-miR5564b	MI0019097	*Sobic.010G113900/ SORBI_3010G113900*	*Protein Rf1, mitochondrial*	0	Cleavage	None	Protein binding	None
sbi-miR5568e	MIMAT0026439	*Sobic.001G193100/ SORBI_3001G193100*	*Protein HLB1*	0	Cleavage	Exocytosis	Protein binding	Early endosome
sbi-miR5569	MI0019105	*Sobic.005G179400/ SORBI_3005G179400*	*UBP1-associated protein 2A*	0	Cleavage	None	RNA binding	None
*sbi-miR6220-3p	MIMAT0026405	*Sobic.002G005400/ SORBI_3002G005400*	*Dirigent protein 11*	0	Cleavage	Phenylpropanoid biosynthetic process	None	Apoplast
sbi-miR6222-3p	MIMAT0026411	*Sobic.004G236600/ SORBI_3004G236600*	*F-box/kelch-repeat protein At1g74510-like*	1	Cleavage	None	Protein binding	Nucleus
*sbi-miR6235-5p	MIMAT0026454	*Sobic.008G095150/ SORBI_3008G095150*	*None*	0	Cleavage	None	None	None

[a]miRNA ID obtained from the miRbase database search against *Sorghum bicolor* accessed on 25 September 2024.
[b]miRNA accession numbers obtained from the miRbase database, accessed on 28 October 2024.
[c]The sobic gene ID represents the target gene ID for each miRNA as obtained from the psRNATarget database. This sobic number was used to search for the latest SORBI gene number of the respective targets on the Gramene database. Both psRNATarget and Gramene databases were accessed on 7 October 2024.
[d]Target descriptions were obtained from the National Center for Biotechnology Information (NCBI) database, accessed on 7 October 2024.
[e]Expect value assigned to the alignment of each miRNA nucleotide sequence and that of its putative target gene on the psRNATarget database. The Expect values range from 0 to 5, where 0 represents a perfect alignment.
[f]Inhibition represents the type of regulation of each miRNA on its putative target gene as obtained on the psRNATarget database.

[g]Gene Ontology (GO) data of each miRNA target gene retrieved from the Gramene database, accessed on 25 September 2024. P stands for biological process, F stands for molecular function, and C stands for cellular component.
*Denotes miRNAs with more than one target gene with perfect alignment as measured by an Expect value of 0.

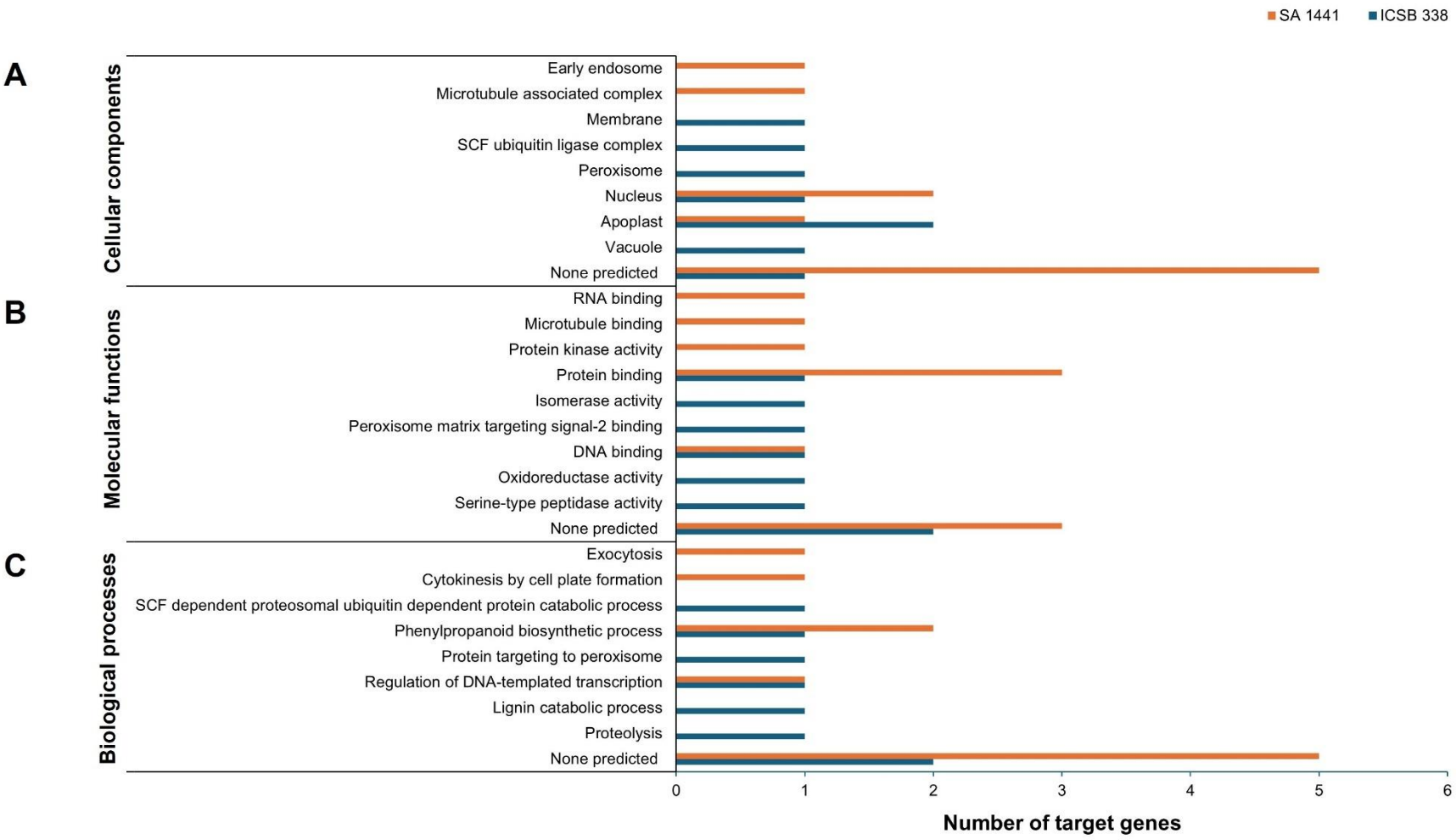

Figure 4.9. Putative Gene Ontology terms of the target genes of constitutively expressed root miRNAs unique to either sorghum variety.

4.3.5 Differentially expressed miRNAs of sorghum roots

Differential expression analysis of the identified miRNAs was conducted using the Differential Expression for RNA-seq tool on the CLC Genomics Workbench (Qiagen) by comparing drought-stressed and well-watered control expression levels for each sorghum variety. The results of the analyses are shown in Tables 4.6 and 4.7 for ICSB 338 and SA 1441 sorghum varieties, respectively. Figure 4.10 illustrates a volcano plot of the differentially expressed miRNAs for both sorghum varieties.

Four positively identified miRNAs in the drought-susceptible ICSB 338 were differentially expressed in response to the drought stress treatment ($p \leq 0.05$) (Table 4.6). Of these four miRNAs, sbi-miR6233-3p and sbi-miR821a were up-regulated, while sbi-miR5566 and sbi-miR6224a-5p were down-regulated (Figure 4.10A). Among the down-regulated miRNAs, the expression level of sbi-miR5566 decreased the most with a $\log_2$ fold change of -2.18. The expression level of sbi-miR821a increased the most with a $\log_2$ fold change of 2.36. *SORBI_3002G113400* (*Uncharacterized LOC8057912*) and *SORBI_3005G123400 (Uncharacterized LOC8055016),* were predicted as the target genes of sbi-miR5566 and sbi-miR6224a-5p, respectively (Table 4.6). *SORBI_3010G217700,* described as a *Putative clathrin assembly protein At5g57200,* was predicted as the target gene for the up-regulated sbi-miR6233-3p (Table 4.6). No descriptive information was available on the NCBI database for the target gene *SORBI_3003G175050* of sbi-miR821a.

Table 4.6. Differentially expressed miRNAs in ICSB 338 sorghum roots under drought stress.

miRNA ID[a]	miRNA accession[b]	Expression pattern[c]	Max group mean[d]	Log_2 fold change[e]	Fold change[f]	P-value[g]	Target ID[h]	Target description[i]
sbi-miR5566	MI0019100	Down	20	-2.18	*-4.52*	*0.032800*	*Sobic.002G113400/ SORBI_3002G113400*	*Uncharacterized LOC8057912*
sbi-miR6224a-5p	MIMAT0026414	Down	322.33	-0.87	*-1.83*	*0.008586*	*Sobic.005G123400/ SORBI_3005G123400*	*Uncharacterized LOC8055016*
sbi-miR6233-3p	MIMAT0026449	Up	30.67	1.09	*2.13*	*0.027095*	*Sobic.010G217700/ SORBI_3010G217700*	*Putative clathrin assembly protein At5g57200*
sbi-miR821a	MI0010925	Up	4.33	2.36	*5.14*	*0.019361*	*Sobic.003G175050/ SORBI_3003G175050*	None

[a]miRNA ID obtained from the miRbase database search against *Sorghum bicolor* accessed on 25 September 2024.

[b]miRNA accession numbers obtained from the miRbase database, accessed on 28 October 2024.

[c]Expression pattern represents the up and down-regulation of miRNAs. "Up" represents the up-regulated and "down" represents down-regulated.

[d]Max mean group represents the average transcripts per million (TPM) calculated by CLC Genomics Workbench software.

[e]Log_2 fold change indicates the expression level of miRNAs. Positive values represent up-regulated and negative values represent down-regulated miRNAs.

[f]The sobic gene ID represents the Target gene ID for each miRNA as obtained from psRNATarget database. This sobic number was used to search for the latest SORBI gene number of the respective targets on the Gramene database. Both psRNATarget and Gramene databases were accessed on 7 October 2024.

[g]Target description was obtained from the National Center for Biotechnology Information (NCBI) database, accessed on 7 October 2024.

For the drought-tolerant SA 1441 sorghum variety, nine miRNAs were differentially expressed in response to drought stress (Table 4.7). Of these miRNAs, only two (sbi-miR5564c-5p and sbi-miR6232b-3p) were up-regulated, while seven (sbi-miR168, sbi-miR2118-5p, sbi-miR395a, sbi-miR5387a, sbi-miR5568c-3p, sbi-miR6229-5p and sbi-miR6235-5p) were down-regulated (Figure 4.10B). One of the up-regulated miRNAs, sbi-miR6232b-3p, with a $\log_2$ fold change of 1.67, targets an *Indole-3-acetaaldehyde oxidase* (*SORBI_3002G043500)* gene that is involved in auxin biosynthesis. However, the other up-regulated sbi-miR5564c-5p, with a $\log_2$ fold change of 1.01, did not have descriptive information available for the target gene *SORBI_3005G027680*.

The down-regulated miRNAs target genes such as the *NAC domain-containing protein 83, Low affinity sulfate transporter 3, Leaf rust 10 disease-resistance locus receptor-like protein kinase-like 2.1, cell division cycle protein 123 homolog, Uncharacterized LOC8067772* and two with no description. Out of the seven down-regulated miRNAs, the expression level of sbi-miR2118-5p, which targets the unknown *(SORBI_3006G084701)* gene decreased the most with a $\log_2$ fold change of -2.49. Conversely, sbi-miR6232b-3p, which targets an *Indole-3-acetaldehyde oxidase* (*SORBI_3002G043500*) gene increased the most with a $\log_2$ fold change of 1.67 (Table 4.7). Overall, the number of down-regulated miRNAs in the drought-tolerant sorghum variety SA 1441, were greater than those in the drought-susceptible ICSB 338 variety (Tables 4.6 and 4.7; Figure 4.10).

Table 4.7. Differentially expressed miRNAs in SA 1441 sorghum roots under drought stress.

miRNA ID[a]	miRNA accession[b]	Expression pattern[c]	Max group mean[d]	Log_2 fold change[e]	Fold change[f]	P-value[g]	Target ID[h]	Target description[i]
sbi-miR168	MI0001556	Down	604	-0.73	*-1.66*	*0.015635*	*Sobic.003G409800/ SORBI_3003G409800*	*NAC domain-containing protein 83*
sbi-miR2118-5p	MIMAT0026426	Down	12.33	-2.49	*-5.61*	*0.040698*	*Sobic.006G084701/ SORBI_3006G084701*	*None*
sbi-miR395a	MI0001534	Down	16.33	-1.03	*-2.04*	*0.007102*	*Sobic.001G470800/ SORBI_3001G470800*	*Low affinity sulfate transporter 3*
sbi-miR5387b	MI0019104	Down	33.33	-0.86	*-1.81*	*0.049725*	*Sobic.003G080000/ SORBI_3003G080000*	*Leaf rust 10 disease-resistance locus receptor-like protein kinase-like 2.1*
sbi-miR5564c-5p	MIMAT0026390	Up	21.67	1.01	*2.01*	*0.002113*	*Sobic.005G027680/ SORBI_3005G027680*	*None*
sbi-miR5568c-3p	MIMAT0026401	Down	5.67	-1.26	*-2.39*	*0.0492*	*Sobic.003G098900/ SORBI_3003G098900*	*Cell division cycle protein 123 homolog*
sbi-miR6229-5p	MIMAT0026434	Down	19.33	-0.77	*-1.71*	*0.018679*	*Sobic.006G028200/ SORBI_3006G028200*	*Uncharacterized LOC8067772*
sbi-miR6232b-3p	MIMAT0026447	Up	5.67	1.67	*3.17*	*0.019341*	*Sobic.002G043500/ SORBI_3002G043500*	*Indole-3-acetaldehyde oxidase*
*sbi-miR6235-5p	MIMAT0026454	Down	5	-1.82	*-3.53*	*0.035479*	*Sobic.008G095150/ SORBI_3008G095150*	*None*

[a]miRNA ID obtained from the miRbase database search against *Sorghum bicolor* accessed on 25 September 2024.
[b]miRNA accession numbers obtained from the miRbase database, accessed on 28 October 2024.
[c]Expression pattern represents the up and down-regulation of miRNAs. "Up" represents the up-regulated and "down" represents down-regulated.
[d]Max mean group represents the average transcripts per million (TPM) calculated by CLC Genomics Workbench software.
[e]Log_2 fold change indicates the expression level of miRNAs. Positive values represent up-regulated and negative values represent down-regulated miRNAs.
[f]The sobic gene ID represents the Target gene ID for each miRNA as obtained from psRNATarget database. This sobic number was used to search for the latest SORBI gene number of the respective targets on the Gramene database. Both psRNATarget and Gramene databases were accessed on 7 October 2024.
[g]Target description was obtained from the National Center for Biotechnology Information (NCBI) database, accessed on 7 October 2024.
*Denotes miRNAs with more than one target gene with perfect alignment as measured by an Expect value of 0.

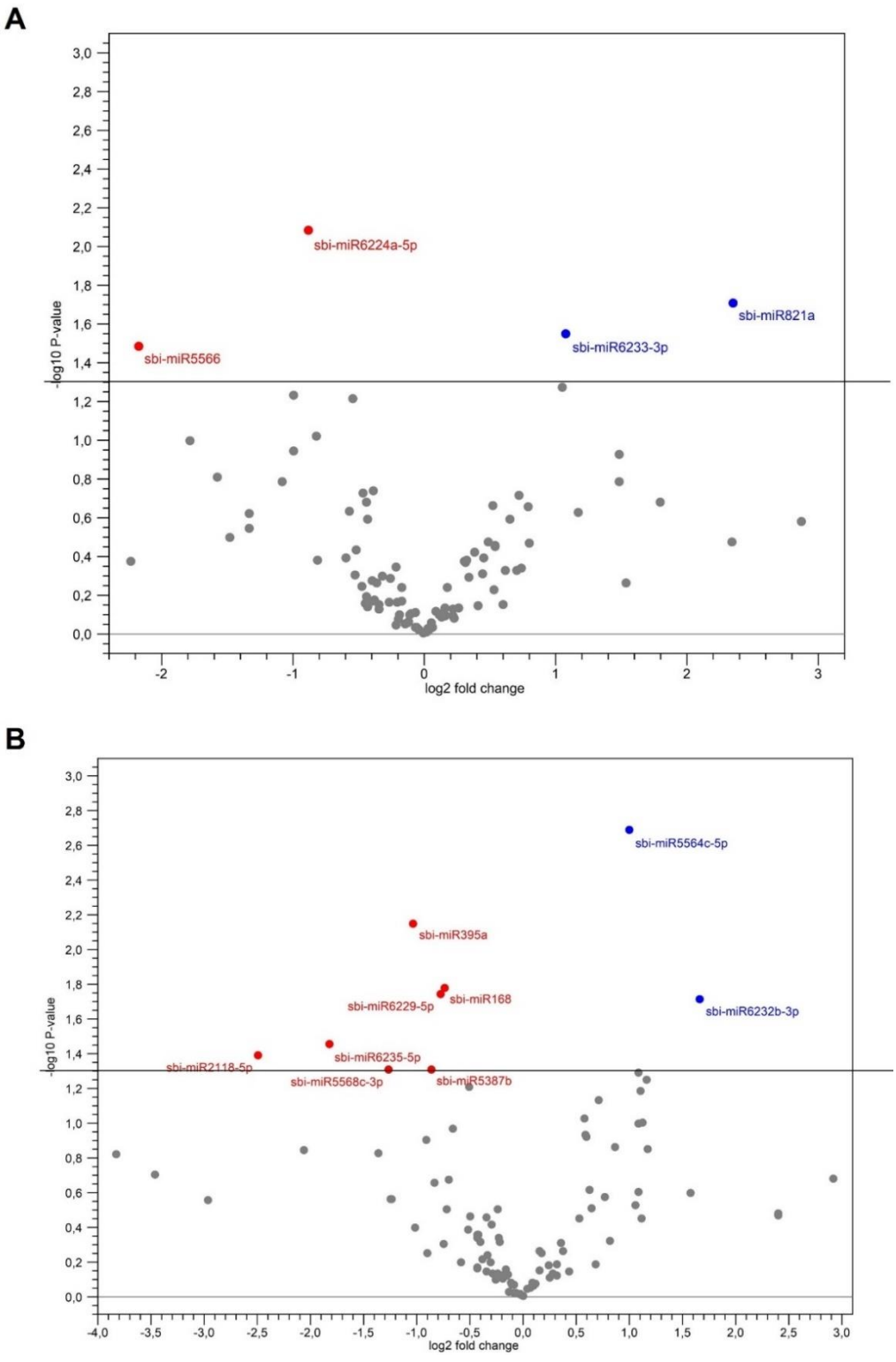

Figure 4.10. Volcano plot of the drought-responsive root miRNAs of two sorghum varieties. (A) is for ICSB 338 and (B) is for SA 1441. Each point in the figures represents a miRNA, with red and blue points indicating down and up-regulated miRNAs, respectively. The grey points indicate miRNAs that were not differentially expressed. The horizontal line is the significance threshold at $p \leq 0.05$.

4.3.6 Experimental challenges encountered in RNA-seq data analyses of sorghum miRNAs

The root total RNA extracted in the study was of good quality (Figure 4.3). The RNA-seq data obtained using the MGI DNBSEQ-G400 sequencing technology had more than 15 million reads for all samples after the quality control workflows (Table 4.2). The PHRED score quality control values ranged from 25-38, representing high quality sequence data (CLC Genomics Workbench) (Table 4.2). However, the three biological replicates in each treatment group per sorghum variety showed wide variation in expression pattern for the number of reads after trimming (Table 4.2) as well as miRNA expression values (Appendix Figure A1 and A2). This resulted in few miRNAs that were identified as drought-responsive (Tables 4.6 and 4.7; Figure 4.10). For this reason, the study did not assign a minimum cut-off value for the log2 fold change.

4.4 Discussion

Next-Generation Sequencing (NGS) technologies are capable of rapidly sequencing millions of nucleic acid fragments simultaneously with high accuracy (Tyagi *et al*., 2022). RNA sequencing (RNA-seq) technologies offer more benefits in terms of sensitivity compared to the more traditional transcriptomic methods such as microarrays and expressed sequence tags (Wang *et al*., 2009). RNA-seq identifies transcripts and provides accurate quantifications of gene expression patterns across samples and treatment groups and are capable of detecting high and low RNA quantities, making it effective for transcriptomic profiling (Chowdhury *et al*., 2020). As such, the current study evaluated the constitutively expressed and drought-responsive miRNAs of ICSB 338 and SA 1441 sorghum root tissues using RNA-seq analysis.

Sorghum is a potentially good model crop used in genomics and transcriptomics studies because of its relatively small and fully sequenced genome compared to other cereals such as wheat and barley (Stein, 2007; Paterson *et al*., 2009). Additionally, sorghum is a drought-tolerant crop, making it an important plant species for research on drought tolerance in arid and semi-arid regions (Hadebe *et al*., 2016). Therefore, understanding plant responses to drought is essential to crop resilience and achieving sustainable crop production (Rivero *et al*., 2022). Comparative transcriptomic studies using contrasting genotypes of a plant species allow for the identification of responses specific to the varieties (Abdel-Ghany *et al*., 2020; Lv *et al*., 2022). In this study, miRNA libraries were constructed and sequenced from the control and drought-stressed root samples of both sorghum varieties using MGI DNBSEQ-G400 sequencing because of its high sequencing accuracy and throughput (Anslan *et al.*, 2021). At the time of sequencing of this data, the MGI DNBSEQ-G400 sequencing technology was much cheaper than the other NGS platforms offered by the ARC-BTP and Core Facility.

Nucleic acid quantification is essential for many downstream processes in the NGS workflow, including RNA gel electrophoresis, RNA library preparation for sequencing, and quantitative PCR analysis of transcripts of interest (Robin *et al*., 2016). The quantification of nucleic acids is commonly performed using the Nanodrop method, which is spectrometry-based and/or the Qubit, which is fluorometry-based (Versmessen *et al*., 2024). Due to the sensitivity of the Nanodrop to contaminants such as proteins and salts from the extracts, Nanodrop measurements tend to overestimate nucleic acid concentrations in samples (Bruijns *et al*., 2022). The Qubit uses fluorescence and is less affected by sample contaminants (Bruijns *et al*., 2022). Despite the disadvantages of the Nanodrop method to overestimate nucleic acid concentrations, it is still widely used for DNA and RNA quantifications in many genomics and transcriptomics workflows (Bruijns et al., 2022).

The Genova Nano quantified samples were higher than those obtained using the Qubit machine (Table 4.2). The discrepancies between the two quantification methods could possibly be attributed to the presence of protein and salt contaminants in the samples, which resulted in higher readings. In this study, the Genova Nano was used to estimate concentrations of the total RNA extracts prior to gel electrophoresis (Figure 4.3) to assess their quality prior to sending to the ARC-BTP and Core Facility for sequencing. Unfortunately, the Genova Nano machine used at the UFS, Qwa-Qwa Campus did not provide the RNA integrity and purity values. In addition, the total RNA integrity and purity results are not presented because I did not ask for the information on time from the service provider (ARC-BTP and Core Facility) as I did not think it was important for presentation in the Master's dissertation.

Reads of high-quality are important for downstream analysis in NGS workflows (Kuster *et al*., 2021). In the current study, the PHRED scores exceeded 30 (Table 4.2), indicating that a percentage accuracy of 99.9% were obtained. This means that the reads are of high quality and will reduce errors in downstream analyses (Kuster *et al*., 2021). All samples had more than 15 million miRNA reads after trimming (Table 4.2) which is sufficient for small RNA analyses.

miRNAs are critical regulators of plant development, with essential roles in signal transduction and adaptation to environmental stress such as drought (Yang *et al*., 2021; Singh *et al*., 2023). miRNAs participate in post-transcriptional gene regulatory processes by cleaving their target mRNAs or inhibiting their translation into proteins (Pu *et al*., 2019). Cleavage of target mRNA reduces the number of the transcripts in plant cells, subsequently preventing their translation into proteins. Alternatively, translation inhibition allows miRNAs to block protein synthesis without degrading the mRNA (Djami-Tchatchou *et al*., 2017). The bioinformatics results of

this study revealed that miRNA inhibition by cleavage was the most abundant mode of inhibition (Tables 4.3 - 4.5; Figure 4.5) in the sorghum lines investigated. Similar findings were reported by Kumar *et al*. (2018), who reported cleavage as the most common mode of regulation for the identified miRNAs in pearl millet (*Pennisetum glaucum* L.).

Constitutively expressed miRNAs are continuously produced under normal growth conditions to regulate cellular processes for the normal growth and development of plants (Wang and Li, 2007). It is important to analyse the pool of miRNAs in contrasting varieties of the same species under controlled conditions to identify the regulatory mechanisms that are common and specific to the genotypes (Dong *et al*., 2022). Among the 81 and 83 constitutively expressed miRNAs in ICSB 338 and SA 1441 identified in the current study, 73 were common to both sorghum varieties, while 8 and 10 were unique to ICSB 338 and SA 1441, respectively (Figure 4.4). This suggest that miRNAs have important functions in the developmental processes of plants (Ferdous *et al*., 2015).

It was also observed that some miRNAs have more than one target gene with a perfect alignment (Table 4.3; Appendix A1). This could be attributed to the short sequence lengths of miRNAs, which allow them to easily pair with other sequences, enabling them to regulate multiple target genes (Ferdous *et al*., 2015). It is also possible that when miRNAs have multiple targets, some targets could be prioritised over others depending on the developmental stage (Naqvi *et al*., 2012). Therefore, the miRNAs may regulate different target genes and thus plant processes at different stages in the plant's life cycle (Naqvi *et al*., 2012; Ferdous *et al*., 2015). Wang *et al*. (2005) demonstrated that in Arabidopsis, miR160 regulates *Auxin response factor (ARF) 10 and 16* (ARF10 and ARF16) for normal growth and development of aerial organs as well as lateral root production. In the current study, sbi-miR160a was predicted to target

ARF22. The sbi-miR160a is amongst the miRNAs identified to have multiple targets (3) that are all *ARFs*, with an Expect value of 0, indicating a perfect alignment (Appendix Table A1).

However, it was also noted that some of the predicted miRNA targets do not belong to the same gene families even though they have an Expect value of 0 in the unique constitutively expressed miRNAs (Table 4.5; Appendix Table A2). This suggests that miRNAs can regulate targets across diverse functional groups or gene families. Ying *et al*. (2008) also reported that different miRNAs can target a single gene. For example, in this study, sbi-miR5568c-3p and sbi-miR6227-5p were predicted to regulate the *Cell division cycle protein 123 homolog*, which is important for cell cycle regulation (Tables 4.3 and 4.7). Similarly, sbi-miR5567 and sbi-miR395a potentially regulate the *Low affinity sulfate transporter 3* gene (Tables 4.3 and 4.7). Then, members of the same miRNA family can regulate the same gene; the *Ethylene-responsive transcription factor RAP2-7* was predicted to be regulated by sbi-miR172a and sbi-miR172f (Table 4.3), while sbi-miR6219-5p and sbi-miR6219-3p regulate *Obtusifoliol 14-alpha demethylase* that is involved in sterol biosynthesis (Table 4.3). Overall, this shows that miRNAs regulates a variety of biological processes (Chen, 2005; Dong *et al*., 2022).

Gene Ontology analysis was performed based on three categories, namely biological processes, molecular functions and cellular components (Ashburner *et al*., 2000). Such GO analyses have been used in other miRNA studies of rice *(Oryza sativa*) under drought stress (Bakhshi *et al*., 2016) to understand their functions. Most of the three GO categories of the target genes were not predicted, highlighting the lack of knowledge about the cellular functions and localizations of the target genes, possibly due to insufficient experimental evidence. The results showed that the three most enriched terms for molecular functions are DNA binding, protein binding and protein kinase activity (Figure 4.9B). The functions are

crucial for plant growth, development and stress response (Shinozaki *et al*., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007).

Analysing miRNA expression profiles under drought conditions can provide insight into their functions in plant stress response (Singh *et al*., 2023). Since miRNA functions depend on their targets, identifying these targets is important for understanding the roles of miRNAs in plants (Liu *et al*., 2012). The differences in the differentially expressed miRNAs between drought-susceptible and drought-tolerant varieties possibly show the unique regulatory patterns that each genotype has in response to drought stress (Liang *et al*., 2024). Up-regulated miRNAs may target genes involved in stress tolerance, leading to plant resilience, while the down-regulated miRNAs could activate genes that help mitigate stress effects (Tiwari and Rajam, 2022). These changes in the expression of miRNAs affect the downstream proteins and metabolites influencing responses to drought stress.

In the drought-susceptible variety ICSB 338, four miRNAs were differentially expressed, with sbi-miR6233-3p and sbi-miR821a being up-regulated, while sbi-miR5566 and sbi-miR6224a-5p were down-regulation in response to the drought stress treatment (Table 4.6). In contrast, the drought-tolerant variety SA 1441 exhibited nine drought-responsive miRNAs, of which two (sbi-miR5564c-5p and sbi-miR6232b-3p) were up-regulated and seven (sbi-miR168, sbi-miR2118-5p, sbi-miR395a, sbi-miR5387b, sbi-miR5568c-3p, sbi-miR6229-5p and sbi-miR6235-5p) were down-regulated (Table 4.7). The down-regulated miRNAs in SA 1441 could possibly play a role in enabling the activation of stress-adaptive genes, which can support the varieties tolerance to drought stress.

Transcription factors are important regulators of gene expression, as they bind to specific DNA molecules to regulate the transfer of genetic information from DNA to RNA (Zhang *et al*., 2008). miRNAs target transcription factors amongst other genes to regulate various plant growth, developmental processes and stress response (Chen, 2005; Zhang *et al*., 2022). The NAC domain-containing proteins are a family of transcription factors that function in embryogenesis, floral development and signalling during stress (Olsen *et al*., 2005; Fuertes-Aguilar and Matilla, 2024). In this study, SA 1441 showed that the NAC domain-containing protein was targeted by the down-regulated sbi-miR168 (Table 4.7). The target gene of the up-regulated sbi-miR6232b-3p is *Indole-3-acetaldehyde oxidase* which is involved in indole-3-acetic acid (IAA) biosynthesis. Indole-3-acetic acid regulates plant growth and development processes. Since the miRNA is up-regulated it decreased the IAA levels impacting the growth of the drought-stressed plants.

Under water limitation, the ability of roots to absorb nutrients decreases which may explain the differentially expressed sbi-miR395a in SA 1441 that targets the *Low affinity sulfate transporter* involved in the uptake of sulfate. Plants have to regulate sulfate movements to maintain a balance across tissues that are affected by the stress. According to Chan *et al*. (2012), during drought stress sulfate increases in the xylem sap. This increase in sulfate level could help with osmotic regulation and it is involved in the synthesis of glutathione, an antioxidant that protects cells from oxidative stress under drought stress (Gallardo *et al*., 2014). Some researchers stated that sulfate also acts as a signal that increases the effect of abscisic acid as it reaches the stomata in leaves, regulating water loss (Ernst *et al*., 2010). Therefore, the down-regulation of sbi-miR395a in the drought-tolerant variety SA 1441 under drought stress (Table 4.7) possibly allows an increase in the expression of *Low affinity sulfate transporter* to improve

the absorption of sulfate. Such a result suggests the importance of the sulfate in drought-response.

4.5 Conclusion

In conclusion, the current study identified miRNAs in the roots of both ICSB 338 and SA 1441 sorghum varieties and some miRNAs were constitutively expressed while others were drought-responsive. Among the constitutively expressed miRNAs, some were common in both varieties and others were unique to each. The drought-responsive miRNAs varied between the two varieties. This shows that the two varieties have unique regulatory mechanisms in response to drought conditions. While the overall count of drought-responsive miRNAs was low compared to other studies, the findings of this Master's study are important for further analyses of plant small RNA under stress.

CHAPTER 5

General Conclusions and Recommendations

5.1 General conclusions

Despite tremendous efforts to increase crop production to feed the growing population, drought continues to pose a significant threat to global agriculture and food security (He *et al*., 2019). Sorghum is a drought-tolerant cereal crop that thrives in arid regions. Its drought adaptive traits can be used to understand drought tolerance in other crops. This study aimed to identify constitutively expressed and drought-responsive miRNAs of the drought-susceptible ICSB 338 and drought-tolerant SA 1441 sorghum varieties. The study revealed that ICSB 338 plants experienced drought stress effects much quicker than the drought-tolerant SA 1441, as revealed by the leaf stomatal conductance (Figure 3.1) and the growth (Figures 3.3C and D) measurements. Although the pot weight readings revealed that SA 1441 lost more water during the 28 days of drought stress treatment (Figure 3.2A), it maintained a higher leaf relative water content (RWC) (Figure 3.4). These findings support the known superior drought phenotype of SA 1441 sorghum plants compared to that of ICSB 338 (Goche *et al*., 2020).

The study also identified constitutively expressed (Tables 4.3 – 4.5) and drought-responsive miRNAs in both sorghum varieties after 15 days of drought stress (Tables 4.6 and 4.7). The miRNAs were sequenced using the MGI-DNBSEQ-G400 machine and the data was analysed using CLC Genomics Workbench software (Qiagen). Due to a lack of knowledge in bioinformatics workflows for RNA-seq data, the learning process has been challenging. However, I managed to identify constitutively expressed (Tables 4.3 - 4.5) and drought-responsive (Tables 4.6 and 4.7) sorghum miRNAs in my samples, which means that I gained knowledge on the miRNA sequencing work.

Bioinformatics analysis of the putative target genes was performed using the psRNATarget database (Dai *et al*., 2018). In this study, most of the constitutively expressed miRNAs were shared between the two sorghum varieties (Table 4.3). This suggests that miRNAs are important in plant developmental processes (Chen, 2005, Zhang *et al*., 2022), however, further studies are required to validate their specific functions. Even though a few drought-responsive miRNAs were identified in both sorghum varieties (Figure 4.10), their presence shows the importance of small RNA in regulating gene expression under drought stress (Chao *et al*., 2022). In addition, seven of the nine drought-responsive miRNAs of SA 1441 were down-regulated. These down-regulated miRNAs possibly result in increased expression of their targets that help mitigate stress effects in plants. For example, down-regulated sbi-miR168 potentially targets the *NAC domain-containing protein 83* gene (Table 4.7) that functions in regulating drought stress responses in plants (Olsen *et al*., 2005; Bakhshi *et al*., 2014; Singh *et al*., 2021). The findings of the current Master's study will contribute to the knowledge on miRNAs in sorghum roots since not much research is available compared to other crops such as rice and maize (Tang *et al*., 2022; Kaur *et al*., 2023). Additionally, the identified miRNAs can help researchers to understand the regulatory mechanisms that influence sorghum tolerance under drought conditions and contribute to the development of resilient crops.

5.2. Recommendations

Recommendations for further studies include the following:

1. Due to the observed wide variations in the expression patterns of biological replicates within a sample group (Section 4.3.6) and as illustrated in the Appendices (Figures A1 and A2) more biological replicates should be re-sequenced before this work can be published. With the re-sequencing data, the False Discovery Rate (FDR ≤ 0.05) and a minimum log2 fold change cutoff of 2 could be used to increase the reliability of the results. Such criteria has been used in other miRNA studies (Ma *et al*., 2015).
2. The differentially expressed miRNAs could be validated using RNA gel blot hybridization. This is a reliable method used for confirming the expression patterns of miRNAs. Due to the short lengths of miRNAs, this method is ableto detect the size and abundance of the identified miRNAs ensuring increased accuracy of the results obtain from RNA sequencing (Ahmad *et al*., 2021).
3. Total RNA expression profiles of leaf and root tissues could also be studied under mild, moderate and severe drought stress conditions to understand the broader transcriptomic changes associated with drought stress in sorghum plants. This will help with identifying gene expression patterns of both coding and non-coding RNAs that will be responsive to drought conditions (Niu *et al*., 2023).
4. Drought-responsive miRNAs could also be identified in leaf and root tissues of both sorghum varieties under mild, moderate and severe drought to analyse common and tissue specific miRNAs. The identified miRNAs will provide insights into specific miRNAs functions in different plant tissues during drought stress (Fileccia *et al*., 2017).

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APPENDICES

Table A1. miRNAs with more than one target gene with Expect values of 0.

miRNA ID	Target ID	Target description
miRNAs common to ICSB 338 and SA 1441		
sbi-miR160a	Sobic.010G236300/ SORBI_3010G236300	Auxin response factor 18
	Sobic.006G149600/ SORBI_3006G149600	Auxin response factor 8
sbi-miR172f	Sobic.001G036800/ SORBI_3001G036800	Floral homeotic protein APETALA 2
	Sobic.002G083600/ SORBI_3002G083600	Floral homeotic protein APETALA 2
sbi-miR5565e	Sobic.002G294000/ SORBI_3002G294000	Probable purine permease 5
	Sobic.003G074801/ SORBI_3003G074801	None
	Sobic.010G228000/ SORBI_3010G228000	Putative serine/threonine-protein kinase
	Sobic.003G106900/ SORBI_3003G106900	Uncharacterized LOC8085053
	Sobic.001G361750/ SORBI_3001G361750	None
	Sobic.003G315500/ SORBI_3003G315500	None
	Sobic.004G262500/ SORBI_3004G262500	Probable L-type lectin-domain containing receptor kinase S.5
	Sobic.008G060500/ SORBI_3008G060500	Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM2
	Sobic.010G265700/ SORBI_3010G265700	Uncharacterized LOC8064991
sbi-miR5565g-5p	Sobic.007G204900/ SORBI_3007G204900	Uncharacterized LOC110437087
sbi-miR5568g-5p	Sobic.002G347600/ SORBI_3002G347600	Pentatricopeptide repeat-containing protein At2g41720
	Sobic.004G135200/ SORBI_3004G135200	Uncharacterized LOC8077788
sbi-miR5568g-3p	Sobic.004G223350/ SORBI_3004G223350	None

	Sobic.001G294150/ SORBI_3001G294150	None
	Sobic.003G317700/ SORBI_3003G317700	Cyclic nucleotide-gated ion channel 4
	Sobic.001G329800/ SORBI_3001G329800	Allene oxide cyclase, chloroplastic
miRNAs unique to SA 1441		
sbi-miR5564b	Sobic.005G027840/ SORBI_3005G027840	None
sbi-miR6220-3p	Sobic.003G017300/ SORBI_3003G017300	CTP synthase
	Sobic.003G28450/ SORBI_3003G28450	None
	Sobic.001G329800/ SORBI_3001G329800	Allene oxide cyclase, chloroplastic
sbi-miR6235-5p	Sobic.002G396200/ SORBI_3002G396200	Peptide deformylase 1A, chloroplastic
	Sobic.004G002600/ SORBI_3004G002600	Thiosulfate sulfurtransferase 18
	Sobic.004G050400/ SORBI_3004G050400	Uncharacterized LOC8076098
	Sobic.009G212400/ SORBI_3009G212400	Mitochondrial outer membrane protein porin 2
	Sobic.003G219400/ SORBI_3003G219400	UBP1-associated protein 2C
	Sobic.004G054700/ SORBI_3004G054700	Ribonuclease H2 subunit C

Table A2. Expression values for the constitutively expressed miRNAs in root tissues of both sorghum varieties.

	miRNA expression value for ICSB 338				miRNA expression value for SA 1441			
miRNA ID[a]	**Control 1**	**Control 2**	**Control 3**	**Average expression value**	**Control 1**	**Control 2**	**Control 3**	**Average expression value**
sbi-miR6219-3p	5890	1972	3298	3720	3519	1111	5291	3307
sbi-miR168	1570	425	687	894	738	359	715	604
sbi-miR5568e-5p	317	181	104	201	189	12	90	97
sbi-miR6224a-5p	284	264	419	322	159	68	214	147
sbi-miR6221-3p	87	89	88	88	65	24	91	60
sbi-miR6229-3p	57	37	43	46	33	19	56	36
sbi-miR6219-5p	45	27	24	32	30	15	43	29
sbi-miR160a	41	30	33	35	36	5	28	23
sbi-miR5387b	38	24	51	38	44	31	25	33
sbi-miR172f	30	3	10	14	11	1	9	7
sbi-miR6226-3p	28	15	20	21	64	4	41	36
sbi-miR172a	28	23	13	21	23	6	23	17
sbi-miR394a	26	19	46	30	111	24	56	64
sbi-miR437x-3p	25	16	15	19	14	7	18	13
sbi-miR6220-5p	24	11	16	17	19	4	26	16
sbi-miR5564c-5p	22	21	13	19	13	3	16	11
sbi-miR5388	22	8	6	12	5	7	14	9
sbi-miR159a	22	9	9	13	12	2	11	8
sbi-miR397-3p	21	11	17	16	16	6	11	11
sbi-miR6229-5p	18	10	6	11	25	8	25	19

sbi-miR5568d-5p	17	8	10	12	10	2	12	8
sbi-miR6232b-5p	17	9	12	13	8	6	15	10
sbi-miR6233-3p	16	12	13	14	26	21	32	26
sbi-miR5384	16	24	39	26	31	11	20	21
sbi-miR5564a	16	3	24	14	22	2	17	14
sbi-miR5568f-5p	15	4	5	8	4	2	4	3
sbi-miR6221-5p	66	89	129	95	65	31	72	56
sbi-miR5565g-5p	13	4	6	8	8	7	10	8
sbi-miR6231-5p	13	6	19	13	12	10	6	9
sbi-miR395a	12	3	8	8	25	9	15	16
sbi-miR6226-5p	11	6	5	7	6	4	11	7
sbi-miR5567	11	4	6	7	9	10	8	9
sbi-miR5565d	10	3	1	5	5	2	10	6
sbi-miR5386	10	1	5	5	7	4	12	8
sbi-miR6223-3p	10	5	3	6	2	2	4	3
sbi-miR6231-3p	9	9	4	7	5	1	6	4
Sbi-miR6227-3p	9	5	12	9	8	2	11	7
sbi-miR2118-3p	9	6	8	8	9	3	8	7
sbi-miR5565e	9	12	4	8	8	4	19	10
sbi-miR6230-5p	9	7	10	9	12	4	10	9
sbi-miR5568g-5p	8	1	9	6	2	2	3	2
sbi-miR5564c-3p	8	4	5	6	6	2	1	3

sbi-miR408	8	3	11	7	6	4	5	5
sbi-miR6224a-3p	8	4	2	5	6	9	4	6
sbi-miR5566	7	3	50	20	16	3	15	11
sbi-miR5568c-3p	7	2	8	6	8	1	8	6
sbi-miR6227-5p	7	3	1	4	3	2	4	3
sbi-miR5568c-5p	7	5	7	6	4	1	9	5
sbi-miR169d-3p	6	7	6	6	5	6	8	6
sbi-miR6228-5p	6	3	4	4	4	2	6	4
sbi-miR6220-3p	6	1	3	3	3	1	1	2
sbi-miR6232b-3p	5	6	1	4	2	2	1	2
sbi-miR2118-5p	5	4	6	5	6	29	2	12
sbi-miR528	5	2	3	3	4	1	6	4
sbi-miR1435a	5	1	5	4	1	3	5	3
sbi-miR5568g-3p	4	1	2	2	1	1	6	3
sbi-miR390	4	2	4	3	3	2	7	4
sbi-miR6222-5p	4	8	4	5	4	5	6	5
sbi-miR167a	4	1	6	4	4	3	8	5
sbi-miR171a	4	2	2	3	2	3	3	3
sbi-miR396a	4	2	4	3	6	1	4	4
sbi-miR6232a-5p	4	2	4	3	2	3	3	3
sbi-miR5568b-5p	4	1	3	3	9	1	6	5
sbi-miR5565a	3	2	5	3	3	1	3	2

sbi-miR6218-3p	3	2	1	2	3	1	1	2
sbi-miR5568f-3p	3	3	3	3	3	1	10	5
sbi-miR6223-5p	3	1	4	3	1	1	2	1
sbi-miR319a	3	3	5	4	5	6	3	5
sbi-miR5382	1	3	3	2	3	1	4	3
sbi-miR5568b-3p	1	2	2	2	1	1	3	2
sbi-miR171e	1	1	2	1	1	1	1	1
sbi-miR6228-3p	1	2	2	2	2	2	1	2
sbi-miR5385	13	3	7	8	19	2	6	9

Table A3. Differential Expression for RNA-seq tool results for constitutively expressed miRNAs of both sorghum varieties (ICSB 338 vs SA 1441).

Name	Identifier	Max group mean	Log_2 fold change	Fold change	P-value	FDR p-value	Bonferroni
*sbi-miR6224a-5p	sbi-miR6224a-5p	322.3333	-1.0662	-2.09391	0.000846	0.017765	0.093902
*sbi-miR6233-3p	sbi-miR6233-3p	26.33333	1.081635	2.116433	0.007798	0.081881	0.865601
*sbi-miR394a	sbi-miR394a	63.66667	1.167766	2.246636	0.013499	0.094492	1
*sbi-miR395a	sbi-miR395a	16.33333	1.240624	2.363007	0.046234	#N/A	1
sbi-miR5568g-5p	sbi-miR5568g-5p	6	-1.18414	-2.27227	0.06159	#N/A	1
sbi-miR5568f-5p	sbi-miR5568f-5p	8	-1.08333	-2.11893	0.073176	#N/A	1
sbi-miR2118-5p	sbi-miR2118-5p	12.33333	1.999656	3.999048	0.075052	#N/A	1
sbi-miR5568b-5p	sbi-miR5568b-5p	5.333333	1.099828	2.143291	0.090948	#N/A	1
sbi-miR6223-3p	sbi-miR6223-3p	6	-1.00033	-2.00045	0.098823	#N/A	1
sbi-miR5564c-5p	sbi-miR5564c-5p	18.66667	-0.6856	-1.60837	0.102997	0.496042	1
sbi-miR6217a-5p	sbi-miR6217a-5p	1.666667	-3.85495	-14.4695	0.131439	#N/A	1
sbi-miR5568e-5p	sbi-miR5568e-5p	200.6667	-1.06061	-2.08581	0.131804	0.496042	1
sbi-miR6235-5p	sbi-miR6235-5p	5	1.214357	2.320373	0.140255	#N/A	1
sbi-miR160a	sbi-miR160a	34.66667	-0.48586	-1.40042	0.141726	0.496042	1
sbi-miR6235-3p	sbi-miR6235-3p	2.333333	-1.52235	-2.87258	0.160276	#N/A	1
sbi-miR5383	sbi-miR5383	3.333333	-1.50543	-2.8391	0.175027	#N/A	1
sbi-miR159a	sbi-miR159a	13.33333	-0.5319	-1.44583	0.176623	#N/A	1
sbi-miR162	sbi-miR162	1.666667	-1.87274	-3.66228	0.179219	#N/A	1
sbi-miR6234a-5p	sbi-miR6234a-5p	1.333333	1.762342	3.392484	0.208044	#N/A	1
sbi-miR397-3p	sbi-miR397-3p	16.33333	-0.42497	-1.34254	0.208223	#N/A	1
sbi-miR5386	sbi-miR5386	7.666667	0.650586	1.569806	0.211057	#N/A	1
sbi-miR437x-3p	sbi-miR437x-3p	18.66667	-0.37821	-1.29973	0.218431	0.530117	1
sbi-miR171c	sbi-miR171c	1	-3.18907	-9.12022	0.219868	#N/A	1
sbi-miR6226-3p	sbi-miR6226-3p	36.33333	0.791885	1.731335	0.22126	0.530117	1
sbi-miR6229-5p	sbi-miR6229-5p	19.33333	0.903144	1.870137	0.244606	0.530117	1
sbi-miR5564c-3p	sbi-miR5564c-3p	5.666667	-0.75607	-1.68889	0.247963	#N/A	1
sbi-miR6232b-3p	sbi-miR6232b-3p	4	-1.07276	-2.10345	0.25139	#N/A	1
sbi-miR6221-5p	sbi-miR6221-5p	94.66667	-0.69393	-1.61768	0.252436	0.530117	1
sbi-miR5567	sbi-miR5567	9	0.499651	1.413871	0.25571	#N/A	1
sbi-miR5568f-3p	sbi-miR5568f-3p	4.666667	0.748569	1.680126	0.264722	#N/A	1
sbi-miR5570	sbi-miR5570	0.666667	2.65048	6.278761	0.277515	#N/A	1
sbi-miR399a	sbi-miR399a	0.666667	2.650223	6.277641	0.277568	#N/A	1
sbi-miR6222-3p	sbi-miR6222-3p	2	1.036431	2.051148	0.28589	#N/A	1
sbi-miR172f	sbi-miR172f	14.33333	-0.84704	-1.79881	0.288575	#N/A	1
sbi-miR6220-3p	sbi-miR6220-3p	3.333333	-0.8209	-1.76651	0.291646	#N/A	1
sbi-miR6231-3p	sbi-miR6231-3p	7.333333	-0.71981	-1.64697	0.300203	#N/A	1
sbi-miR6224a-3p	sbi-miR6224a-3p	6.333333	0.751966	1.684086	0.300222	#N/A	1
sbi-miR437a	sbi-miR437a	2	-1.37784	-2.59878	0.305264	#N/A	1
sbi-miR166a	sbi-miR166a	7	0.520666	1.434617	0.31118	#N/A	1
sbi-miR167a	sbi-miR167a	5	0.568925	1.483418	0.31663	#N/A	1
sbi-miR6229-3p	sbi-miR6229-3p	46	-0.35165	-1.27602	0.329295	0.628655	1
sbi-miR6233-5p	sbi-miR6233-5p	1	1.384403	2.610639	0.339769	#N/A	1

sbi-miR6225-3p	sbi-miR6225-3p	1	1.384263	2.610386	0.339801	#N/A	1
sbi-miR6223-5p	sbi-miR6223-5p	2.666667	-0.81274	-1.75654	0.34208	#N/A	1
sbi-miR408	sbi-miR408	7.333333	-0.40421	-1.32336	0.40255	#N/A	1
sbi-miR5569	sbi-miR5569	3	0.684908	1.607599	0.404604	#N/A	1
sbi-miR168	sbi-miR168	894	-0.31343	-1.24265	0.409891	0.685227	1
sbi-miR6232a-3p	sbi-miR6232a-3p	1	-1.20692	-2.30843	0.412511	#N/A	1
sbi-miR5568e-3p	sbi-miR5568e-3p	2.666667	0.968274	1.956498	0.42227	#N/A	1
sbi-miR5566	sbi-miR5566	20	-0.73731	-1.66707	0.424188	0.685227	1
sbi-miR5381	sbi-miR5381	4.333333	-0.73589	-1.66543	0.430872	#N/A	1
sbi-miR164a	sbi-miR164a	1.666667	0.788594	1.72739	0.431438	#N/A	1
sbi-miR5568a	sbi-miR5568a	0.333333	1.845549	3.593897	0.473263	#N/A	1
sbi-miR169a	sbi-miR169a	0.333333	-1.91597	-3.77368	0.487662	#N/A	1
sbi-miR1432	sbi-miR1432	0.333333	-1.91583	-3.77331	0.487698	#N/A	1
sbi-miR5568d-5p	sbi-miR5568d-5p	11.66667	-0.39932	-1.31888	0.49567	#N/A	1
sbi-miR6234a-3p	sbi-miR6234a-3p	1.333333	-0.77306	-1.70889	0.508401	#N/A	1
sbi-miR319a	sbi-miR319a	4.666667	0.472613	1.387621	0.521869	#N/A	1
sbi-miR390	sbi-miR390	4	0.386975	1.307648	0.526486	#N/A	1
sbi-miR5565e	sbi-miR5565e	10.33333	0.341973	1.267489	0.537988	#N/A	1
sbi-miR6232b-5p	sbi-miR6232b-5p	12.66667	-0.24654	-1.18636	0.548525	#N/A	1
sbi-miR398	sbi-miR398	3	-0.42887	-1.34618	0.566708	#N/A	1
sbi-miR172a	sbi-miR172a	21.33333	-0.19699	-1.1463	0.591139	0.886709	1
sbi-miR5385	sbi-miR5385	9	0.384839	1.305714	0.591143	#N/A	1
sbi-miR5565a	sbi-miR5565a	3.333333	-0.36251	-1.28566	0.603871	#N/A	1
sbi-miR5564b	sbi-miR5564b	1.333333	0.489377	1.403838	0.639917	#N/A	1
sbi-miR393a	sbi-miR393a	1.333333	0.488892	1.403367	0.640289	#N/A	1
sbi-miR5565g-3p	sbi-miR5565g-3p	2.666667	-0.51305	-1.42706	0.647806	#N/A	1
sbi-miR6228-3p	sbi-miR6228-3p	1.666667	0.417687	1.335784	0.653579	#N/A	1
sbi-miR5565d	sbi-miR5565d	5.666667	0.401554	1.32093	0.659488	#N/A	1
sbi-miR5565g-5p	sbi-miR5565g-5p	8.333333	0.257506	1.19541	0.660036	#N/A	1
sbi-miR5568c-5p	sbi-miR5568c-5p	6	-0.21949	-1.16432	0.668599	#N/A	1
sbi-miR5382	sbi-miR5382	2.666667	0.311718	1.241185	0.669997	#N/A	1
sbi-miR528	sbi-miR528	3.666667	0.264986	1.201624	0.670615	#N/A	1
sbi-miR5568g-3p	sbi-miR5568g-3p	2.666667	0.312052	1.241472	0.681107	#N/A	1
sbi-miR5384	sbi-miR5384	26.33333	-0.24832	-1.18782	0.681516	0.920937	1
sbi-miR5389	sbi-miR5389	5.333333	0.357373	1.281091	0.692226	#N/A	1
sbi-miR6227-3p	sbi-miR6227-3p	8.666667	-0.16622	-1.12211	0.696052	#N/A	1
sbi-miR396a	sbi-miR396a	3.666667	0.265114	1.201731	0.696575	#N/A	1
sbi-miR6221-3p	sbi-miR6221-3p	70.66667	-0.11094	-1.07993	0.738416	0.920937	1
sbi-miR6230-3p	sbi-miR6230-3p	1	-0.4025	-1.3218	0.742822	#N/A	1
sbi-miR6230-5p	sbi-miR6230-5p	8.666667	0.137838	1.100255	0.757895	#N/A	1
sbi-miR169d-3p	sbi-miR169d-3p	6.333333	0.136888	1.09953	0.770103	#N/A	1
sbi-miR5388	sbi-miR5388	12	-0.27341	-1.20866	0.774122	#N/A	1
sbi-miR5568c-3p	sbi-miR5568c-3p	5.666667	0.135778	1.098685	0.783542	#N/A	1
sbi-miR6232a-5p	sbi-miR6232a-5p	3.333333	-0.17761	-1.13101	0.792106	#N/A	1
sbi-miR6231-5p	sbi-miR6231-5p	12.33333	-0.14259	-1.10389	0.802012	#N/A	1
sbi-miR5568d-3p	sbi-miR5568d-3p	1.333333	-0.25983	-1.19734	0.804309	#N/A	1
sbi-miR171e	sbi-miR171e	1.333333	-0.2596	-1.19715	0.804486	#N/A	1

sbi-miR5387b	sbi-miR5387b	37.66667	0.09589	1.068724	0.805218	0.920937	1
sbi-miR1435a	sbi-miR1435a	3.666667	-0.14653	-1.1069	0.818978	#N/A	1
sbi-miR6227-5p	sbi-miR6227-5p	3.666667	-0.14677	-1.10709	0.821691	#N/A	1
sbi-miR6220-5p	sbi-miR6220-5p	17	0.081436	1.058071	0.825833	0.920937	1
sbi-miR6219-3p	sbi-miR6219-3p	3720	-0.0811	-1.05783	0.833229	0.920937	1
sbi-miR529	sbi-miR529	3	0.131401	1.095357	0.844478	#N/A	1
sbi-miR6218-5p	sbi-miR6218-5p	1.666667	-0.17636	-1.13003	0.849951	#N/A	1
sbi-miR171a	sbi-miR171a	2.666667	0.130147	1.094405	0.854205	#N/A	1
sbi-miR6226-5p	sbi-miR6226-5p	7.333333	0.071282	1.05065	0.871784	#N/A	1
sbi-miR6218-3p	sbi-miR6218-3p	2	-0.12183	-1.08811	0.885767	#N/A	1
sbi-miR397-5p	sbi-miR397-5p	1.666667	0.122478	1.088603	0.889643	#N/A	1
sbi-miR5568b-3p	sbi-miR5568b-3p	1.666667	0.122423	1.088562	0.88969	#N/A	1
sbi-miR2118-3p	sbi-miR2118-3p	7.666667	-0.06105	-1.04323	0.89028	#N/A	1
sbi-miR821b	sbi-miR821b	1	0.111074	1.080032	0.920491	#N/A	1
sbi-miR821a	sbi-miR821a	0.666667	0.098281	1.070497	0.940911	#N/A	1
sbi-miR6225-5p	sbi-miR6225-5p	0.666667	0.097957	1.070257	0.941102	#N/A	1
sbi-miR6222-5p	sbi-miR6222-5p	5.333333	0.044372	1.031234	0.943452	#N/A	1
sbi-miR6219-5p	sbi-miR6219-5p	32	0.015171	1.010572	0.966235	0.973808	1
sbi-miR5565f	sbi-miR5565f	0.333333	0.066553	1.047212	0.969521	#N/A	1
sbi-miR6228-5p	sbi-miR6228-5p	4.333333	0.021815	1.015236	0.969641	#N/A	1
sbi-miR5387a	sbi-miR5387a	0.333333	0.066277	1.047012	0.96965	#N/A	1
sbi-miR5564a	sbi-miR5564a	14.33333	0.022007	1.015371	0.973808	0.973808	1
sbi-miR437x-5p	sbi-miR437x-5p	0	#N/A	#N/A	#N/A	#N/A	#N/A
sbi-miR156a	sbi-miR156a	0	#N/A	#N/A	#N/A	#N/A	#N/A

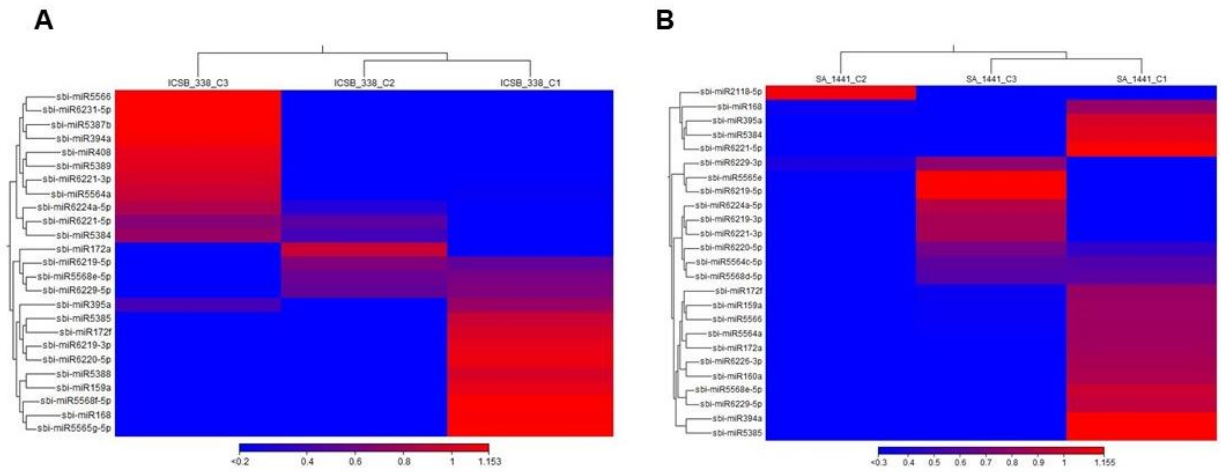

Figure A1. Heatmaps of root miRNAs expression values of sorghum control samples. (A) shows the three biological replicate controls of ICSB 338 and (B) show the three biological replicate controls of SA 1441.

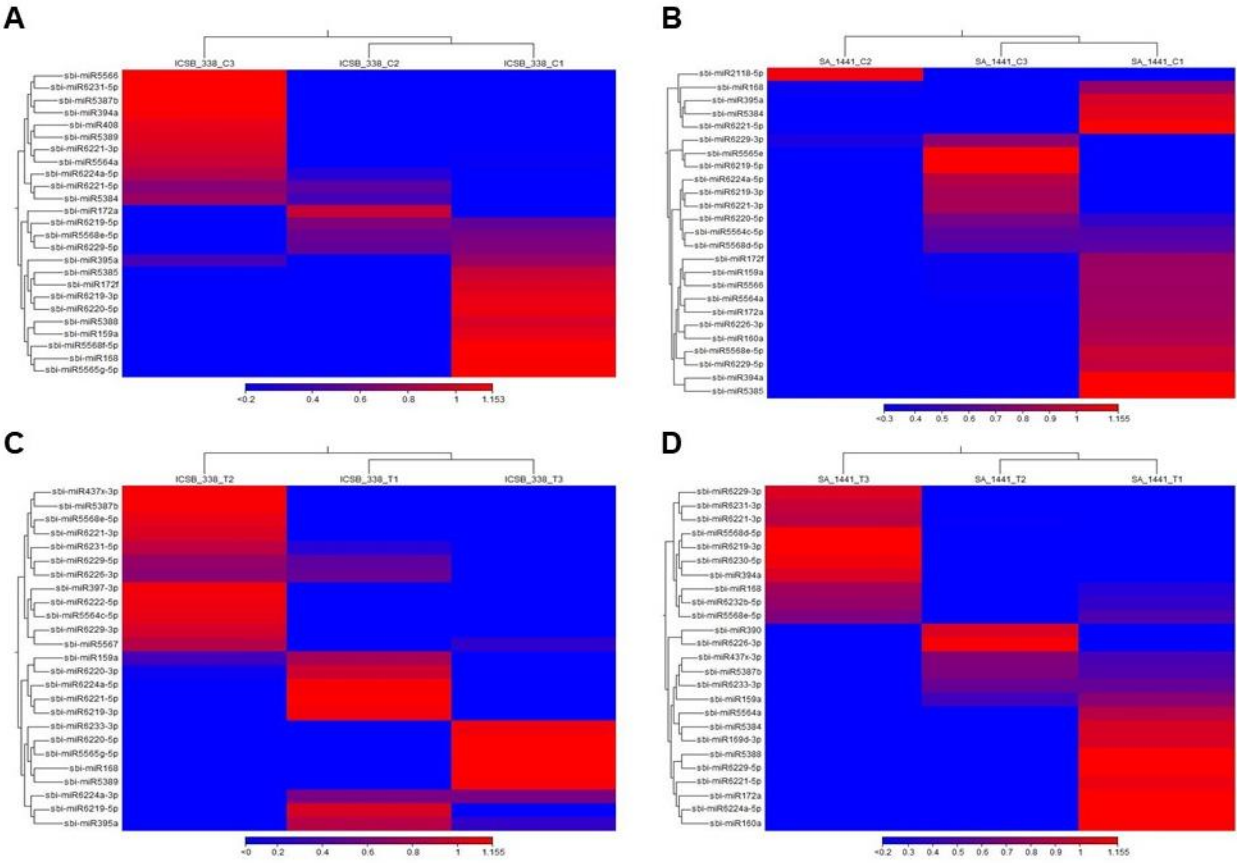

Figure A2. Heatmaps of root miRNAs expression values of sorghum control and drought-stressed samples. (A) and (C) shows the three biological replicate controls and drought-stressed sample of ICSB 338, respectively. (B) and (D) shows the three biological replicate controls and drought-stressed sample of SA 1441, respectively.